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Multi-targeted tyrosine kinase inhibitors in the treatment of cancer and neurodegenerative disorders

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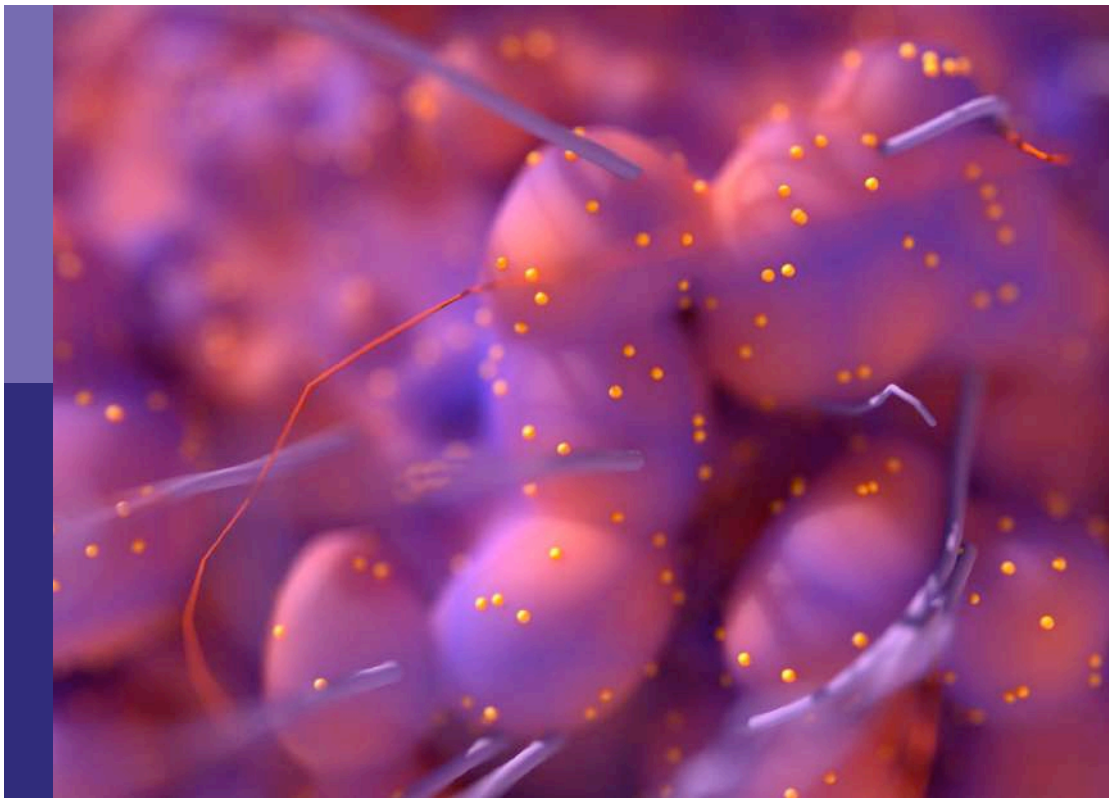

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Multi-targeted tyrosine kinase inhibitors in the treatment of cancer and neurodegenerative disorders

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Editorial: Multi-targeted tyrosine kinase inhibitors in the treatment of cancer and neurodegenerative disorders

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tyrosine kinase Inhibition, cancer, apoptosis, downstream signalling, Abl and Src tyrosine kinases, epidermal growth factor receptor (EGFR), HER-2 (ErbB2)

Editorial on the Research Topic
Multi-targeted tyrosine kinase inhibitors in the treatment of cancer and neurodegenerative disorders

Tyrosine kinases, which catalyse the phosphorylation of tyrosine residues in target proteins using ATP, play a plethora of roles in the regulation of diverse cellular functions including growth, motility, differentiation, and metabolism. Since their activity is tightly regulated in normal cells, in cancer due to emerging mutations, overexpression and autocrine paracrine stimulation, they can acquire transforming functions (Riegel et al., 2022). The pathogenesis of neurodegenerative diseases is also related with protein kinases (Kawahata and Fukunaga, 2023).

Tyrosine kinases are mainly classified as receptor tyrosine kinases and non-receptor tyrosine kinases, including crucial members. Epidermal growth factor receptor (EGFR) belongs to the ERbB family of receptor tyrosine kinases along with three other closely related receptors, namely, HER-2, HER-3, and HER-4. EGFR and HER-2 lead to autophosphorylation of the intracellular domain through tyrosine kinase activity and subsequent stimulation of downstream cascade that may result in proliferation, suppression of apoptosis, metastasis and angiogenesis. On the other hand, c-Abl (Abl-1) is a non-receptor tyrosine kinase, which is also essential in the regulation of several anti-apoptotic and proliferative signal transduction pathways. They have mainly been identified as important targets for several types of cancer such as EGFR for non-small-cell lung cancer (NSCLC), glioma and colorectal cancer; HER-2 for breast and colorectal cancers and c-Abl for chronic myeloid leukemia (CML).

One of the major platforms that they have participated in is neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral

sclerosis (ALS). Aberrant activity of tyrosine kinases, in particular EGFR and c-Abl, have been reported to induce neuronal apoptosis and cell cycle arrest in response to a wide range of stimuli resulting in neurodegeneration and neuroinflammation.

The main goal of the Research Topic entitled "*Multi-targeted Tyrosine Kinase Inhibitors in the Treatment of Cancer and Neurodegenerative Disorders*" is to identify new and promising tyrosine kinase inhibitors to be effective in cancer and neurodegenerative disorders.

In this Research Topic, twelve high-quality papers have been published (three of them original research articles, two reviews, one systematic review, five case reports and one clinical trial), which focused mainly on cancer. In these studies, the authors presented their latest results across a wide spectrum of research dealing with usage of multi-target tyrosine kinase inhibitors in the microsatellite stability subtype of colorectal cancer (Liu et al.), discussing the effects of anlotinib combined with chemotherapy in patients with metastatic triple-negative breast cancer (Huang et al.), reviewing the treatment of advanced hepatocellular carcinoma with multikinase inhibitor axitinib (Jiang et al.), as well as discussing the discontinuation and clinical outcomes in patients with B-cell lymphoproliferative diseases treated with bruton tyrosine kinase inhibitors (BTKi) in China (Yan et al.). Intriguing are work of (Li et al.), who demonstrated safety and efficacy of transarterial chemoembolization combined with tyrosine kinase inhibitors and camrelizumab in the treatment of patients with advanced unresectable hepatocellular carcinoma and work of (Wang et al.), who showed effective combined treatment of sunitinib (tyrosine kinase inhibitor) with immune checkpoint inhibitors. The role of tyrosine kinase inhibitors was also highlighted in pulmonary carcinoma by work of (Yang et al.), who used aumolertinib for effective treatment for asymptomatic pulmonary giant cell carcinoma with EGFR L858R mutation and by work of (Li et al.), who demonstrated the response to fifth-line brigatinib plus entrectinib in an anaplastic lymphoma kinase (*ALK*)-rearranged lung adenocarcinoma with an acquired ETV6-NTRK3 fusion. The successful and safety response to tyrosine kinase inhibitors was found in treatment of metastatic renal cell carcinoma (Krawczyk et al.) and in combination of two inhibitors bevacizumab with erlotinib for a novel FH gene mutation hereditary leiomyoma and renal cell carcinoma (Bai et al.). In the work of (Liu et al.), the molecular and microenvironment changes upon midostaurin treatment in mast cell leukemia at single-cell level were revealed. The effectiveness of using tyrosine kinase inhibitors and their challenges in glioblastoma treatment was discussed in the work of (Rahban et al.).

Together the published papers in this collection highlight the important role which tyrosine kinases play in pleotropic number of cellular processes in physiological and pathological conditions. The papers reveal the specificity and the effectiveness of using different tyrosine kinases inhibitors as a successful treatment of different cancers. It is hoped that the reader will find useful and appreciate this Research Topic.

Author contributions

BS: Writing–original draft, Writing–review and editing. LS: Writing–original draft, Writing–review and editing. RT: Writing–original draft, Writing–review and editing. VO: Writing–original draft, Writing–review and editing. HC: Writing–original draft, Writing–review and editing.

Conflict of interest

Author HC was employed by Science Farm Ltd.

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Case Report: MSS colorectal extrahepatic (non-liver) metastases as the dominant population for immunotherapy combined with multi-target tyrosine kinase inhibitors

Jiayin Liu[1], Dan Li[1], Jing Han[1], Yin Zhang[2], Xue Zhang[1], Zhisong Fan[1], Li Feng[1], Long Wang[1], Hui Jin[1], Jing Zuo[1] and Yudong Wang[1]*

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Background: The microsatellite stability(MSS) subtype of Colorectal Cancer (CRC) represents approximately 95% of mCRC cases. Immunotherapy was not as encouraging as the data for MSS mCRC cancer. We report the treatment of a series of patients with extrahepatic metastasis of MSS colorectal cancer, which can provide reference and guidance for the treatment of non-hepatic metastasis of MSS colorectal.

Case presentation: This report describes 8 typical cases of successful MSS treatment with lung metastases of CRC. We systematically reviewed the clinical data and detailed medical history of one of these patients with extrahepatic metastasis from MSS colorectal cancer, and reviewed the literature to analyze and discuss the related epidemiological features, mechanisms and recent research findings of the special subgroup of the population.

Conclusions: Although MSS colon rectal cancer is still known as a cold tumor in the industry, immunotherapy combined with multi-targeted anti-vascular tyrosine kinase inhibitors had brought clinical benefits to patients with non-hepatic metastases from MSS colorectal cancer.

KEYWORDS

MSS, extrahepatic (non-liver) metastases, dominant population, immunotherapy combined, multi-target tyrosine kinase inhibitors

Introduction

According to the most recent cancer report published by the National Cancer Center of China (1), colorectal cancer (CRC) has become the second most common malignant tumor after lung cancer, with about 408,000 new cases. The incidence and mortality of CRC ranks among the top second malignant tumors and has become one of the main cancers that can endanger life and health.

About 20% of patients present distant metastases at the time of initial diagnosis, of which the liver and lung are the most representative sites. The lung is the organ most likely to metastasize after the liver, and 5–15% of patients will eventually develop lung metastases. Treatment of lung metastases has become an integral part of the comprehensive treatment of CRC. The general treatment of CRC multiple pulmonary metastases mainly involves drug therapy, including systemic chemotherapy and targeted drug therapy. After drug treatment, some patients may achieve a reduction in the size of original unresectable lesions and achieve resectable conditions. Patients with resectable metastases have a 5-year survival rate of approximately 30–40%.

The MisMatch Repair-deficient(dMMR)/microsatellite instability-high(MSI-H) subtype of CRC represents approximately 15% of all cases and 5% of mCRC cases. Due to the high mutation rate of dMMR/MSI-H, tumors are highly immunogenic, enabling them to activate the antitumor response of the immune system. dMMR/MSI-H patients have been reported to be more responsive to immune checkpoint inhibitor (ICI)-based immunotherapy. In the KEYNOTE-016 trial, investigators found that multiple tumors in dMMR benefited from pembrolizumab immunotherapy, and in mCRC, pembrolizumab monotherapy resulted in an objective response rate 57% in cases of dMMR, while the ORR was 0 in patients with pMMR (2).

Furthermore, KEYNOTE-164 and 158 studies confirmed that pembrolizumab produced an ORR of 33% and a long-term survival benefit in previously treated patients with advanced MSI-H CRC (3, 4). Based on the excellent results of five studies including KEYNOTE-016, 164, and 158 trials, the FDA approved pembrolizumab in 2017 for the treatment of patients with solid tumors with MSI-H/dMMR, including mCRC. Although the above studies affirmed the benefit of immunotherapy in patients with MSI-H/dMMR mCRC, it is not recommended as first-line treatment of advanced patients.

For 95% of MSS bowel cancer patients, immunotherapy was not as encouraging therapy as the data for advanced MSI-H/dMMR bowel cancer. In contrast, MSS bowel cancer is still called a cold tumor in the industry, and single-drug immunotherapy has little effect on advanced bowel cancer. Basic research suggests that the level of lymphocytes infiltrating the MSS tumor microenvironment is low, and the immune response is weak. The KEYNOTE016 phase II study and the KEYNOTE-028 IB study also showed that patients with normal mismatch repair (pMMR) CRC did not respond to pembrolizumab therapy. How to change the immune microenvironment and how to turn a cold tumor into a hot tumor has become the biggest bottleneck in the immunotherapy of advanced CRC. However, recent studies related to combination therapy have raised the possibility of improving the efficacy of immunotherapy in this population. The combination of immunotherapy and small-molecule antiangiogenic targeted therapy has made significant progress. This report describes a typical case series of successful MSS treatment with lung metastases of CRC.

Case presentation

A 60-year-old female was referred to our hospital in June 2016 for increased stool frequency for more than six months. Her mother died of "rectal cancer". Colonoscopy showed ulcerated neoplasia of the rectum 8-13 cm from the anal verge, invading nearly half of the circumference. Pathology on bite-examination was adenocarcinoma of the rectum. The abdominal MRI showed a rectal wall mass consistent with rectal cancer, with multiple enlarged lymph nodes around the rectal mesentery and superior rectal artery; the rest of the pelvis was not abnormal. The patient's imaging stage was cT3N2M0.

Radical bowel cancer surgery: In June 2016, the patient underwent a radical Dixon operation for rectal cancer. The postoperative pathology showed grade II adenocarcinoma invading the mesentery, with no clear choroidal aneurysm embolus or nerve invasion. Lymph nodes: peri-intestinal 0/10, mesenteric 0/4, mesenteric root 0/4 metastases. The patient was diagnosed with stage IIB (pT4aN0M0) adenocarcinoma. Genetic testing suggested KRAS E2p.G13D mutation (Figure 1A). In July 2016, the patient started postoperative treatment with the XELOX protocol for one cycle. Patients are not tolerated due to adverse reactions and are reviewed regularly (Figure 1A).

In June 2018, CT scan suggests nodule in upper lobe of right lung, metastasis? Not excluding primary lung cancer. Disease-free survival after bowel cancer surgery was 2 years (DFS).

Upper lobe of right lung surgery: In June 2018, the patient underwent right upper lung lobectomy. Immunohistochemistry suggested TTF-1 (-), CDX2 (+), Napsin A (-), PDL1 (DAKO 22C3) (0% positive), PD-L1 (VENTANA SP263) (0% positive), ALK Negative (-), BRAF (-), MLHI1 (+), PMS2 (+), MSH2 (+), MSH6 (+). Pathology suggests adenocarcinoma, considered of intestinal origin. In July 2018, the patient was given capecitabine chemotherapy for one cycle. Patients are not tolerated due to adverse reactions and are reviewed regularly (Figure 1A). Disease-free survival after excision of lung metastases was 13 months(DFS).

1st-line treatment: In July 2019, CT scan suggested multiple nodules in both lungs and metastases were considered. A whole-body bone scanning indicated bone metastases. The patient started 1st-line treatment with mFOLFOX6+bevacizumab, lasting for seven cycles, and the disease was stable which achieved stable disease (SD) of the lung lesion (Figure 1A). Subsequently, the patient was not diagnosed and treated as planned due to the impact of the epidemic and remained on SD until March 2020 with a PFS of 8 months.

2nd-line treatment:In March 2020, CT scan showed enlarged multiple metastases in both lungs, the patient developed a progressive disease (PD). The patient started 2nd-line treatment with FOLFIRI +Bevacizumab, lasting for eight cycles, and the disease was stable

which yielded a partial response partial response (PR) of the lung lesion (Figure 1A). 2nd-line maintenance therapy was given with irinotecan and bevacizumab, lasting for two cycles and until November 2020 with a PFS of 7 months.

3rd-line treatment : Starting from November 2020, patients received a combinatorial treatment with furoquinitinib (5mg d1-14 q21d), camrelizumab (200mg d1 q21d),lasting 23 cycles so far. The patient received 3rd-line treatment achieved maintain partial response (PR)(Figure 1B).

Discussion

Currently, the lungs are the second most common site of CRC metastases after the liver. As rectal cancer patients are prone to lung metastases (5) and the proportion of rectal cancer cases in China (nearly 50%) is significantly higher than that reported in western countries (around 30%) (6), the diagnosis and treatment of lung metastases in CRC indicated more significant clinical problems in China.

MSI-H patients account for only 5% of advanced CRC patients, and the remaining 95% of CRC patients are generally genotyped as MSS. To date, the efficacy of immunotherapy among mCRC patients with MSS has not been satisfactory, but significant research is currently underway (7).

Recent research has focused on improving clinical response rates and generalizing these treatments to all CRCs (8). Cancer patients with liver metastases demonstrate significantly worse outcomes than those without liver metastases when treated with anti PD-1 immunotherapy. The research report that patients with both hepatic and extrahepatic metastasis showed more favorable survival and higher response to dual ICB than those with hepatic metastasis only (9). Cancer cells invading the liver may trigger liver specific tolerance mechanisms that reduce systemic antitumor immunity and cancer immunotherapy efficacy. Clinical data reveals that liver metastasis patients are less responsive to treatment with anti PD-1 antibodies than patients without liver metastases, which is confirmed from basic research studies (10, 11). The liver facilitates distant immune suppression of tumor antigens independently of tumor burden. Tregs undergo specific priming in the presence of liver tumor, and enhanced Tregs can modify tumor-antigen specific MDSCs that migrate to distant sites, ultimately suppressing antigen-specific CD8+ T cell activation *via* clonal anergy. Furthermore, liver metastasis is correlated with a decreased efficacy of immunotherapy in cancer patients. Liver metastases can attract $CD8^+$ T cells from systemic circulation. Within the liver, activated antigen-specific $FasL^+CD8^+$ T cells undergo apoptosis following their interaction with $FasL^+CD11b^+F4/80^+$ monocyte-derived macrophages.

NCCN guidelines and CSCO guidelines recommend regorafenib or fruquintinib or TAS-102 as limited treatment options, but the survival benefits after the third line is not ideal. The recently published REGONIVO study (12), which explored the combination of immunotherapy (nivolumab) with an antiangiogenic targeted therapy (regorafenib), showed that the combined regimen achieved an ORR of 33% in MSS/pMMR mCRC. The researchers believed that the efficacy of combination therapy indicates that anti-angiogenesis therapy may improve the immune status of the tumor microenvironment and relieve the immunosuppressive effect, which enhances outcomes of immunotherapy. In addition, patients who benefited from this combined treatment were all male and had lung metastases, which may have implications for the selection of the treatment population.

One study (13) suggests that the combination of fruquintinib and sintilimab reduced angiogenesis and reprogramed the liver vascular structure, enhanced infiltration of CD8+ T cells ($p<0.05$), CD8+ TNFa+ ($p<0.05$) T cells, and CD8+ IFNg+ ($p<0.05$) T cells and reduced the ratios of MDSCs and macrophages in mice. Furthermore, fruquintinib can correct the immune escape

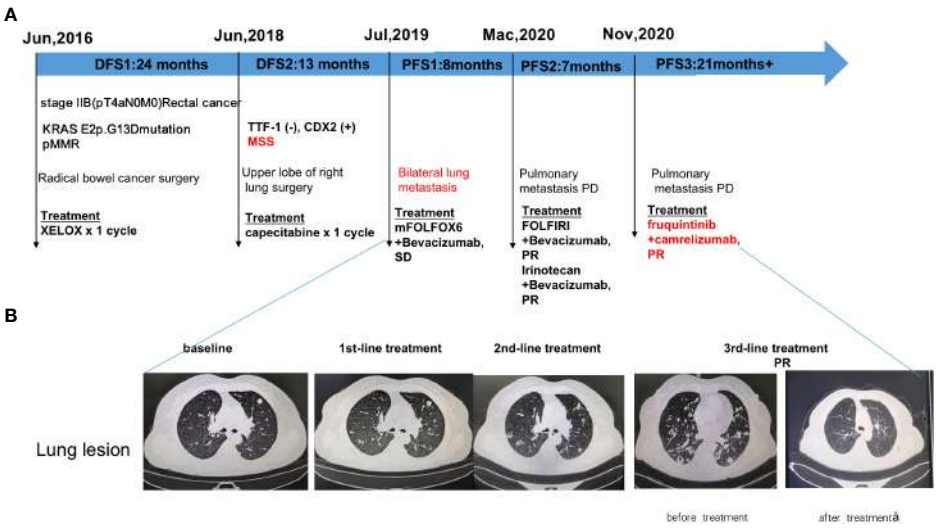

FIGURE 1
Diagram of patient's treatment history and clinical course. **(A)** Timeline of treatment; **(B)** Imaging changes before and after treatment. PR: partial response; SD: stable disease; PFS: progression-free survival.

microenvironment of tumor cells, mainly by inhibiting PD-L1 expression, inhibiting tumor release of inflammatory factors and immunosuppressive factors such as IL6/IL-10/VEGFR, and inhibiting bone marrow-derived suppressor cells. In turn, the secretion of T-reg cells is inhibited and the microenvironment is conducive to synergistic immunotherapy.

Based on the above clinical studies, we select the appropriate patients in the clinic. From the eight patients selected, the combination of immunotherapy and targeted therapy in the treatment could benefit (Table 1). These data have also been confirmed in our clinical practice.

Meanwhile, we analyzed the hematological data of 8 patients before and after treatment, including blood routine, biochemical indexes (Table 2) and tumor markers. We analyzed the changes of tumor markers (including CEA, CA199 and CA724) before and after treatment in 8 patients (Supplementary Material). It can be seen that there is a downward trend in tumor markers before and after treatment.

TABLE 1 Demographic features, clinical characteristics, and therapeutic regimens.

Items	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Gender	Female	Female	Female	Female	Male	Female	Female	Female
Age (years)	65	60	68	76	54	57	55	61
location	Colon Cancer	Colon Cancer	Colon Cancer	Rectal Cancer	Colon Cancer	Colon Cancer	Colon Cancer	Rectal Cancer
PS	1	1	1	1	1	1	1	1
Treatment Lines	3 lines	3 lines	3 lines	3 lines	3 lines	3 lines	3 lines	3 lines
Metastasis Site	Lung	Lung, bone	Lung	Lung	Peritoneal	Peritoneal	Lung	Lung
KRAS Status	mutation	mutation	Wild	mutation	mutation	mutation	mutation	mutation
MMR/ MSS	pMMR	MSS	pMMR	pMMR	pMMR	pMMR	MSS	pMMR
1 Lines	XELOX+ Bevacizumab	XELOX+ Bevacizumab	mFOLFOX6+ cetuximab	X+Bevacizumab	XELOX+ Bevacizumab	XELOX+ Bevacizumab	mFOLFOX6+ Bevacizumab	mFOLFOX6+ Bevacizumab
2 Lines	FOLFIRI+ Bevacizumab	FOLFIRI+ Bevacizumab	XELIRI+ cetuximab	FOLFIRI+ Bevacizumab	FOLFIRI+ Bevacizumab	FOLFIRI+ Bevacizumab	FOLFIRI+ Bevacizumab	FOLFIRI+ Bevacizumab
3 lines	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab	Fruquintinib camrelizumab
Circles	12	23	15	6	7	13	14	16
Best Response	PR	PR	PR	PR	SD	SD	PR	PR
PFS	12M+	21M+	17M+	7M+	7M+	14M+	15M+	18M+

Description: The deadline for statistics is August 2022.
Patient 2 in the table is the case presentation patient in the article

TABLE 2 Baseline blood routine and biochemical indicators.

Items	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Gender	Female	Female	Female	Female	Male	Female	Female	Female
Age (years)	65	60	68	76	54	57	55	61
3 lines Start time	2021.8	2020.11	2021.3	2022.1	2022.1	2021.6	2021.5	2021.2
WBC X10^9/L	4.26	8.37	4.05	6.25	8.37	3.64	4.41	4.55
RBC X10^12/L	3.86	4.6	3.84	4.16	4.63	4.28	3.36	3.89
HGB g/L	125.9	145.8	137	136	139	135	109	115

(Continued)

TABLE 2 Continued

Items	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
Gender	Female	Female	Female	Female	Male	Female	Female	Female
NE X10^9/L	2.71	4.5	2.18	2.93	4.52	1.79	2.75	2.95
PLT X10^9/L	154	244	165	124	168	259	259	230
LY X10^9/L	1.11	2.75	1.5	2.59	2.19	1.24	1.29	1.93
NLR(NE/LY)	2.44	1.63	1.45	1.13	2.06	1.44	2.13	1.52
PLR(PLT/LY)	138.73	88.72	110	109.7	76.71	215.8	200.77	119.17
ALT U/L	17.1	20.4	21.3	24	23.6	21.4	17.7	23
AST U/L	22	22.7	24.9	28	24	23.2	21.3	25
ALB g/L	41.8	39.5	42.5	39.8	40.1	43.4	42.2	39
CREA umol/L	58.7	54.5	48.2	80	83.7	50.5	44	51
LDH U/L	188	155	176	146.5	236	164	143	156

Conclusions

Sample population selection is also critical when formulating treatment regimens. Our case suggests that MSS colorectal extrahepatic (non-liver) metastases as the dominant population for immunotherapy combined with multi-target tyrosine kinase inhibitors. Therefore, the combined antivascular immunotherapy is promising, and we can look forward to a new treatment landscape for MSS CRC in the future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration (as revised in 2013). This study was conducted with Fourth Hospital of Hebei Medical University Research Ethics Board approval (2021KS005). Written informed consent was obtained from the patient's family for publication of this case report and accompanying images. A copy of the written consent is available for review by the editorial office of this journal.

Author contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Supplementary material

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A single-arm phase II clinical trial of anlotinib combined with chemotherapy for the treatment of metastatic triple-negative breast cancer

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Background: Anlotinib is a novel oral small-molecule tyrosine kinase inhibitor (TKI), which can inhibit angiogenesis. The purpose of this study was to evaluate the efficacy and safety of anlotinib combined with chemotherapy in patients with metastatic triple-negative breast cancer (TNBC).

Methods: This phase II clinical trial included 40 patients with metastatic TNBC who had previously received anthracycline and/or taxane treatment. All patients received anlotinib combined with chemotherapy. The primary endpoint was progression-free survival (PFS). The secondary endpoints included overall survival (OS), objective response rate (ORR), clinical benefit rate (CBR), disease control rate (DCR) and safety.

Results: During May 1, 2019 and April 30, 2022, there were 40 patients enrolled in this study. The median PFS and median OS were 8.8 months (95% confidence interval [CI] 6.5-11.1 months) and 19.0 months (95% CI, 12.1–25.9 months), respectively. The ORR, CBR and DCR were 40.0% (16/40), 85.0% (34/40) and 95.0% (38/40), respectively. Cox univariate and multivariate analyses demonstrated that having more than 3 metastatic sites (p = 0.001; p = 0.020) was an independent and meaningful unfavorable prognostic factor for PFS. 37.5% of patients had grade 3 to 4 treatment-related adverse events (TRAEs). The grade 3 to 4 TRAEs included neutropenia (22.5%), leukopenia (20.0%), secondary hypertension (10.0%), hand-foot syndrome (5.0%), vomiting (5.0%), proteinuria (5.0%) and thrombocytopenia (2.5%). None of the patients withdrew from the study or died due to TRAEs.

Conclusion: In this single-arm study, the treatment of metastatic TNBC with anlotinib combined with chemotherapy showed certain efficacy, and its toxicity was acceptable.

KEYWORDS

anlotinib, angiogenesis, chemotherapy, triple-negative breast cancer (TNBC), tyrosine kinase inhibitor (TKI)

1 Introduction

Among women, breast cancer is the cancer with the highest incidence rate worldwide at present, and it is also one of the main causes of cancer death. The 2020 global cancer statistics showed that there were about 2.26 million women were newly diagnosed with breast cancer, and 684,996 women died of breast cancer (1). In China, breast cancer is also the most common diagnosed cancer in females, with 429,105 new cases per year and 124,002 deaths (2). Despite advances in cancer treatment, 20% to 30% of early breast cancer patients will still relapse or metastasize (3). The median overall survival (OS) period of metastatic breast cancer is generally only 2 to 3 years (4).

Triple-negative breast cancer (TNBC) is defined as the absence of estrogen receptor (ER) and progesterone receptor (PR) expression and non-amplified human epidermal growth factor receptor 2 (HER2) expression; it accounts for about 12-20% of all invasive breast cancers (5–7). TNBC has a poor clinical prognosis, and has the characteristics of highly heterogeneous, strong invasion and high degree of malignancy. It is prone to recurrence and metastasis. The most important systemic treatment of TNBC is chemotherapy, however, the effective rate of chemotherapy alone is unsatisfactory.

Angiogenesis is a key factor in the processes of growth, invasion and metastasis of malignant tumors (8). Therefore, antitumor angiogenesis strategies can be used as an effective means to treat cancer (9, 10). Anti-angiogenic drugs mainly include antibodies and small-molecule tyrosine kinase inhibitors (TKIs). Bevacizumab (a macromolecular monoclonal antibody) can block tumor angiogenesis, which has been shown to be effective in metastatic breast cancer (11–14). Sorafenib, sunitinib and apatinib are anti-angiogenic TKIs that are mainly used to treat advanced liver cancer, metastatic renal cell carcinoma, metastatic gastric cancer, etc. (15–17). In terms of metastatic breast cancer, some clinical studies have also been carried out on anti-angiogenic TKI drugs. Sorafenib monotherapy could not prolong progression-free survival (PFS) in advanced breast cancer (18, 19). However, sorafenib combined with capecitabine could improve PFS in patients with HER2-negative advanced breast cancer (20). Sunitinib has a serious adverse event (AE) in the treatment of advanced breast cancer, so its application is limited (21). Apatinib monotherapy or combined with chemotherapy has shown efficacy in metastatic breast cancer (22–24).

Anlotinib is a novel oral anti-angiogenic TKI that blocking vascular endothelial growth factor receptor (VEGFR)1-3, fibroblast growth factor receptor (FGFR)1-4, platelet-derived growth factor (PDGFR)-α, PDGFR-β, and stem cell factor receptors, which inhibit tumor angiogenesis and tumor cell proliferation (25, 26). Many clinical studies have shown that anlotinib has encouraging efficacy and controllable toxicity in some solid tumors, such as non-small cell lung cancer, soft tissue sarcoma, small cell lung cancer, and medullary thyroid cancer (27–30). Preclinical studies have shown that anlotinib can inhibit the proliferation of breast cancer cells (31, 32). Anlotinib combined with TQB2450 (a humanized monoclonal antibody targeting programmed death-ligand 1) showed an acceptable safety profile and promising activity in advanced TNBC patients who were previously treated with anthracyclines and/or taxanes (33). A phase II clinical trial shows that anlotinib alone is effective for advanced breast cancer (34). A real-world study shows that single or combined treatment of anlotinib is effective for heavily pretreated HER2 negative metastatic breast cancer, with low toxicity (35).. These studies showed that anlotinib is effective in metastatic breast cancer, especially in HER2-negative subtypes or TNBC. However, to date, there is no prospective clinical study on the treatment of TNBC with anlotinib combined with chemotherapy. The purpose of this study was to evaluate the efficacy and safety of anlotinib in combination with chemotherapy of the physician's choice in pretreated patients with metastatic TNBC. To our knowledge, this should be the first prospective report on the results of metastatic TNBC treated with anlotinib combined with chemotherapy.

2 Materials and methods

2.1 Patients

The current prospective study enrolled 40 Chinese female patients with pretreated metastatic TNBC who received anlotinib combined with chemotherapy at the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College during May 1, 2019 and April 30, 2022.

Eligible patients had to meet the following criteria (1): female patients ≥18 years old; (2) histologically confirmed TNBC (defined as ER negative and PR negative on immunohistochemistry [IHC] and negative HER2 status, defined as 0 or 1+ based on IHC; patients with HER2 2+ by IHC were subjected to a fluorescence *in situ* hybridization (FISH) test for the HER2 gene and the result was non-amplification) for the primary or metastatic lesion; (3) presence of at least one measurable metastatic lesion; (4) performance score of 0-1 according to the Eastern Cooperative Oncology Group (ECOG) scoring criteria; (5) relapsed or failed after previous anthracycline and/or taxane treatment in the (neo)adjuvant or metastatic setting; and (6) adequate organ function (mainly including liver function, kidney function, heart function, lung function, etc.). The exclusion criteria were as follows: (1) other malignant tumors have been diagnosed in the past 5 years; (2) abnormal laboratory test results or organ dysfunction; and (3) previously received treatment with anlotinib.

This study involving human participants was reviewed and approved by the institutional review boards and ethics committees (ethical code: 2019-33-2) of the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College and was conducted in accordance with the Declaration of Helsinki. All patients signed the written informed consent to participate in this study.

2.2 Study design and treatment

All patients participating in this study were treated with anlotinib (8 mg orally once daily) and a chemotherapeutic agent (oral vinorelbine, orally on days 1 and 8 of each 21-day cycle, with doses of 60 mg/m^2 for the first cycle and 80 mg/m^2 for the subsequent cycles; or albumin bound paclitaxel, 260 mg/m^2 intravenously on day 1 of each 21-day cycle; or gemcitabine, 1000 mg/m^2 intravenously on days 1 and 8 of each 21-day cycle; or eribulin, 1.4 mg/m^2 intravenously on days 1 and 8 of each 21-day cycle; or capecitabine, 1000 mg/m^2 orally twice daily on days 1 to 14 of each 21-day cycle; or oxaliplatin, 130 mg/m^2 intravenously on day 1 of each 21-day cycle; or docetaxel, 75 mg/m^2 intravenously on day 1 of each 21-day cycle).

The patients were followed up until October 31, 2022. At baseline and every two cycles (every 6 weeks) during treatment, tumor evaluation was conducted for evaluable lesions through computed tomography (CT) or magnetic resonance imaging (MRI).

The primary endpoint was progression-free survival (PFS), defined as the time from the start of oral anlotinib treatment to objective tumor progression or death from any cause, whichever occurred first. The secondary endpoints included OS (defined as the time from the start of treatment to the date of mortality from any cause), overall response rate (ORR, defined as the proportion of patients who achieved a confirmed complete response or confirmed partial response), clinical benefit rate (CBR, defined as the proportion of patients who achieved a confirmed complete response or confirmed partial response or stable disease for ≥ 24 weeks), disease control rate (DCR, defined as the proportion of patients who achieved a confirmed complete response or confirmed partial response or stable disease for ≥4 weeks), and safety. It should be noted that confirmed complete response/partial response were defined as complete response/partial response in at least 2 continuous tumor evaluation. The efficacy was evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, while the safety was assessed in accordance with the Common Terminology Criteria for Adverse Events (CTCAE), version 5.0.

2.3 Statistical analyses

All data were statistically analyzed using SPSS software (IBM Corp., Armonk, NY, USA; version 26.0) and GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA). PFS and OS of patients were estimated by the Kaplan-Meier method. In addition, univariate and multivariate analyses were used to determine the impact of variables on PFS and OS by the Cox proportional hazards regression model. P-value < 0.05 was considered as statistically significant.

3 Results

3.1 Clinical characteristics

Forty patients with metastatic TNBC participated in this study. The baseline characteristics of patients are presented in Table 1. The median age at enrollment in the clinical study of the patients was 50 years (range from 26 to 72 years), and all patients were female. Sixteen patients (40.0%) had an ECOG performance status score of 0, and 24 patients (60.0%) had an ECOG performance status score of 1. Furthermore, 16 patients (40.0%) had grade I–II tumor histology, 24 (60.0%) had grade III tumor histology. A total of 23 patients (57.5%) had stage I–II disease at initial diagnosis, 17 patients (42.5%) had stage III-IV disease at initial diagnosis. Twenty-nine patients (72.5%) received one or two lines of treatment, and 11 patients (27.5%) received ≥ 3-line treatment. The majority of patients (30, 75.0%) had visceral metastasis, and 17 patients (42.5%) had more than 3 metastatic sites. All patients had received treatment with anthracycline and/or taxane before enrollment. In this study, the combined chemotherapeutic agents included oral vinorelbine (12, 30.0%), albumin bound paclitaxel (11, 27.5%), gemcitabine (9, 22.5%), eribulin (4, 10.0%), capecitabine (2, 5.0%), oxaliplatin (1, 2.5%) and docetaxel (1, 2.5%).

3.2 Efficacy

The patients were followed up until October 31, 2022, and the median follow-up time was 12.6 months (range from 3.0 to 36.8 months). At the end of the follow-up, 31 patients discontinued the study treatment due to disease progression, no patients stopped the treatment permanently due to toxicity, 22 patients died from the disease progression, and no death caused by other reasons. As demonstrated in Figure 1A and Table 2, the median PFS was 8.8 months (95% CI, 6.5–11.1 months), and 9 patients still did not have disease progression at the last follow-up. The median OS was 19.0 months (95% CI, 12.1–25.9 months), and 18 patients were still alive to the end of follow-up (Figure 1B, Table 2).

Among 40 patients, a total of 16 achieved PR as the best response, with an ORR of 40.0%. Thirty-eight patients achieved PR or SD, with a DCR of 95.0%. Additionally, 34 patients achieved PR or SD for more than 24 weeks, so the CBR of this study was 85.0%. None of the patients achieved CR (Table 2).

As presented in Table 3 and Figure 2, univariate analysis of a total of 40 patients showed that ECOG performance status score of 1 (p = 0.039), stage III–IV disease at diagnosis (p = 0.048), received third-line or above treatment (p = 0.001), had more than 3 metastatic sites (p = 0.001), and had liver metastasis (p = 0.004) may exhibit a higher risk of disease progression.

The univariate analysis (Table 3) indicated that the higher risk variables for death were as follows: ECOG performance status score of 1 (p = 0.005), stage III–IV disease at diagnosis (p = 0.008), third-line or above treatment (p = 0.002), more than 3 metastatic sites (p = 0.004), liver metastasis (p = 0.002), and brain metastasis (p = 0.048). The PFS, OS and corresponding 95% CIs for these factors that were statistical significant in univariate analysis are shown in Table 4.

In addition, multivariate Cox analysis of variables with statistical significance in univariate analysis was conducted (Table 5). We carried out multivariate analysis on 5 factors influencing PFS in univariate analysis and found that having more than 3 metastatic sites (HR, 3.030; 95% CI, 1.193 to 7.692; p

TABLE 1 Patient characteristics at baseline.

Characteristic	n (%)
Age of enrollment, years	
<50	20 (50.0)
≥50	20 (50.0)
Location	
Left	20 (50.0)
Right	20 (50.0)
ECOG performance status	
0	16 (40.0)
1	24 (60.0)
Histopathologic grade	
I-II	16 (40.0)
III	24 (60.0)
TNM stage at initial diagnosis	
I-II	23 (57.5)
III-IV	17 (42.5)
DFS duration, months	
≤24	27 (67.5)
>24	13 (32.5)
Lines of treatment, lines	
<3	29 (72.5)
≥3	11 (27.5)
Type of metastatic site	
Non-visceral	10 (25.0)
Visceral	30 (75.0)
Metastatic sites	
Liver	11 (27.5)
Lung	24 (60.0)
Bone	20 (50.0)
Brain	8 (20.0)
Number of metastatic sites, n	
≤3	23 (57.5)
>3	17 (42.5)
Previous chemotherapy	
Anthracycline	36 (90.0)
Taxane	39 (97.5)
Anthracycline or Taxane	40 (100.0)
Combined chemotherapeutic drug	
Oral vinorelbine	12 (30.0)
Albumin bound paclitaxel	11 (27.5)
Gemcitabine	9 (22.5)
Eribulin	4 (10.0)
Capecitabine	2 (5.0)
Oxaliplatin	1 (2.5)
Docetaxel	1 (2.5)

ECOG, Eastern Cooperative Oncology Group; TNM stage, the stage of tumor, node and metastasis; DFS, disease free survival.

= 0.020) was an independent and meaningful unfavorable prognostic factor for PFS. The median PFS times were 6.3 months (95% CI, 4.1 to 8.5 months) in the subgroup with more than 3 metastatic sites and 11.9 months (95% CI, 9.9 to 13.9 months) in the subgroup with 1-3 metastatic site(s) (Figure 3). Multivariate analysis showed that there were no significant unfavorable prognostic factors for OS.

3.3 Safety

Table 6 summarizes the treatment-related adverse events (TRAEs) that occurred in our study, including all grades. Among all 40 patients with toxicity records, 97.5% of patients (n=39) developed TRAEs of varying degrees and the incidence of grade 3-4 TRAEs was 37.5%. The non-hematological TRAEs included hand-foot syndrome (47.5%), secondary hypertension (45.0%), vomiting (40.0%), fatigue (40.0%), proteinuria (37.5%), diarrhea (37.5%), nausea (35.0%), oral mucositis (20.0%) and hemorrhage (5.0%). The hematological TRAEs were leukopenia (75.0%), neutropenia (70.0%), aspartate aminotransferase increase (20.0%), alanine aminotransferase increase (17.5%), thrombocytopenia (15.0%), hypertriglyceridemia (15.0%), anemia (12.5%) and hypercholesterolemia (12.5%). In addition, Grade 3-4 TRAEs were neutropenia (22.5%), leukopenia (20.0%), secondary hypertension (10.0%), hand-foot syndrome (5.0%), vomiting (5.0%), proteinuria (5.0%) and thrombocytopenia (2.5%). Most TRAEs were limited to patients with Grade 1-2 and were therefore tolerable and manageable. Two patients stopped taking anlotinib for 3 to 7 days due to grade 3 hand-foot syndrome and were able to continue taking anlotinib orally in subsequent cycles and tolerated the treatment well. None of the patients withdrew from the study because of treatment-related toxicity, and no deaths due to TRAEs occurred.

4 Discussion

As we know, this study should be the first prospective study to explore the activity and safety of anlotinib combined with chemotherapy in patients with metastatic TNBC. In this study, the median PFS of all 40 patients was 8.8 months (95% CI, 6.5–11.1 months), while the median OS was 19.0 months (95% CI, 11.8–26.2

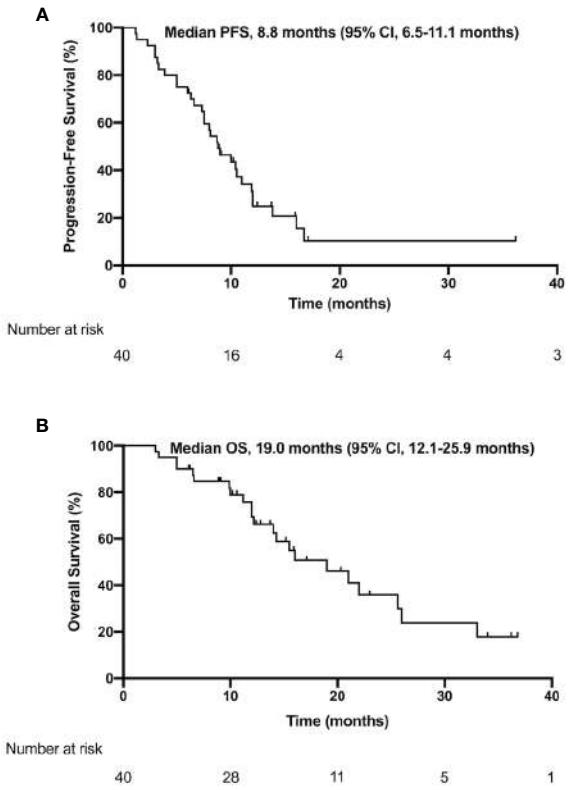

FIGURE 1
Kaplan–Meier estimates of PFS **(A)** and OS **(B)** in the patients with metastatic triple negative breast cancer who received anlotinib and chemotherapy. PFS, progression-free survival; OS, overall survival; CI, confidence interval.

months). In addition, the ORR was 40.0% (16/40), the DCR was 95.0% (38/40) and the CBR was 85% (34/40). These results indicated that the combination of anlotinib and chemotherapy has good activity in the treatment of metastatic TNBC.

TABLE 2 Efficacy outcomes (N=40).

Endpoint	
Primary endpoint	
Median progression-free survival, months (95% CI)	8.8 (6.5-11.1)
Secondary endpoints and other best clinical response	
Median overall survival, months (95% CI)	19.0 (12.1-25.9)
Complete response, no. (%)	0 (0)
Partial response, no. (%)	16 (40.0)
Stable disease, no. (%)	22 (55.0)
Disease progression, no. (%)	2 (5.0)
Objective response rate, no. (%)	16 (40.0)
Clinical benefit rate, no. (%)	34 (85.0)
Disease control rate, no. (%)	38 (95.0)

CI, confidence interval.

Chemotherapy is very important for controlling the disease progression of patients with metastatic TNBC. The median PFS of first-line combined chemotherapy was between 5.5 months and 9.8 months (36–38). However, the efficacy is worse in patients with heavily pretreated metastatic TNBC, and a study showed that capecitabine combined with cisplatin in pretreated metastatic TNBC had a PFS of 3.68 months (39). 304 Study showed eribulin or vinorelbine were used as a multi-line treatment for patients with advanced breast cancer, the PFS was only 2.8 months (40). Therefore, the efficacy of chemotherapy alone (whether a combination of two drugs or a single-drug regimen) in the treatment of advanced TNBC is limited. In recent years, with the application of immunotherapy or PARP inhibitors combined with chemotherapy, the treatment efficacy of metastatic TNBC has been improved (41). Currently, patients with metastatic TNBC still have fewer treatment options than patients with other subtypes of breast cancer.

Anti-angiogenic drugs have shown certain efficacy in the treatment of some solid tumors. Studies have shown that the median PFS of bevacizumab, sorafenib, sunitinib and apatinib monotherapy for the treatment of advanced breast cancer was 2.0–4.0 months, and the ORR was 0%–16.7% (15, 19, 20, 22–24). In previous clinical studies, anlotinib monotherapy was also proven to be effective in the multi-line treatment of advanced breast cancer. Hu et al. (34) reported a phase II study of anlotinib monotherapy in pretreated HER2-negative metastatic breast cancer. Following the results, the median PFS of the population was 5.22 months, and the ORR was 15.38%. In subgroup analysis, the median PFS of TNBC patients was 4.04 months. It seems that the PFS of anlotinib monotherapy is longer than that of other anti-angiogenic drug monotherapies, but the efficacy of all anti-angiogenic drug monotherapies is still very limited. Anti-angiogenic drugs combined with chemotherapy may improve the effect of antitumor treatment in advanced breast cancer. The ORRs of bevacizumab, sorafenib, sunitinib and apatinib for the treatment of advanced breast cancer were significantly increased to 23.2%–51.3% after combination with chemotherapy, with median PFS of 4.4-11.8 months, and the result was better than monotherapy (12–14, 21, 25). In a real-world study of anlotinib monotherapy or combined with chemotherapy in multi-line therapy in patients with advanced breast cancer, the median PFS of monotherapy was 3.0 months, and that of combined treatment was 5.5 months. In subgroup analysis, the median PFS of TNBC patients was 3.5 months (35). In our study, the median PFS of TNBC patients who had previously received at least two lines of treatment in the metastatic setting was 5.0 months, which was longer than that of patients with metastatic TNBC reported by Hu et al. (4.04 months) (34) and Shao et al. (3.5 months) (35). In addition, the median PFS of anlotinib combined with chemotherapy as first-line or second-line treatment was 10.5 months, indicating that it was better than the existing reports on metastatic TNBC. Although these findings come from different study populations and evaluations, with consistent findings in metastatic TNBC, the combination of anlotinib and chemotherapy has good antitumor activity for early- or late-line treatment.

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TABLE 3 Cox univariate regression analysis for progression-free survival and overall survival.

	Progression-free survival			Overall survival		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
Age of enrollment, years (<50 vs. ≥50)	0.912	0.447-1.860	0.800	1.111	0.475-2.598	0.809
ECOG performance status (0 vs. 1)	2.231	1.042-4.776	**0.039**	5.928	1.702-20.647	**0.005**
Location (left vs. right)	1.877	0.915-3.849	0.086	1.960	0.818-4.699	0.131
Histopathologic grade (I-II vs. III)	1.155	0.543-2.460	0.708	0.678	0.278-1.655	0.394
TNM stage at initial diagnosis (I-II vs. III-IV)	2.062	1.007-4.222	**0.048**	3.509	1.395-8.829	**0.008**
DFS duration, months (≤24 vs. >24)	1.118	0.500-2.502	0.786	1.331	0.498-3.556	0.569
Lines of treatment, lines (≤2 vs. >2)	3.614	1.673-7.807	**0.001**	4.802	1.784-12.925	**0.002**
Number of metastatic sites (≤3 vs. >3)	4.074	1.845-8.993	**0.001**	3.934	1.552-9.970	**0.004**
Visceral metastasis (no vs. yes)	2.201	0.844-5.739	0.107	1.217	0.406-3.642	0.726
Liver metastasis (no vs. yes)	3.031	1.412-6.507	**0.004**	4.314	1.731-10.756	**0.002**
Lung metastasis (no vs. yes)	1.325	0.634-2.771	0.455	0.703	0.293-1.691	0.432
Bone metastasis (no vs. yes)	1.220	0.599-2.486	0.584	1.714	0.723-4.061	0.221
Brain metastasis (no vs. yes)	1.795	0.763-4.223	0.180	2.548	1.006-6.453	**0.048**

HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; TNM stage, the stage of tumor, node and metastasis; DFS, disease free survival.
Bold values indicate a p-value of < 0.05.

At the 2022 ASCO Annual Meeting, Liu et al. (42) reported a prospective clinical trial study of eribulin versus eribulin plus anlotinib in the treatment of patients with advanced or metastatic breast cancer. According to the published abstract results, the median PFS of patients with advanced TNBC treated with anlotinib plus eribulin reached 9.7 months. In addition, Yin et al. (43) also reported a single-arm phase II clinical study on the treatment of metastatic HER2 negative breast cancer with anlotinib and eribulin. However, the median PFS of this study was only 4.7 months. In our study, the median PFS of anlotinib combined with chemotherapy was 8.8 months, and the median PFS in the third-line treatment or above setting was 5.0 months. There were 11 patients with the third-line or beyond treatment (including 3 third-line patients, 1 fourth-line patient, 3 fifth-line patients, 3 sixth-line patients, and 1 tenth-line patient; all patients had visceral metastasis). Therefore, our study shows that anlotinib combined with chemotherapy has potential efficacy for TNBC patients were heavily pretreated and with visceral metastasis.

In our study, the most common TRAEs were leukopenia, neutropenia, hand-foot syndrome, secondary hypertension, vomiting, fatigue, proteinuria, etc. Among them, hematological toxicity and gastrointestinal reactions were mainly caused by chemotherapy drugs, while hand-foot syndrome, secondary hypertension and proteinuria were mainly caused by the anlotinib. The majority of TRAEs in patients receiving anlotinib combined with chemotherapy were grades 1-2, and the incidence

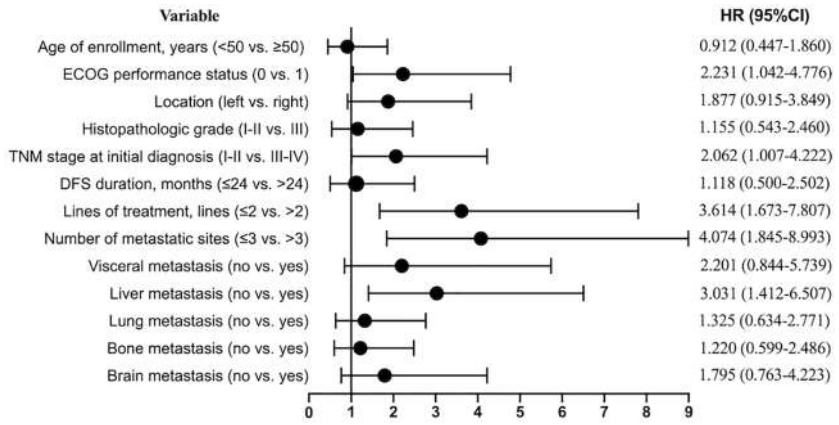

FIGURE 2
Forest plot of cox univariate regression analysis for progression-free survival. HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; TNM stage, the stage of tumor, node and metastasis; DFS, disease free survival.

TABLE 4 PFS and OS for subgroup analysis.

Characteristic	PFS (95% CI)	OS (95% CI)
ECOG performance status		
0	12.0 (11.0-13.0)	NE (NE-NE)
1	7.5 (3.8-11.2)	14.0 (9.1-18.9)
TNM stage at initial diagnosis		
I-II	11.0 (8.1-13.9)	33.0 (12.0-54.0)
III-IV	7.5 (3.7-11.3)	12.2 (9.6-14.8)
Lines of treatment, lines		
≤2	10.5 (9.0-12.0)	25.6 (18.2-33.0)
>2	5.0 (1.4-8.6)	12.0 (10.0-14.0)
Number of metastatic sites		
≤3	11.9 (9.9-13.9)	26.0 (12.4-40.0)
>3	6.3 (4.1-8.5)	12.0 (10.7-13.3)
Liver metastasis		
No	10.5 (9.0-12.0)	25.6 (18.7-32.5)
Yes	6.0 (2.7-9.3)	12.0 (10.1-13.9)
Brain metastasis		
No	10.0 (7.1-12.9)	21.0 (14.2-27.8)
Yes	6.3 (2.1-10.5)	12.0 (8.1-15.9)

PFS, progression-free survival; OS, overall survival; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; TNM stage, the stage of tumor, node and metastasis; NE, not evaulated.

was similar to that in previous relevant clinical trials (34, 35). Anti-angiogenic drugs are likely to increase the probability of hemorrhage. In our study, two patients suffered from hemorrhage, one from gum bleeding, and the other from chest wall tumor bleeding, both of which were very mild, without causing massive bleeding or anemia. No serious bleeding events were observed in the whole study, such as hemoptysis, gastrointestinal bleeding, hematuria and intracranial hemorrhage. RIBBON-2 trial showed that bevacizumab combined with chemotherapy could significantly prolong PFS of second-line treatment for patients with advanced breast cancer, but could not improve OS (13). In terms of safety, the AEs in the bevacizumab group that led to the discontinuation of the study were more than those in the placebo group (13.3% versus 7.2%), but in fact there is no difference in the number of treatment-related deaths between the two groups (6 patients in the bevacizumab group versus 5 patients in the placebo group) (13). In the subgroup analysis of TNBC, compared with placebo group, bevacizumab group could prolong PFS (6.0 months versus 2.7 months) and there is a trend to improve OS (17.9 months versus 12.6 months) (44). Two patients in both groups have treatment-related deaths (2% in bevacizumab group versus 4% in placebo group) (44). Similarly, in our study, anlotinib combined with chemotherapy showed potential efficacy and good tolerance. Only two patients (5%) temporarily stopped taking anlotinib due to

TABLE 5 Cox multivariate regression analysis for progression-free survival and overall survival.

	Progression-free survival			Overall survival		
Variable	HR	95% CI	P-value	HR	95% CI	P-value
ECOG performance status (0 vs. 1)	1.158	0.412-3.256	0.781	2.251	0.360-14.072	0.386
TNM stage at initial diagnosis (I-II vs. III-IV)	1.376	0.467-4.053	0.562	1.657	0.366-7.494	0.512
Lines of treatment, lines (≤2 vs. >2)	1.962	0.733-5.250	0.180	1.667	0.444-6.249	0.449
Number of metastatic sites (≤3 vs. >3)	3.030	1.193-7.692	**0.020**	1.993	0.615-6.464	0.250
Liver metastasis (no vs. yes)	1.094	0.349-3.426	0.877	1.414	0.411-4.864	0.583
Brain metastasis (no vs. yes)	–	–	–	1.395	0.440-4.427	0.572

HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; TNM stage, the stage of tumor, node and metastasis.
Bold values indicate a p-value of < 0.05.

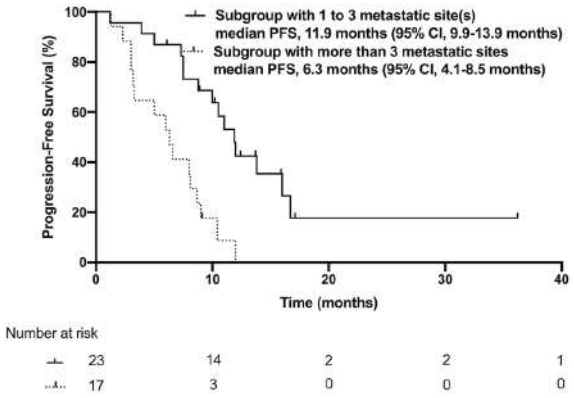

FIGURE 3
Kaplan–Meier estimates of progression-free survival in the subgroup with 1 to 3 metastatic site(s) or with more than 3 metastatic sites. PFS, progression-free survival; CI, confidence interval.

grade 3 hand-foot syndrome (after active supportive treatment, their symptoms are relieved and they continued to take anlotinib orally), and there were no treatment-related deaths.

The current study is a small sample phase II clinical study, which from a single center in China. The limitation of this study is that it only enrolled a small number of patients and lacked a standard control group. However, anlotinib combined with chemotherapy is still a potential and effective alternative for patients with metastatic TNBC. We look forward to the conduction of more multicenter randomized controlled trials can be conducted in a larger cohort to further verify the efficacy and safety of anlotinib combined with chemotherapy.

5 Conclusions

In summary, the findings of this single-arm clinical trial showed that anlotinib combined with chemotherapy appeared to be

TABLE 6 Treatment-related adverse events.

Adverse events	All grade, n (%)	≥ Grade 3, n (%)
Non-hematologic		
Hand-foot syndrome	19 (47.5)	2 (5.0)
Secondary hypertension	18 (45.0)	4 (10.0)
Vomiting	16 (40.0)	2 (5.0)
Fatigue	16 (40.0)	0 (0)
Proteinuria	15 (37.5)	2 (5.0)
Diarrhea	15 (37.5)	0 (0)
Nausea	14 (35.0)	0 (0)
Oral mucositis	8 (20.0)	0 (0)
Hemorrhage	2 (5.0)	0 (0)
Hematologic		
Leukopenia	30 (75.0)	8 (20.0)
Neutropenia	28 (70.0)	9 (22.5)
Aspartate aminotransferase increase	8 (20.0)	0 (0)
Alanine aminotransferase increase	7 (17.5)	0 (0)
Thrombocytopenia	6 (15.0)	1 (2.5)
Hypertriglyceridemia	6 (15.0)	0 (0)
Anemia	5 (12.5)	0 (0)
Hypercholesterolemia	5 (12.5)	0 (0)

efficacious for metastatic TNBC, with acceptable toxicity. It provides a potential and effective alternative for patients with metastatic TNBC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the institutional review boards and ethics committees (ethical code: 2019-33-2) of the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College and was conducted in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by J-YH, X-FX, X-LC, Q-YZ, L-PC and XB. Writing-reviewing and editing were performed by J-YH, X-FL, LS and J-FG. The first draft of the manuscript was written by J-YH and C-WD edited it critically. All authors reviewed the results and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The multikinase inhibitor axitinib in the treatment of advanced hepatocellular carcinoma: the current clinical applications and the molecular mechanisms

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Advanced hepatocellular carcinoma (HCC) is a formidable public health problem with limited curable treatment options. Axitinib, an oral tyrosine kinase inhibitor, is a potent and selective second-generation inhibitor of vascular endothelial growth factor receptor (VEGFR) 1, 2, and 3. This anti-angiogenic drug was found to have promising activity in various solid tumors, including advanced HCC. At present, however, there is no relevant review article that summarizes the exact roles of axitinib in advanced HCC. In this review, 24 eligible studies (seven studies in the ClinicalTrials, eight experimental studies, and nine clinical trials) were included for further evaluation. The included randomized or single-arm phase II trials indicated that axitinib could not prolong the overall survival compared to the placebo for the treatment of advanced HCC, but improvements in progression free survival and time to tumor progression were observed. Experimental studies showed that the biochemical effects of axitinib in HCC might be regulated by its associated genes and affected signaling cascades (e.g. VEGFR2/PAK1, CYP1A2, CaMKII/ERK, Akt/mTor, and miR-509-3p/PDGFRA). FDA approved sorafenib combined with nivolumab (an inhibitor of PD-1/PD-L1) as the first line regimen for the treatment of advanced HCC. Since both axitinib and sorafenib are tyrosine kinase inhibitors as well as the VEGFR inhibitors, axitinib combined with anti-PDL-1/PD-1 antibodies may also exhibit tremendous potential in anti-tumoral effects for advanced HCC. The present review highlights the current clinical applications and the molecular mechanisms of axitinib in advanced HCC. To move toward clinical applications by combining axitinib and other treatments in advanced HCC, more studies are still warranted in the near future.

KEYWORDS

axitinib, hepatocellular carcinoma, survival, mechanism, tyrosine kinase

Background

Liver cancer, a hypervascular tumor, ranks as the 6th most common malignancy worldwide (1). Besides, it is the third most common cause of cancer-associated mortalities (1). According to the Cancer statistics 2023 on the category of liver cancer, it is predicted to have 41,210 new cases and 29,380 deaths in the United States (2). Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer, comprising over 80% of cases. HCC is prevalent in Eastern Asia and Africa, where mortality and incidence are highest (3). In patients with distant metastases of HCC, the 5-year survival rate is only 2.4%. There are several factors that contribute to the development of HCC, including viral infections (the main cause), alcohol-related liver cirrhosis, genetic mutations, diabetes mellitus, obesity, smoking, exposure to chemical carcinogens, and non-alcoholic steatohepatitis, etc (4, 5). It frequently occurs in patients with chronic liver disease. Several factors play an important role in the pathophysiology of HCC, these include but are not limited to genetic predisposition, cellular microenvironment, and immune cells (6).

For early-stage HCC patients, resection, transplantation, and local ablation are the preferred treatments. In patients with intermediate stages of the disease, transarterial chemoembolization (TACE), local ablation treatments (i.e., radiofrequency ablation), and radiotherapy (RT) techniques are recommended, while those with advanced disease should first receive systemic treatments (5). Multiple randomized studies demonstrated that a better survival rate was observed in patients treated with TACE versus those treated only symptomatically (7). In the case of advanced or unresectable HCC, systemic therapies are an effective treatment modality. Specifically, sorafenib is a first-line systemic treatment for unresectable HCC (8). In recent years, immunotherapy (i.e., ramucirumab, nivolumab, and pembrolizumab) also play an important role in treating patients with advanced HCC who failed the treatment of sorafenib (9).

Axitinib is a selective tyrosine kinase inhibitor (TKI) of vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 (10). This anti-angiogenic drug was found to have promising activity in various solid tumors. Axitinib has been approved for the treatment of advanced renal cell carcinoma (RCC) after prior systemic therapies have failed (11). According to mounting evidence, angiogenesis is confirmed to contribute to the pathogenesis and progression of HCC *via* several signal pathways, e.g. VEGF/VEGF receptor (VEGFR) signalling (12). Since both HCC and RCC are hypervascular cancers that can be effectively controlled by angiogenesis inhibitors, axitinib has been studied as a second-line treatment option in advanced HCC after sorafenib failed (13).

At present, numerous phase I/II clinical trials on axitinib have been completed and some of them have been published. Nevertheless, there is still no relevant review for summarizing all the clinical and experimental evidence of the effects of axitinib on advanced-stage HCC. Therefore, we conducted this study, which might better illustrate the status of axitinib in treating advanced HCC.

Overview of axitinib

Axitinib, known as AG 013736 (Inlyta; Pfizer Inc, New York, New York), an oral tyrosine kinase inhibitor, is a potent and selective second-generation inhibitor of VEGFR1, 2, and 3 (14). As engagement of these VEGFRs, cell growth and angiogenesis are stimulated, leading to tumor growth. The VEGF/VEGFR pathway plays an essential role in normal vascular development. Besides, it is also associated with the survival, growth and metastasis of tumors (15). Axitinib is a multitarget tyrosine kinase inhibitor that can not only suppress EGFR1/2/3 and the gene cKIT, but also the platelet-derived growth factor receptor (PDGFR) (16). Axitinib is an indazole derivative synthesized by chemical synthesis with a molecular weight of 386,47 Da (17). It can bind to the inactive conformation of the catalytic domain of VEGF receptor tyrosine kinases (RTKs). Taking axitinib orally produces rapid absorption and a maximum plasma concentration within four hours. When taken at therapeutic doses, axitinib has a high protein binding rate exceeding 99%, preferring albumin over other proteins. Metabolism of axitinib occurs predominantly in the liver by CYP3A4/5, but to a lesser extent by CYP1A2, 2C19, and UGT1A1, producing pharmacologically inactive metabolites (17). The majority of axitinib is excreted in the feces as a result of hepatobiliary excretion, while less than 20% is excreted by the kidney. The plasma concentration of axitinib increased significantly in patients with moderate hepatic impairment (Child-Pugh B), but not in patients with mild impairment (Child-Pugh A) (17, 18).

One of the on-target effects of VEGFR-tyrosine kinase inhibitors include an increase in blood pressure. As a result, an increase in diastolic blood pressure > 90 mm Hg was associated with axitinib's effectiveness in solid tumors (19). Higher exposure and diastolic blood pressure were found to be independently correlated with longer PFS as wells as OS and a higher probability of partial response (20).

A starting dose of 5 mg twice daily of axitinib is recommended with continuous daily administration, while dose adjustments are recommended according to individual tolerability. It is recommended to raise the dose to 7 mg after two weeks in patients who have shown good tolerance (e.g. no adverse events > grade 2, no increases in BP > 150/90 mm Hg or introduction of antihypertensive treatment) (17). If adverse events occur, a dose reduction could be necessary followed by reintroduction of treatment.

Axitinib was approved by both American and European agencies in 2012 for the treatment of advanced renal cell carcinoma following one prior systemic therapy failure (21). VEGF inhibitors (e.g., bevacizumab) and VEGFR inhibitors (e.g., sorafenib, sunitinib, pazopanib, and axitinib) are effective against advanced renal cell carcinoma. They are approval for treating advanced RCC either as monotherapy or in combination with interferon-alpha (bevacizumab) (14). In addition to kidney cancer, axitinib was also found to improve the outcomes in patients with other malignancies, included head and neck tumors, thyroid cancer, breast cancer, non-small-cell lung cancer, pancreatic cancer, melanoma, and HCC (16, 22, 23).

Relevant studies reporting the roles of axitinib in HCC

To identify the eligible studies, we searched several electronic databases, including MEDLINE (PubMed), the Cochrane Library databases, EMBASE (OVID), PsychINFO, SCOPUS, and ISI, from their inception until December 31, 2022. Among the studies included, only those studies reported using English were considered to be eligible. PubMed search keywords with various combinations were as follows: (((((“Axitinib”[Mesh]) OR (AG 013736)) OR (AG013736)) OR (AG-013736)) OR (Inlyta)) AND ((((((((((((((((((“Carcinoma, Hepatocellular”[Mesh]) OR (Carcinomas, Hepatocellular)) OR (Hepatocellular Carcinomas)) OR (Liver Cell Carcinoma, Adult)) OR (Liver Cancer, Adult)) OR (Adult Liver Cancer)) OR (Adult Liver Cancers)) OR (Cancer, Adult Liver)) OR (Cancers, Adult Liver)) OR (Liver Cancers, Adult)) OR (Liver Cell Carcinoma)) OR (Carcinoma, Liver Cell)) OR (Carcinomas, Liver Cell)) OR (Cell Carcinoma, Liver)) OR (Cell Carcinomas, Liver)) OR (Liver Cell Carcinomas)) OR (Hepatocellular Carcinoma)) OR (Hepatoma)) OR (Hepatomas)). In addition, registered studies in the ClinicalTrials (https://clinicaltrials.gov/) were also included for data analyzing.

Studies in the ClinicalTrials

As listed in the page in ClinicalTrials.gov, seven clinical studies have been registered focusing on the safety and the efficacy of Axitinib in treating advanced HCC (Table 1). Six out seven (86%) of these trials have been completed, while one trial was withdrawn. All these studies were either Phase I or Phase II trial. The areas that these trials conducted included Canada, Hong Kong, Chinese Taipei, and Multi-center in multiple countries. The sample size in these studies ranged from 9 to 224 participants. The age among these participants were >18 years. The dosage regimens for Axitinib administration were 5 mg twice daily orally. The treatment of Axitinib continued until progressive disease, intolerable toxicity, or patient withdrawal. The treatment cycle included 4 weeks, 8 weeks, and 3-6 months. The combination therapies with Axitinib, including TACE, radiotherapy, Avelumab, and Crizotinib. The responsible party included Pfizer, National Taiwan University Hospital, Chinese University of Hong Kong, and Shin Kong Wu Ho-Su Memorial Hospital. In the study of NCT01210495 (Phase 2), the outcomes of the clinical trial were non-significant OS [12.7 (10.2 to 14.9) for Axitinib vs 9.7 (5.9 to 11.8) for Placebo, HR=0.907, 95% CI: 0.646-1.274, *P*=0.2872] but a significant median PFS [3.6 (2.3 to 4.6) for Axitinib vs 1.9 (1.9 to 3.5) for Placebo, HR=0.618, 95%CI: 0.438-0.871, *P*=0.0039] as well as a significant Time to Disease Progression (TTP) (HR=0.621, 95%CI: 0.434-0.889, *P*=0.006). In another study of NCT03289533 (Phase 1), the investigators demonstrated that patients received avelumab 10 mg/kg Q2W in combination with axitinib 5 mg BID turned out to be a TTP of 5.52 months (1.91 to 7.39), an objective response of 13.6% (2.9 to 34.9), a disease control of 68.2% (45.1 to 86.1), and a Time to Tumor Response (TTR) of 1.91 (1.9 to 3.7). Five out of seven studies did post the outcomes. Serious adverse events were reported at 46.6% [Axitinib: 62/133 (46.6%) vs Placebo: 16/68 (23.5%)] and36.36% (8/22).

Experimental (preclinical) studies

At present, there were eight experimental studies had reported the molecular biological effects of axitinib in HCC development (Table 2). These included studies were conducted in the USA, China, Australia, Canada, and Singapore. The cell lines used in these studies included various types of HCC cells. It has been shown that axitinib inhibits cellular phosphorylation of VEGFR-2, VEGF-induced endothelial cell survival, tube formation, and vascular permeability in preclinical studies (24). A significant delay in the growth of human xenograft tumors was observed when VEGFR-2 was expressed, suggesting a therapeutic potential for this protein (18, 24). Therefore, inhibition of VEGFR-2, like axitinib, might be effective for treating multi-blood vessel solid tumor (e.g., HCC). In an early preclinical study developed by Ma et al. (25), the author found that axitinib modulated the anti-tumor activity of metronomic cyclophosphamide in multiple ways. Axitinib caused a rapid decrease of blood vessel perfusion, exhibiting a transient pro-apoptotic activity on the cancer cells.

Klotho is an aging suppressor gene, while its molecular mechanisms in hepatocarcinogenesis remains unclear (26). Chen et al. (27) demonstrated that Axitinib was an efficient VEGFR2 inhibitor for Klotho-mediated anoikis resistance. Axitinib exhibited the anti-tumor function of Klotho in suppressing anoikis resistance and anchorage-independent growth *via* inhibiting VEGFR2/PAK1 signaling, which provide a therapeutic intervention for those HCC patients with high Klotho expression. Targeting Klotho/VEGFR2/PAK1 signaling pathway by Axitinib might be an effective treatment for the hepatoma resistance to anoikis in hepatocarcinogenesis, thus providing an intervention with HCC metastasis (27).

In recent years, tyrosine kinase inhibitors (TKIs) and multikinase inhibitors (MKIs) have gained increasing importance as oncology drugs that improve the treatment of many types of tumors (28), including HCC. MKI axitinib was found to be effective in inhibiting CYP1A2-catalyzed O-deethylation of 7-ethoxyresorufin by cDNA-expressed enzymes and human liver microsomes (29). As a result, co-administering axitinib with alternate substrates of CYP1A2 may result in interactions of each other, together contributed to improving the efficacy of pharmacological treatments.

As an alternative to traditional surgery, radiofrequency ablation (RFA) is widely used for the treatment of HCC (30). Compared with surgical resection, RFA is a simpler modality that inflicts less injury to the liver. Mounting studies have reported that RFA in combination with immunosuppressant could increase the clinical efficacy of HCC. Liu et al. (31) found that insufficient RFA enhanced HCC cancer cell proliferation by activating CaMKII/ERK-dependent VEGF overexpression, while 5 μM Axitinib could significantly suppress HCC cell proliferation and viability *via* inhibiting VEGFR.

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TABLE 1 Studies registered in the ClinicalTrials.

Clinical Trials ID	Study area	Status	Cancer type	Number of patients	Age (years)	Therapies (Axitinib)	Time Frame	Responsible Party	Outcomes	Serious Adverse Events (%)
NCT01334112, Phase 2	Canada	Completed	Advanced HCC	30	Over 18	5 mg, BID, Orally, in cycles of 4 weeks	January 2011 to March 2018	University Health Network, Toronto; Pfizer	No Results Posted	No Results Posted
NCT01273662, Phase 2	Multi-center	Completed	Advanced HCC	45	Over 18	5 mg, BID, Orally	April 2011 to December 2016	National Taiwan University Hospital	No Results Posted	No Results Posted
NCT01210495, Phase 2	70 centers (13 countries)	Completed	Advanced HCC	224	Over 18	1 mg, 5 mg BID, Orally vs Placebo; Duration: 3-6 months	December, 2010 to December, 2016	Pfizer	Median OS: 12.7 (10.2 to 14.9) for Axitinib vs 9.7 (5.9 to 11.8) for Placebo, HR=0.907, 95% CI: 0.646-1.274, P=0.2872; Median PFS: 3.6 (2.3 to 4.6) for Axitinib vs 1.9 (1.9 to 3.5) for Placebo, HR=0.618, 95% CI: 0.438-0.871, P=0.0039; TTP: HR=0.621, 95%CI: 0.434-0.889, P=0.006	Axitinib: 62/133 (46.6%) vs Placebo: 16/68 (23.5%)
NCT01352728, Phase 2	Hong Kong	Completed	Unresectable HCC	50	Over 18	TACE+ 5 mg Axitinib, Daily, Orally	May, 2011 to June, 2018	Chinese University of Hong Kong	No Results Posted	No Results Posted
NCT02814461, Phase 1	Chinese Taipei	Completed	Advanced HCC	9	20-85	1mg, 2mg, 3mg, BID, Orally; Combination with radiotherapy; Duration: total 8 weeks	June, 2016 to November 2018	Shin Kong Wu Ho-Su Memorial Hospital	No Results Posted	No Results Posted
NCT03289533, Phase 1	NA	Completed	Advanced HCC	22	Over 20	avelumab 10 mg/kg Q2W in combination with axitinib 5 mg BID	September, 2017 to September, 2020	Pfizer	TTP=5.52 (1.91 to 7.39); Objective Response= 13.6 (2.9 to 34.9); Disease Control= 68.2 (45.1 to 86.1) TTR= 1.91 (1.9 to 3.7)	8/22 (36.36%)
NCT01441388, Phase 1	NA	Withdrawn	Advanced HCC, Glioblastoma	NA	Over 18	Crizotinib plus axitinib	September 27, 2011 to December 20, 2011	Pfizer	No Results Posted	No Results Posted

NA, Not available; HCC, Hepatocellular carcinoma; HR, Hazard ratio; CI, Confidence interval; OS, Overall Survival; PFS, Progress Free Survival. TACE, Transarterial Chemoembolisation; TTP, Time to Disease Progression; TTR, Time to Tumor Response.

During tumor angiogenesis, tumor vessels have structural and functional abnormalities, and they are essential for tumor growth, progression, and metastasis (32). Inhibiting tumor vasculature may be an effective cancer therapy target. Lv et al. (33) established a VX2 liver tumor–bearing rabbit model and further demonstrated that axitinib exhibited antitumor efficacy on HCC animal model. In this study, the authors also found that tumor growth was significantly suppressed by Axitinib compared with the controls. In addition to the above findings, they further suggested that the therapeutic effects of Axitinib in suppressing VX2-mediated HCC in rabbits could be noninvasively and quantitatively monitored with CT spectral imaging parameters.

TABLE 2 Experimental (preclinical) studies reported that biological effects of axitinib in HCC.

Study	Study area	Materials	Signaling pathway	Molecular mechanisms
Chen et al., 2013	China	Cell lines and human hepatoma tissues	VEGFR2/ PAK1	Axitinib is an efficient inhibitor for Klotho-mediated anoikis resistance, which might provide a therapeutic intervention for those HCC patients with high Klotho expression.
Gu et al., 2013	Australia	Human liver tissue	CYP1A2	Axitinib potently suppressed CYP1A2-dependent 7-ethoxyresorufin O-deethylation activity at a low level.
Liu et al., 2015	China	Human HCC cell line	ERK, VEGFR	5 μM Axitinib significantly inhibited cancer cell proliferation and viability; Insufficient radiofrequency ablation enhanced liver cancer cell proliferation by activating CaMKII/ERK-dependent VEGF overexpression.
Lv et al., 2016	China	liver tumor – bearing rabbits	NA	Axitinib exhibits antitumor efficacy on liver tumor; tumor growth was significantly suppressed by Axitinib compared with the control, tended to be smaller with higher dosages.
Chiew et al., 2017	Singapore	HCC cell line, HUVEC, and mouse model	Akt/mTor	Axitinib induces HUVEC apoptosis and reduced vascular networks, but is unable to kill liver cancer cells; Axitinib had a lower anti-angiogenic effect than sunitinib.
Amin et al., 2019	Canada	Cells and clinical samples	Glycolysis and the citric acid cycle	Under axitinib treatment, 5,6-dihydrouracil and glycopyranose increased, while glutamic acid, glutamine, and a lactose derivative decreased with treatment-response.
Lin et al., 2019	China	Rats and cells	NA	Axitinib inhibited the metabolism of loperamide noncompetitively *in vitro* and affect the pharmacokinetic characteristics of loperamide *in vivo*.
Li et al., 2020	China	HCC cells	miR-509-3p/ PDGFRA	LINC00467 promoted the proliferation and invasion of the HCC cells. Besides, high level of LINC00467 contributed to Axitinib resistance of HCC through miR-509-3p/PDGFRA axis.

NA, not available.

At present, treatment efficacy is commonly monitored by computed tomography (CT) scans and magnetic resonance imaging (MRI). Treatment effects for solid tumors are typically characterized by the reduction in tumor size or tumor attenuation (34). Amin et al. (35) demonstrated that 5,6-dihydrouracil and glycopyranose increased after treatment with axitinib, while glutamic acid, glutamine, and a lactose derivative decreased with treatment-response. In Amin et al.'s study, the clinical samples were collected at 2-4 weeks after initiation of axitinib. Since such a phenomenon of the treatment-related changes in the metabolome was detected, the clinicians may identify individuals who are not benefiting from a chemotherapeutic agent, which may serve as a part of an adaptive treatment algorithm.

Among axitinib patients, diarrhea is the most common adverse reaction, with a mean frequency in all grades exceeding 50% (36). Loperamide, an opioid receptor agonist, is widely prescribed to treat chronic diarrhea and acute diarrhea as well (37). Chemotherapy-induced diarrhea is commonly treated with high-dose loperamide, which is considered the standard first-line treatment. As a result, concurrent use of loperamide and axitinib may ensure the efficacy of antitumor properties as well as minimize the side effects. However, loperamide is a substrate of CYP2C8, CYP3A4 and P-gp, suggesting there may be a direct correlation between axitinib and loperamide (38). Lin et al. (39) showed that axitinib inhibited the metabolism of loperamide noncompetitively *in vitro* and affected the pharmacokinetic characteristics of loperamide *in vivo*. The peak time of loperamide increased while blood clearance decreased under the impact of axitinib. As a result, since the pharmacokinetics of LOP have been altered remarkably, it is recommended to avoid the combination of axitinib and LOP, even if the two drugs are administered at therapeutic doses.

The PDGFR gene has been reported to be highly expressed in HCC (40). PDGFR can be regulated by Axitinib, while PDGFRA and PDGFRB are two isoforms of PDGFR. High expression of PDGFRA/B was found to be closely associated with low OS in HCC patients (41). A recent study conducted by Li et al. (42) indicated that LINC00467 promoted the proliferation and invasion of the HCC cells. The authors also found that high level of LINC00467 contributed to Axitinib resistance of HCC through miR-509-3p/PDGFRA axis. LINC00467, one of the lncRNAs being detected, has been found to serve as an oncogene in multiple cancers, including neuroblastoma, lung cancer, and colorectal cancer (43). In Li et al.'s study (42), LINC00467 was upregulated in HCC samples as compared to the normal live tissues by analyzing TCGA database. The authors further observed that LINC00467 inhibition might suppress the proliferation and invasion but promoted the apoptosis of the HCC cells. They also suggested that LINC00467 might involve in the Axitinib resistance of HCC.

Inconsistent with the above studies, Chiew et al. (44) reported that Axitinib induced HUVEC apoptosis and reduced vascular networks *via* the Akt/mTOR signaling pathway, but is unable to kill the HCC cells. They concluded that Axitinib had a lower anti-angiogenic effect than sunitinib for treating HCC in a 3D co-culture spheroid of tumor cells.

Taken together, the above experimental/preclinical studies demonstrated that the molecular mechanisms of anti-tumor action of the Axitinib presents itself as a multifaceted process. The associated signaling molecules included VEGFR2/PAK1,

CYP1A2, CaMKII/ERK, Akt/mTor, and miR-509-3p/PDGFRA. Figure 1 showed the molecular mechanism underlying the roles of axitinib in advanced HCC. The biochemical mechanisms might be associated with glycolysis and the citric acid cycle. These *in vitro* and *in vivo* studies may improve the understanding of the biological functioning of Axitinib in the treatment of HCC, indicating that Axitinib is worthy to be popularized in clinical practice.

Published clinical trials

After a systematically search in the six common databases, we have identified nine published clinical trials (Table 3). The year of publication among the included studies ranged from 2015 to 2021. The study location/area/region included Japan, Canada, China, and multi-center involving multiple countries. The study design included phase I, phase II, and case report, either single arm or randomized. The sample size ranged from 1 to 202 participants. The tumor stage of HCC included advanced, metastatic, and inoperable HCC. The age of the participants ranged from 18 to 84. The administration of Axitinib was mainly 5 mg twice daily orally, while 1 mg, 2mg, 3mg, and 7mg twice daily were also investigated. The treatment period included 4 weeks, 8 week, and 16 weeks. The combination therapies included transarterial chemoembolization, radiotherapy, and avelumab intravenously. The most common adverse event reported in the nine included studies was hypertension. Other side-effect included diarrhea, decreased appetite, thrombocytopenia, ulcerative oral mucositis, rash, polyhidrosis, fatigue, emesis, hyperbilirubinemia, high transaminases, abdominal pain, asthenia, palmar-plantarerythrodysesthesia syndrome, proteinuria, alkaline phosphatase, and bilirubin.

A randomized phase II study developed by Kang et al. (45) in 2015 recruited 134 advanced HCC patients under axitinib treatment (5 mg BID in 4-week cycles, orally) and 68 patients received placebo. The investigators found that Axitinib did not improve OS over placebo (HR=0.907, 95%CI: 0.646-1.274, median months: 12.7 vs 9.7). But Axitinib could significantly improve the PFS, TTP, and BCR (all P<0.05). Interestingly, though the OS between the axitinib and placebo group did not reach statistical significance, the authors found the OS was better in some subgroups, such as Eastern Cooperative Oncology Group performance status (ECOG PS) 1, excluding those intolerant to prior antiangiogenic therapy, as well as those patients with baseline α-fetoprotein ≥400 ng/ml. In regard to PFS, the survival time was significantly better in patients under axitinib treatment at Asian, but not non-Asian. In this study, the authors also investigated several serum soluble protein biomarkers to predict the survival of the patients. The results showed that patients with low baseline serum level of E-selectin or stromal cell-derived factor-1 had a significantly higher OS than those with a high level. In addition, the investigators further found a prognostic association between lower baseline levels of circulating IL-6 or angiopoeitin-2 and longer OS (all P<0.05). The adverse events in this study included diarrhoea (54%), hypertension (54%), and decreased appetite (47%). This study indicated that axitinib could prolong rather the PFS and TTP than the OS of patients with advanced HCC, with an acceptable safety profile. E-selectin, stromal cell-derived factor-1, IL-6, and

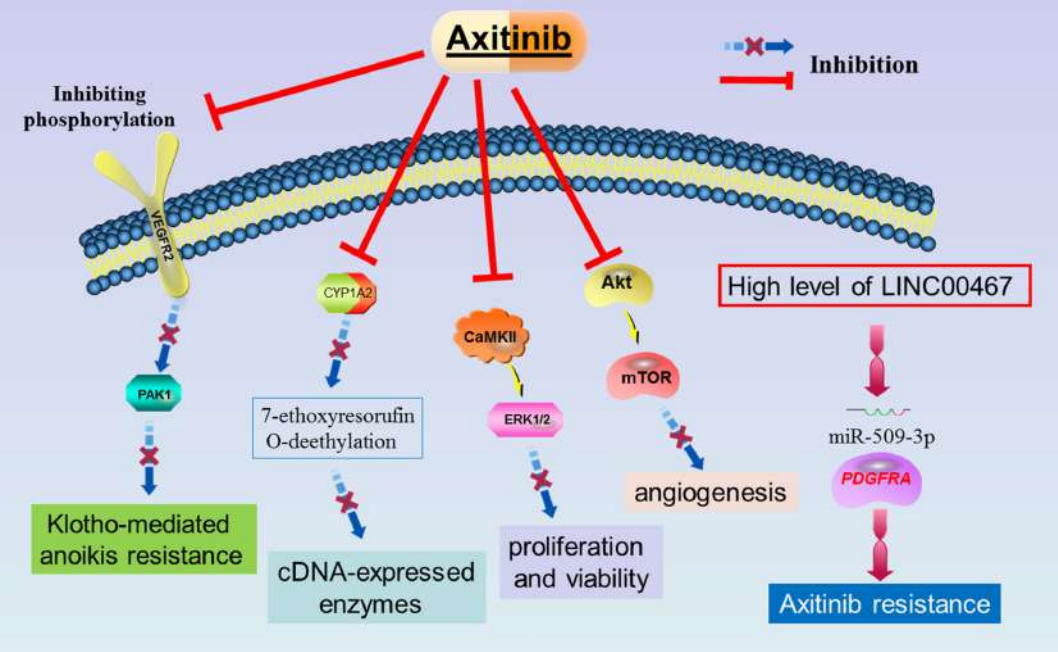

FIGURE 1
The molecular mechanism underlying the roles of axitinib in advanced HCC.

TABLE 3 Published studies reported the effect of axitinib in advanced HCC.

Study and references	Study area	Clinical phase	Cancer type, Number of patients	Age (years)	Axitinib	Therapeutic effects or molecular mechanisms	Adverse events (%)
Kang et al., 2015	Japan	II/ Randomized	Advanced HCC, 202	25-84	5 mg BID in 4-week cycles, orally	Axitinib did not improve OS over placebo (HR=0.907, 95%CI: 0.646-1.274, median months: 12.7 vs 9.7). But it could significantly improve the PFS, TTP, and BCR.	Diarrhoea (54%), hypertension (54%), decreased appetite (47%).
McNamara et al., 2015	Canada	Single-arm phase II	Advanced HCC, 30	27-85	5 mg twice daily orally, 16 weeks	Tumor control rate at 16 weeks was 42.3% (95% CI: 22.3%-63.1%).	Hypertension (16.7%), thrombocytopenia (13.3%), diarrhea (10.0%)
Zhang et al., 2015	China	Case report	Metastatic HCC, 1	64	7 mg twice daily orally	Combined axitinib and cabozantinib (50 mg, qd, po) after failed sorafenib treatment. The patient had a total survival of 10 months.	Ulcerative oral mucositis, rash, polyhidrosis, fatigue, loss of appetite, emesis, and elevated blood pressure
Lo et al., 2016	Multi-center Clinical Trial	Single arm phase II	Advanced HCC, 15	18-78	5 mg twice daily orally	No significant association was found in the PFS (P=0.310) or progression at 16 wk (P=0.849). But a borderline statistically significant OS was observed (P=0.050).	NA
Chan et al., 2017	China	Single arm phase II (follow-up: 39.9 months)	Inoperable HCC, 50	61.8 ± 7.6	5 mg twice daily orally combined with transarterial chemoembolization	The 2-year OS rate was 43.7%, and the median OS was 18.8 months. The median TTP was 10.4 months (95% CI: 5.4-12.7) and the PFS was 8.4 months (95% CI: 3.9-11.2).	Hyperbilirubinemia (14%), increase in transaminases (44%), and abdominal pain (24%)
Kudo et al., 2018	Multi-center Clinical Trial	II/ Randomized	Advanced HCC, 202	65	5 mg twice daily orally in 4-week cycles	Median OS in the axitinib and the placebo arm, respectively, was 12.3 months and 11.2 months in non-Asia (P=0.465) and 13.5 months and 6.3 months in Asia (P=0.226). PFS significantly longer in the axitinib group (3.6 months) than in the placebo arm (1.8 months) in Asia (HR 0.556, 95% CI 0.370–0.835; P= 0.0023) but not in non-Asia group.	Hypertension, diarrhea, asthenia, fatigue, etc.
Lin et al., 2020	China	II/ Randomized	Advanced HCC, 45	32-76	5 mg twice daily orally	The disease control rate was 62.2%. Median PFS and OS were 2.2 months and 10.1 months, respectively.	Fatigue (60%), anorexia (57%), hypertension (56%), and rash (49%), etc.
Kudo et al., 2021	Japan	Phase Ib	Advanced HCC, 22	20-84	5 mg twice daily orally combined avelumab 10 mg/kg intravenously every 2 weeks	The objective response rate was 13.6% (95% CI: 2.9–34.9%) per RECIST 1.1 and 31.8% (95% CI: 13.9–54.9%) per mRECIST for HCC.	Hypertension (50.0%), palmar-plantarerythrodysesthesia syndrome (22.7%), and decreased appetite (13.6%).
Yang et al., 2021	China	Phase I	Advanced HCC, 9	37-83	1 mg, 2mg, and 3mg twice daily for 8 weeks in combination with radiotherapy	Overall response rate was 66.7%. 1–year OS was 66.7% and median PFS was 7.4 months.	Hypertension, proteinuria, increased alanine transaminase, alkaline phosphatase, and bilirubin.

NA, Not available; HCC, Hepatocellular carcinoma; HR, Hazard ratio; CI, Confidence interval; OS, Overall Survival; PFS, Progress Free Survival; RECIST, Response Evaluation Criteria in Solid Tumors.

angiopoeitin-2 were the potential prognostic and predictive biomarkers in the action of axitinib on advanced HCC.

In a previous single-arm phase II study in Canada, McNamara et al. (46) recruited 30 advanced HCC patients and all the patients received Axitinib 5 mg twice daily orally for16 weeks (without a placebo group). The authors found the tumor control rate at 16 weeks was 42.3% (95% CI: 22.3%-63.1%) through the standards of RECIST1.1. The median duration of tumor control on Axitinib treatment was 3.6 months (95% CI, 2.8-9.2 months). PFS (P= 0.0005) and OS (P = 0.04) were found to be associated with the greatest percentage change from baseline in the sum of the diameters of tumor lesions by using RECIST 1.1. Similar trend

was found in Choi criteria but not for mRECIST criteria. For different race of the investigated patients, the median OS in Asians was 9.7 months vs 6.6 months for non-Asian (P=0.19), no significant different was found. In this study, no biomarker was identified that could be predicted the PFS and the OS (all P>0.05). The adverse events reported in this trial included hypertension (16.7%), thrombocytopenia (13.3%), and diarrhea (10.0%). In the further analysis, median PFS in patients who developed grades 1-3 hypertension was 10.7 months vs 2.8 months in those who did not (P=0.0004). In aspect of the OS, patients who developed hypertension was 17.2 months vs 6.0 months in those who did not (P < 0.0001). This study suggested that axitinib could encourage tolerable clinical activity in HCC patients, which need more potential biomarkers to evaluate the responses of axitinib treatment.

At present, in addition to McNamara et al.'s study, there were another two single arm phase II studies had published. Lo et al. (13) conducted a multi-center clinical trial which investigated 15 advanced HCC patients in Australia, Canada, and UK. The researchers reported that no significant association was found in the PFS (P=0.310) or progression (P=0.849) after the treatment of axitinib 5 mg twice daily orally at 16 weeks. However, they observed a borderline statistically significant on the OS (P=0.050), even though limited by a small sample size. On the other hand, the authors also indicated that dynamic contrast-enhanced ultrasound (DCE-US) might be potentially useful in monitoring early tumor response of advanced HCC to axitinib treatment. Besides, tumor fractional blood volume measurement using the DCE-US infusion technique might be a promising imaging biomarker to predict OS in patients with advanced HCC who under axitinib treatment. Another single arm phase II trial developed by Chan et al. (47) had recruited 50 inoperable HCC patients who treated with 5 mg Axitinib twice daily orally combined with transarterial chemoembolization with a follow-up of 39.9 months. This study showed that the 2-year OS rate was 43.7% and the median OS was 18.8 months after the treatment of the combination of Axitinib and transarterial chemoembolization. The median TTP was 10.4 months (95% CI: 5.4-12.7) and the PFS was 8.4 months (95% CI: 3.9-11.2). In this study, the common adverse events of grade 3 were increase in transaminases (44%), abdominal pain (24%), and hyperbilirubinemia (14%), which might be associated with the treatment of transarterial chemoembolization. Under the treatment of Axitinib, the side-effect included hypertension (24%) and hand-foot skin reaction (14%). The authors also investigated the predictive parameters of efficacy of the treatment. They found that patients who developed hypertension had a better median PFS when compared to those without a hypertension (11.6 months vs 4.5 months, P= 0.0017). Similarly, the median OS was also better in those with hypertension than the absence of hypertension (25 months vs 14.1 months, P= 0.0222). Interestingly, a higher grade of hypertension also was correlated to a better median PFS (P= 0.004). In the multivariate analyses, the presence and grading of hypertension and Eastern Cooperative Oncology Group (ECOG) performance status were the independent prognostic factors for OS (all P<0.05). The above studies revealed that combining axitinib with TACE or DCE-US might effective for patients with inoperable HCC.

A previous phase II randomized multi-center clinical trial (48) demonstrated that the median OS in the axitinib (5 mg twice daily orally in 4-week cycles) and the placebo arm, respectively, was 12.3 months and 11.2 months in non-Asia (P=0.465) and 13.5 months and 6.3 months in Asia (P=0.226). These results indicated axitinib did not prolong the OS of the advanced HCC patients. However, PFS significantly longer in the axitinib group (3.6 months) than in the placebo arm (1.8 months) in Asia (HR 0.556, 95% CI 0.370–0.835; P= 0.0023) but not in non-Asia group (P>0.05). In line with the above findings, the adverse events were hypertension, diarrhea, asthenia, and fatigue, etc. This study revealed that the PFS in the axitinib/BSC arm could be affected by different population, showing that longer PFS was identified in patients from Asia rather than non-Asia. In a phase II randomized trial developed in China, Lin et al. (49) reported the disease control rate was 62.2% in 45 advanced HCC patients received 5 mg axitinib twice daily orally. Median PFS and OS of the participants were 2.2 months and 10.1 months, respectively. Of note, the side-effects were detected as fatigue (60%), anorexia (57%), hypertension (56%), and rash (49%), which were higher than that of other phase II studies. This study observed that Axitinib is moderately active and has acceptable toxicity for patients with advanced HCC who have failed to respond to sorafenib monotherapy in the first instance.

In addition to the above clinical trials, Zhang et al. (16) presented a case report on the topic of the combination of axitinib and cabozantinib after failed sorafenib treatment in a metastatic HCC patient. This male patient aged at 64 y old, complained of discomfort in upper abdomen. This patient was diagnosed with lung and bone metastases of stage D primary HCC with chronic type B hepatitis. The author reported that this advanced HCC patient received the combination of axitinib (7 mg twice daily orally) and cabozantinib (50 mg, qd, orally) after failed sorafenib treatment. However, the patient had a total survival of 10 months after these treatments. The patient exhibited disease progression after treating with sorafenib for 2.5 months. Then, he was treated with treated with angiogenesis inhibitor axitinib and c-Met inhibitor cabozantinib, but turned to be a poor outcome. This study highlighted that options of appropriate therapies and timing needed enhanced communication and collaboration of relevant disciplines, which might facilitate to improve the therapeutic efficacy.

Directions for future research

Most of the above included studies were designed for treating the advanced HCC patients with axitinib alone. The outcomes turned out to be no significant benefit from axitinib treatment on the OS. Even though this agent could prolong the OS of the sufferers, there was no significant difference between axitinib treatment and the placebo. However, we should note that patients received axitinib treatment had a significant better PFS as compared

to the controls. Therefore, many scholars believed that Axitinib might serve as the second-line treatment for patients with advanced HCC who failed sorafenib treatment.

Mounting evidence demonstrated that the combination of immunotherapy (included the combination of different immunosuppressors) and radiotherapy/chemotherapy/ablation may significantly promote the therapeutic efficacy in patients with unresectable or metastatic HCC (50–53). Combination therapy may have better antitumor properties than monotherapy. Though monotherapy with axitinib showed no significant survival benefits, the combination of anti-PD-1/PD-L1, anti-CTLA-4, or other tyrosine kinase inhibitors (TKIs) may be a promising regimen for advanced HCC. Immunotherapy is a promising therapy option for unresectable and advanced HCC. Immune checkpoint inhibitors (ICIs) have been shown to be highly effective in the treatment for this type of cancer. PD-1/PD-L1 is one of the widespread applications of ICI, having the potent anticancer effect for advanced HCC. According to the lately evidence, ICIs combined with kinase inhibitors exhibit a potential superior anti-tumor effect on advanced HCC. Mechanistically, PD-1/PD-L1 blockade with its antibody can restore T-cell function, while axitinib is an inhibitor against multiple types of VEGFR. Both anti-PD-1 and anti-VEGFR enhance the therapeutic anti-tumor effects. Current evidence indicates that single-agent axitinib showed none of significant overall survival benefit for patients with advanced HCC. However, promisingly, axitinib combined with anti-PD-1/PDL-1 agents have exerted a potential anticancer effect on advanced HCC. Within the topic of this review, Kudo et al. (54) earlier detected axitinib plus anti-PD-1/PDL-1 agents had the possible antitumor potential effect on advanced HCC. In this study, axitinib combined with anti-PD-L1 monoclonal antibody (avelumab) presented with a manageable toxicity profile. 72.7% of the advanced HCC patients complicated with Grade 3 treatment-related adverse events, including hypertension, palmar-plantar erythron dysesthesia syndrome, and loss of appetite. However, no Grade 4 treatment-related adverse events or treatment-related deaths occurred under these regimens. Though the objective response rate appeared to be numerically lower compared to other similar trials in the first-line HCC setting, a combination treatment of axitinib and avelumab exerted with clinical activity as first-line treatment. The authors suggested that the inconsistent results might be correlated to the limited patient numbers and differences in trial design. Several undergoing phase 1 and phase 3 studies (55–57) have investigated the anti-PD-1 monoclonal antibody plus anti-VEGF multikinase inhibitor in patients with advanced HCC. The objective response rates were up to 36% per RECIST and 46% per mRECIST, indicating the promising anti-tumor effects on advanced HCC. Furthermore, Kudo et al. (54) also found that patients without baseline vascular invasion or with baseline extrahepatic spread presented with a higher ORR per RECIST 1.1 and favorable OS. In addition, patients with PD-L1+ tumors had longer OS than those with PD-L1- tumors. Therefore, anti-VEGF multikinase inhibitor plus anti-PD-1 monoclonal antibody may be more suitable for the patients who without baseline vascular invasion, with baseline extrahepatic spread, or with PD-L1+ tumors. The United States FDA approved sorafenib and nivolumab as the first anti-PDL-1/PD-1 antibodies for the treatment of HCC. Since both sorafenib and axitinib are the important tyrosine kinase inhibitors, axitinib combined with anti-PDL-1/PD-1 antibodies may also have promising outcomes in advanced HCC treatment, which is waiting for more well-designed RCT to prove it.

At present, there were two phase I trials have been published recently within the topic of the combination of axitinib and other therapies. Both of the two studies were conducted in Asia (China and Japan) and published in the year of 2021. Kudo et al. (54) recruited 22 advanced HCC patients who aged 20-84 in a phase I study. The authors found that advanced HCC patients received 5 mg axitinib twice daily orally combined avelumab 10 mg/kg intravenously every 2 weeks had a moderate objective response rate (13.6%, 95% CI: 2.9–34.9%, per RECIST 1.1 and 31.8%, 95% CI: 13.9–54.9%, per mRECIST). The adverse events included hypertension (50.0%), palmar-plantarerythrodysesthesia syndrome (22.7%), and decreased appetite (13.6%). In another recent phase I trial, Yang et al. (58) demonstrated that nine advanced HCC patients under 1 mg, 2mg, and 3mg axitinib twice daily for 8 weeks in combination with radiotherapy had an overall response rate of 66.7%. The 1−year OS was recorded at 66.7% and median PFS was 7.4 months. The side-effects included hypertension, proteinuria, increased alanine transaminase, alkaline phosphatase, and bilirubin. The two phase I study suggested that axitinib in combination with other therapies exhibited a promising antitumor efficacy on advanced HCC and underwent with a comparable adverse events of axitinib monotherapy. However, these results were derived from phase I or phase Ib trial with a small sample size. Therefore, more multi-center randomized trials with large sample size are still warranted for validating the therapeutic efficacy of the combination of axitinib and other treatments in managing patients with advanced HCC.

Conclusion

The present review highlights the current clinical applications and the molecular mechanisms of axitinib in advanced HCC. The included randomized or single-arm phase II trials indicated that axitinib could not prolong OS as compared to placebo for the treatment of advanced HCC, but improvements in PFS and time to tumor progression were observed. Experimental studies showed that the biochemical effects of axitinib in HCC might be regulated by its associated genes and affected signaling cascades. Axitinib combined with axitinib exerts promising antitumor efficacy on advanced HCC. Future directions should focus on the identification of precise biomarkers and the development of novel immunotherapy agents. To move toward clinical applications by combining axitinib and other treatments in advanced HCC, more studies are still warranted in the near future.

Author contributions

HJ and SX contributed to conceive and design the study. JL, CJ, and JM performed the article searching. HJ and SX extracted the

data. HJ and SX wrote the manuscript. JM and LW supervised the manuscript. All of the authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Real-world treatment patterns, discontinuation and clinical outcomes in patients with B-cell lymphoproliferative diseases treated with BTK inhibitors in China

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Introduction: Bruton tyrosine kinase inhibitor (BTKi) has demonstrated substantial efficacy in treating B-cell lymphoproliferative diseases (BLPD). Nonetheless, the significant discontinuation rates due to toxicity or financial reasons cannot be overlooked. In China, empirical evidence on the usage of BTKi remains scarce.

Methods: To address this, a retrospective cohort study was conducted focused on 673 Chinese patients with BLPD who underwent at least one month of BTKi therapy.

Results: Median age at BTKi initiation was 60 years. The median duration on BTKi treatment of the whole cohort was 36.4 months. The median post-BTK survival was not reach. BTKi-based treatment was permanently discontinued in 288 (43.8%) patients during follow-up, mostly attributed to progressive disease. Within the first 6 months of BTKi treatment, 76 patients (26.3%) had early treatment discontinuation. Patients with early discontinuation had extreme worse outcome with a median post-discontinuation survival of only 6.9 months. On multivariate analysis, withdrawal BTKi by toxicity and withdrawal BTKi within 6 months retained to be independent predictors of post-BTK survival, after taking account of the response depth, lines of therapy and baseline cytogenetics including 17p deletion. The decision between BTKi monotherapy and combination therapy, along with the preference for first or second-generation BTKi, exerted no significant impact on survival.

Discussions: These observations contribute valuable real-world insights into the utilization of BTKi in China. We concluded that BTKi is an effective and well-tolerated treatment for long-term use in Chinese patient population. However, it is imperative to stress that a proportion of patients discontinue BTKi early, leading

to suboptimal outcomes. This study underscores the importance of adherence to BTKi therapy for improved clinical outcomes in real-world patients.

KEYWORDS

treatment patterns, adverse events, BTK inhibitor, discontinuation, outcome

1 Introduction

The advent of Bruton tyrosine kinase inhibitors (BTKi) revolutionized the management of patients with B-cell lymphoproliferative diseases (BLPD), especially for chronic lymphocytic leukemia (CLL), Waldenstrom macroglobulinemia/lymphoplasmacytic lymphoma (WM/LPL) and mantle cell lymphoma (MCL). Ibrutinib was first approved by the USA Food and Drug Administration (FDA) in 2014 for the treatment of patients with previous treated CLL. The approval was expanded to the first-line CLL setting irrespective of the patient's 17p deletion status in 2016. Ibrutinib was approved in adults with symptomatic WM/LPL by the FDA in 2013 and the European Medicine Agency (EMA) in 2015. Furthermore, ibrutinib has been widely accepted as a standard-of-care for patients with relapsed/refractory (R/R) MCL, but it remains no consensus on the ideal timing for its introduction within the treatment algorithm (1, 2).

The use of BTKi has significantly improved the prognosis for patients with BLPD, however, one of the commonalities of this disease category is incurability. Over half of the patients relapse within five years of initiating BTKi treatment (3–5). Given the indolent nature and extended survival of BLPD patients, choosing a treatment regimen must consider the delicate balance between efficacy and tolerability. The pivotal role of BTKi is undisputed, however, there are ongoing questions for its real-world usage as follows.

First, patients involved in clinical trials are under close scrutiny and are highly selected, therefore, they may not fully embody the real-world treatment dynamics. In China, concrete real-world evidence supporting BTKi usage is sparse. Second, there is a lack of real-world study that focus on the selection of different BTKi and comparing the efficiency and toxicity of BTKi among various BLPD subtypes. Additionally, it is crucial to identify the clinically relevant predictors of post-BTKi survival to guide optimal treatment decisions. Third, it is known that the incidence of BLPD is considerably lower in Asian populations compared to in Western countries, especially for CLL (6). Previous studies have suggested that Chinese CLL patients are generally younger, exhibit more mutated immunoglobulin heavy-chain variable genes (IGHV), as well as with a unique mutation landscape (7, 8). Chinese CLL patients had higher frequency mutations of *KMT2D*. *KMT2D*-mutated CLL showed impaired H3K4 methylation activity and decreased sensitivity to ibrutinib *in vitro* (7). Moreover, the ibrutinib responses in WM/LPL are affected by *MYD88* mutation status (9). There is a relatively low percentage of *MYD88* mutation in Chinese WM/LPL as reported (10, 11). These data indicate a unique biology of BLPD in Eastern populations, potentially implying a less necessity and efficacy of BTKi treatment in Chinese CLL patients. Consequently, the efficacy and clinical outcomes of BTKi treatment in Chinese patients setting needs to be further explored.

In light of these observations, real-world evidence on the efficacy and safety of BTKi on Chinese patients is important to help guide treatment planning. The principal focus of this retrospective observational study was to outline the rate of BTKi adherence, duration of BTKi exposure and reasons for discontinuation in Chinese real-world setting of BLPD. Furthermore, this study aimed to ascertain whether these factors had any impact on post-BTKi survival.

2 Methods

2.1 Patients

This is a single-center, real world, retrospective study performed at the Institute of Hematology and Blood Disease Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, diagnosed from January 2006 to October 2022. The diagnosis was established using the WHO classification criteria (12). This study included all of the patients who received at least one dose of BTKi at our hospital from January 2014 to October 2022. The specific inclusion and exclusion criteria are specified in Supplementary Figure 1. Discontinuation was defined as a gap of ≥90 days in treatment. Demographic data of the study cohort, lactate dehydrogenase (LDH) level, previous treatment lines, treatment regimen, adverse events (AE), and mortality data were collected. The study examined the impact of treatment duration and reasons for discontinuation on survival. Treatment response was evaluated according to standard definitions. Fluorescence *in situ* hybridization studies with specific probes for 17p13 (LSI TP53) and 11q22 (LSI ATM) and chromosome karyotype studies were performed within 6 months before starting BTKi. This study was approved by the Ethics Committee of our hospital (Institute of Hematology and Blood Disease Hospital, Tianjin, China). All patients enrolled provided written informed consent before starting treatment.

2.2 Outcome

Demographic and clinical data of the study cohort were evaluated with descriptive statistics. Toxicity and outcome data were collected during variable follow-up period (minimum 3 months). The response to ibrutinib therapy was assessed according to the 2014 Lugano criteria (13). Survival curves were generated by the Kaplan-Meier method and compared by using the 2-sided log-rank test. Post discontinuation survival (PDS) was defined as the period from the discontinuation of BTKi therapy until death due to any cause or until the date of the last follow-up examination. Post-BTKi overall survival (post-BTKi OS) of the patients was calculated from the first dose of BTKi to either the date of death or the date of the last follow-up examination. Post-BTKi failure-free survival (post-BTKi FFS) was defined as the interval from initial dose of BTKi to disease progression, relapse, changing treatment regimen, death, or the last follow-up evaluation.

2.3 Statistical analysis

Variables used for univariate analyses included: age>65, gender, line of therapy, best response, elevated LDH level, the presence of complex karyotype, the presence of 17p deletion or 11q deletion, usage of commercially available BTKi or participation in a clinical trial, the choice of first or second-generation BTKi, clinical trial participation, BTKi exposure duration and reason for discontinuation. Only those variables identified as significant at the $P < 0.05$ level based on univariate analysis were subsequently assessed using stepwise multivariable logistic regression. Hazard ratios (HRs) for post-BTKi survival were calculated using Cox proportional hazards models. A two-sided Fisher exact test or X-squared test were used to compare categorical parameters. Student t or Mann-Whitney U tests were used to examine differences between two continuous variables. Statistical analyses were performed using SPSS version 21.0 (IBM, Chicago, IL), Graphpad Prism 7 and R package version 3.5.1. $P<0.05$ was considered as statistically significant.

3 Results

3.1 Study population characteristics

A total of 6177 patients with BLPD hospitalized at least once at our institute from January 2006 to October 2022. At the time of data cutoff, 673 of these patients (11.4%) were included in the study, with a median follow-up of 28.8 months from the initiation of BTKi. A flow diagram of the case selection process is presented in Supplementary Figure 1. The median age was 60 years, 71.0% of patients were male (Table 1). The most common diagnosis in BTKi treated BLPD patients was CLL (62.0%), followed by WM/LPL (28.4%), MCL (13.2%) (Figure 1). The cohort included 288 relapsed-refractory (R/R) and 385 treatment-naïve patients. The baseline characteristics and follow-up data for these groups were depicted in Table 1. The R/R group had a higher proportion of patients receiving monotherapy than previous untreated group (84% vs. 47%, $P<0.001$).

A total of 471 (70.0%) patients were treated with commercially available drug/off study. Ibrutinib was the most common choice of BTKi (451/673, 67.0%), following by zanubrutinib (24.5%) and orelabrutinib (4.0%). BTKi monotherapy was the most common regimen (Mono, 63.6%), followed by combinations of BTKi with fludarabine, cyclophosphamide and rituximab (BTKi with FCR, 13.2%), cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)-like chemotherapy (BTKi with CHOP-like, 5.9%), rituximab only (BTKi with R, 5.6%), bendamustine rituximab (BTKi with BR, 4.9%), and other regimens (Other, 6.7%). 202 patients (30.0%) participated non-blind clinical trials, which included 13.8% investigator-initiated trials and 16.2% industry-sponsored trials. We compared the baseline characteristics of patients participating in clinical trials with those receiving commercially available BTKi. Patients in clinical trial were younger, more frequently using next generation BTKi, less likely to have elevated LDH and more likely with R/R disease compared to those using commercial BTKi (Supplementary Table 1).

3.2 BTKi discontinuation time and reason

At the last follow-up, 288 (42.8%) patients had discontinued BTKi-based treatment. The reasons for discontinuing BTKi were grouped into four categories. First, 89 of the 288 patients discontinued BTKi due to toxicity (30.9%). Among those who discontinued due to toxicity, the most common causes for discontinuation were infection (40.4%), followed by thrombocytopenia or bleeding (16.9%), skin rash (10.1%), neutropenia (9.0%), cardiac arrhythmia (9.0%), anemia (4.5%), and reactivation of hepatitis B (4.5%). Second, 140 of the 288 patients discontinued BTKi due to progression, transformation or death (48.6%). 98 of the 140 patients (70.0%) were R/R patients. Third, 11.8% patients (34/288) withdrew BTKi due to unaffordable insurance or patients' preference. Fourth, 8.7% patients (25/288) discontinued BTKi according to the professional suggestions. Of those, 16 patients finished the treatment course of BTKi+FCR and reached minimal residual disease (MRD)-negative complete remission (CR). Following the physician's advice, these patients discontinued BTKi and started regular post-withdrawal follow-up checks. Other patients discontinued BTKi or changed treatment regimens in preparation for surgery or allo-transplantation.

Patients treated with ibrutinib had a higher risk of discontinuing treatment during follow-up compared to those treated with zanubrutinib (47.2% vs. 29.1%, $P<0.001$, Figure 2A). The discontinuation difference between the two BTKi was largely due to toxicity (15.3% vs. 9.1%, $P=0.047$). The frequency of BTKi discontinuation varied by regimen, with the highest rate observed among patients treated with BTKi combined with a CHOP-like regimen (60.0% vs. 41.7% for other regimens, $P=0.027$). Patients treated with BTKi combined with BR/FCR showed a comparable rate of discontinuation than patients treated with BTKi monotherapy (Figure 2B). Thus, BR/FCR appeared to be

TABLE 1 Baseline demographics and clinical characteristics of patients.

Measures	Previous untreated (n=385)	Relapsed/refractory (n=288)	Total (n=673)
Median age at diagnosis, years (range)	61 (26–88)	60 (17-84)	60 (17-88)
Gender (Male: Female)	2.5/1	2.3/1	2.4/1
Disease, n (%)			
CLL	255 (65)	162 (52)	417 (62)
WM/LPL	83 (21)	84 (27)	167 (25)
MCL	47 (12)	42 (14)	89 (13)
Treatment regimen, n (%)			
Monotherapy	182 (47)	246 (84)	428 (64)
Combination therapy	203 (53)	42 (16)	245 (36)
BTKi option, n (%)			
Ibrutinib	277 (72)	174 (60)	451 (67)
Zanubrutinib	94 (24)	71 (25)	165 (25)
Orelabrutinib	9 (2)	18 (6)	27 (4)
Other	5 (1)	25 (9)	30 (4)
Elevated LDH, n (%)	105 (28)	82 (29)	187 (29)
Cytogenetics, n (%)			
17p deletion	47 (13)	36 (14)	83 (14)
11q deletion	38 (12)	30 (13)	68 (12)
Complex karyotype	76 (23)	44 (19)	120 (22)
Time, months			
Median follow-up time from BTKi initiation	26.4	34.1	28.8
Median time from diagnosis to BTKi	16.7	53.6	31.8
Median time on BTKi treatment	70.3	28.6	36.4
Median FFS post BTKi therapy	70.3	34.5	50.9
2-year survival post BTKi therapy, %	88.5	75.0	82.6
5-year survival post BTKi therapy, %	74.4	57.3	65.0
Continuation events during follow-up, n (%)	126 (33)	162 (56)	288 (43)
Death events during follow-up, n (%)	48 (12)	82 (28)	130 (19)

CLL, chronic lymphocytic leukemia; WM/LPL, Waldenstrom macroglobulinemia/lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; BTKi, Bruton tyrosine kinase inhibitors; LDH, lactate dehydrogenase; FFS, failure-free survival.

relatively tolerable and effective choice as BTKi combination regimen rather than CHOP-like treatment. During the first year on BTKi, patients had a significantly higher rate of discontinuation due to toxicity compared to subsequent years (8.6% for first year, vs. 2.8% for second year, 1.5% for third year, 0.3% for fourth year or more, $P<0.001$, Figure 3B). Since the second year on BTKi treatment, progression had become the predominate reason for BTKi withdrawal. The cumulative incidence of discontinuation due to progression increased year by year, while the occurrence curve of other reasons-related discontinuation tended to be horizontal after the third year on BTKi treatment (Figure 3A).

The median time to BTKi discontinuation of the whole cohort was 36.4 months. At 1 year, 77.2% of patients in our cohort remained on BTKi treatment, and at 2 years 60.7% remained on treatment. We also evaluated the influencing factors of duration time on BTKi in different subgroups. Among the three disease categories, patients with CLL or WM/LPL had significantly longer periods of BTKi use (45.7 and 36.0 months, respectively) compared to the patients with MCL (16.0 months) ($P<0.001$, Figure 4A).

Statistically, significant longer time of BTKi adherence were observed in patients with first-line therapy, those who responsed to BTKi, and those receiving monotherapy or combined R/BR/FCR treatment (Figure 4). Notably, there was no significant difference in BTKi adherence time by monotherapy group *versus* combination therapy group (Supplementary Figure 2). The duration of BTKi treatment remained similar when comparing the choice of different

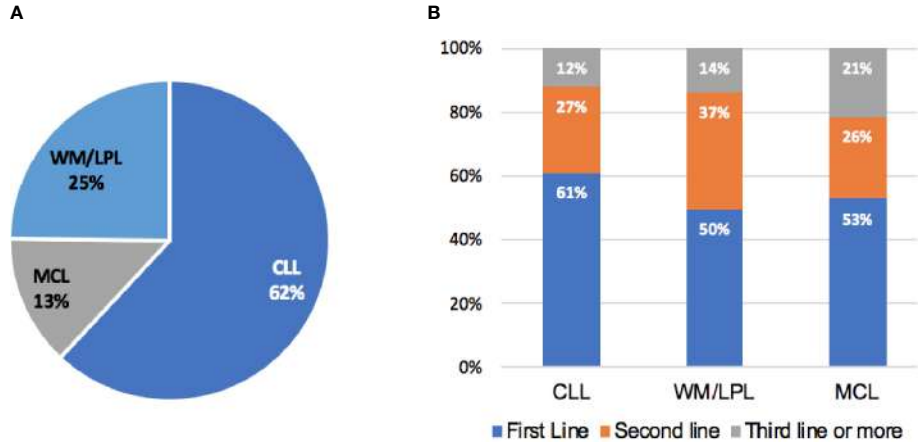

FIGURE 1
Patients distribution. **(A)** Disease distribution in patients receiving BTKi therapy. **(B)** Percentage of patients by line of therapy in which BTKi was initiated.

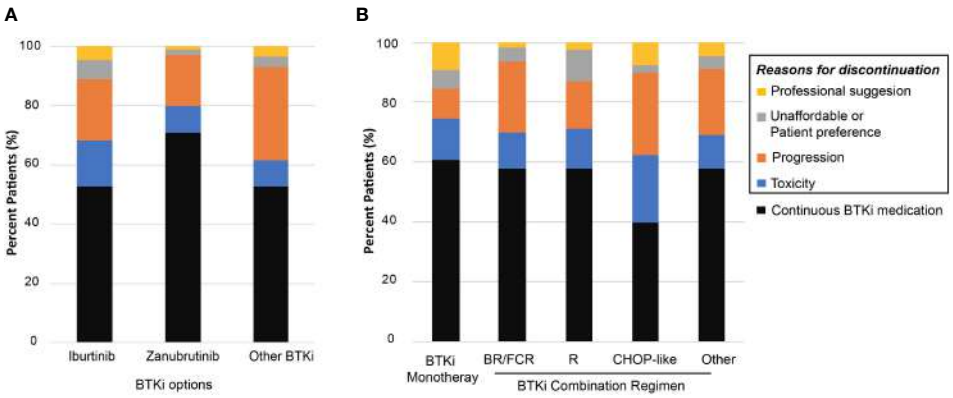

FIGURE 2
Reasons for BTKi discontinuation. **(A)** Distribution of BTKi discontinuation reasons among different BTKi option **(B)** Distribution of BTKi discontinuation reasons in patients receiving specific treatment.

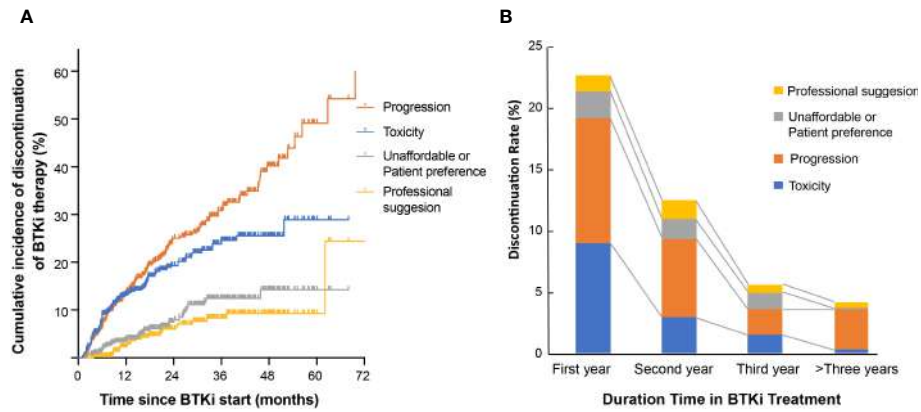

FIGURE 3
Reason for BTKi discontinuation. **(A)** Cumulative incidence of discontinuation stratified by reason for discontinuation. **(B)** The distribution of discontinuation reasons for patients who withdraw BTKi in the first year, second year, third year or who received BTKi for more than three years.

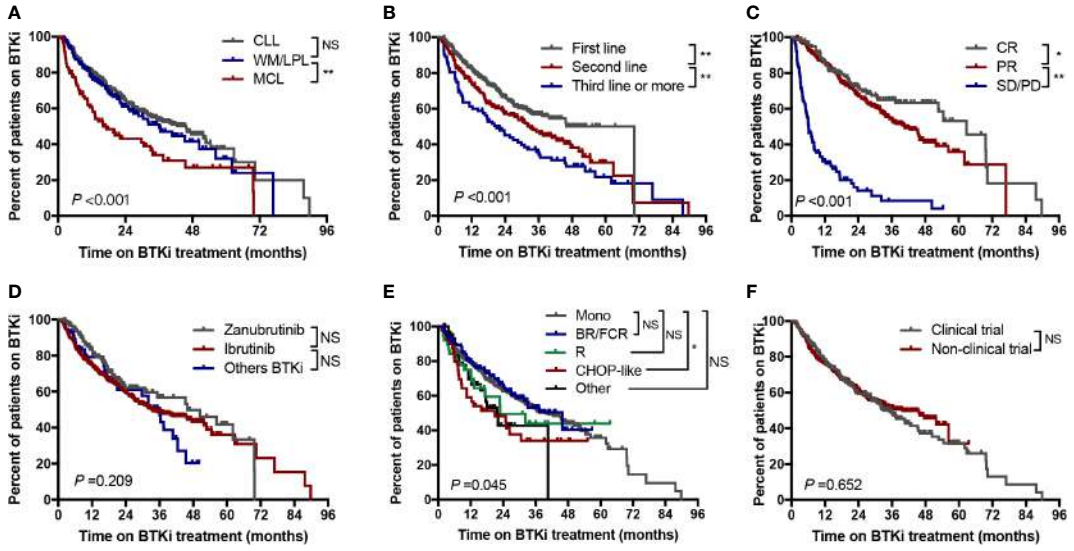

FIGURE 4
Time to BTKi discontinuation stratified by **(A)** Disease subtypes; **(B)** Line of therapy; **(C)** Depth of response; **(D)** Selectivity of BTKi; **(E)** Treatment regimen; **(F)** Clinical trial participation. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; Mono, monotherapy; NS, no significance; *P<0.05; **P<0.01.

generations of BTKi (Figure 4D) and and between clinical trial participation versus commercial use (Figure 4F).

3.3 Response to treatment and outcome

The best overall response rate (ORR) of the entire cohort was 82.8%, with a CR rate of 20.2%. The combination therapy group showed significantly higher CR rates than the monotherapy group (37.1% vs. 11.0%, *P*<0.001), however the ORR rate was similar between the two groups (85.3% vs. 83.9%, *P*=0.620). The CR rate was higher in untreated subgroup than relapse/refractory group (27.8% vs. 11.1%, P<0.001). The median time from diagnosis to BTKi starting was 31.8 months.

With a median follow-up time of 28.8 months, 221 patients (77 in first-line group, and 144 in previously treated group) had experienced BTKi treatment failure. The estimated median post-BTKi FFS of the entire cohort was 50.9 months, with 2-year and 5-year FFS of 70.8% and 42.3%, respectively (Figure 5A). The median post-BTKi OS was not reached, with 2-year and 5-year OS of 82.6% and 65.0%, respectively (Figure 5B). Inferior outcome was observed in patients who discontinued BTKi by any cause. The median PDS

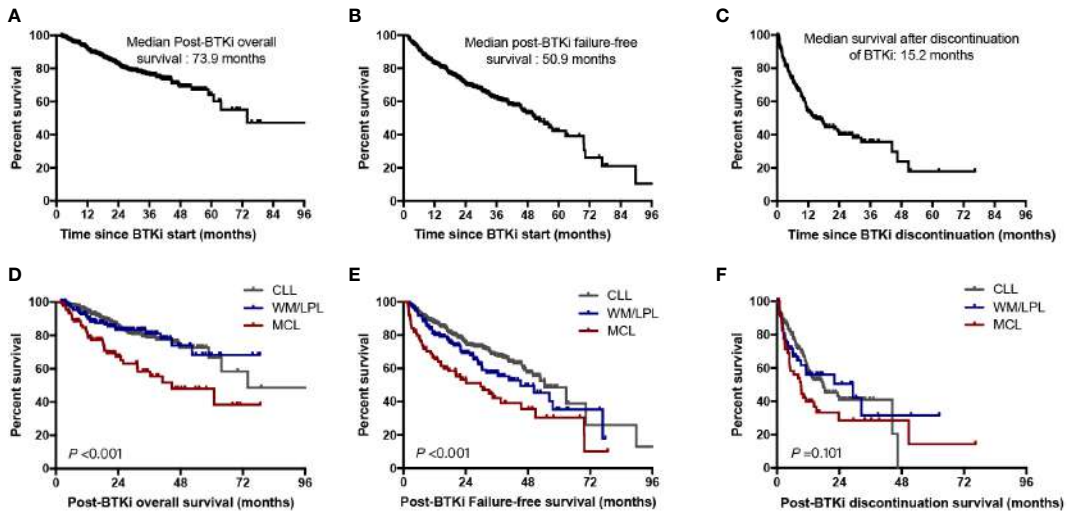

FIGURE 5
Outcomes for the entire cohort. **(A)** Overall survival for the whole cohort of patients since BTKi treatment start; **(B)** Failure-free survival since BTKi treatment start; **(C)** Overall survival from date of BTKi discontinuation; **(D)** Post-BTKi overall survival by disease subtypes; **(E)** Post-BTKi failure-free survival by disease subtypes; **(F)** Post-BTKi discontinuation survival by disease subtypes.

was 15.2 months, which means that more than half of the patients died within 2 years after discontinuation of BTKi (Figure 5C). According to the variety of disease in patients on BTKi treatment, we found patients with CLL and WM/LPL had superior post-BTKi survival than patients with MCL (P<0.001, Figure 5D). Different diseases had distinct timeframes to BTKi failure, with median post-BTKi FFS of 54.5 months in CLL, 45.2 months in WM/LPL, 29.8 months in MCL (P<0.001, Figure 5E). This variation in the efficacy of BTKi across different disease subtypes aligns with previous clinical trial findings (Figures 5D–F).

Among the 288 patients who had discontinued BTKi during follow-up, 26.4% of those discontinued within 6 months on BTKi identified as early discontinuation. The major reason for early discontinuation was toxicity (48.7%), followed by disease progression (42.1%). Patients with early discontinuation had extreme worse outcome with a median PDS of only 6.9 months. While patients who discontinued after 24 months on BTKi had a significantly longer survival whatever the withdrawal cause (median PDS: 46.5 months vs. 13.2 months for those discontinued in 12-24 months, P=0.003, Figure 6A). When we looked into the effect of discontinuation reason on survival, patients who discontinued due to toxicity had similar post discontinuation survival to those who discontinued due to disease progression (median PDS 10.8 and 11.1 months, P=0.776). Patients who withdrew BTKi due to economic reasons or professional suggestion had relatively longer survival after BTKi discontinuation compared to other reasons (median PDS 46.5 and not reach, respectively, Figure 6B). In our subgroup analysis of specific disease subtypes and treatment statuses, we observed that patients with CLL and WM/LPL, as well as both treatment-naïve and relapsed/refractory patients, who discontinued BTKi due to toxicity or within 6 months had significantly shorter post-BTKi survival (Supplementary Figure 3). However, the reason for discontinuation held no prognostic significance in patients with MCL (Supplementary Figure 3E).

3.4 Prognostic factors of post-BTKi survival

Patients were stratified by BTKi regimen (monotherapy versus combination therapy), choice of BTKi (Ibrutinib versus Zanubrutinib versus others), line of therapy (front-line versus R/R), depth of response (CR versus PR versus less than PR), clinical trial participation versus commercial use, reasons for discontinuation (intolerance versus progressive disease versus other reasons), and timing of discontinuation events (within 6 months versus more than 6 months). Besides, we also analyzed the impact of clinical characteristics on post-BTKi survival, such as age, LDH level and cytogenetic abnormalities.

We found line of therapy, depth of response less than PR, elevated LDH level with complex karyotype, with 17p deletion, withdrawal BTKi by toxicity and withdrawal BTKi within 6 months were associated with inferior post BTKi FFS and OS (Supplementary Figure 4; Figure 7). Besides, the following variables were also associated with inferior post BTKi OS: age>65 and commercial BTKi use other than participating a clinical trial (Figure 7). However, no significant impact on outcome was identified among patients receiving different generations of BTKi (P=0.491, Figure 7B). Similar conclusions were reached when we considered only patients with CLL (Supplementary Figure 5). Besides, patients with combination had a trend of better post-BTKi FFS than those with monotherapy therapy but with no statistical significance (P=0.058).

To determine the independent factors associated with post BTKi survival, we included all the factors significant in univariate analyses in a multivariate Cox model. Finally, category of disease (hazard ratio [HR] = 1.8, P=0.021), age>65 (HR = 1.6, P=0.045), with 17p deletion (HR = 2.7, P<0.001), not first-line of therapy (HR = 2.3, P<0.001), withdrawal BTKi by toxicity (HR = 2.4, P<0.001) and withdrawal BTKi within 6 months (HR = 6.4, P<0.001) were independent predictors of inferior post-BTKi survival (Table 2).

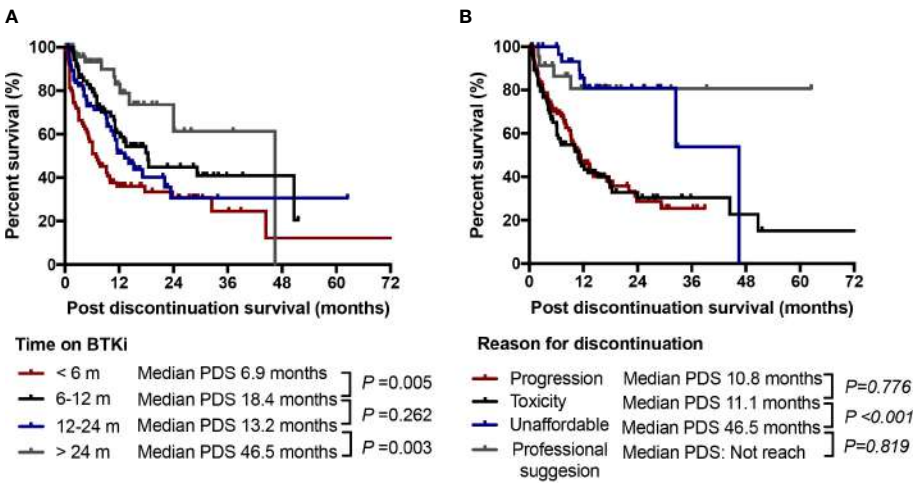

FIGURE 6
Discontinuation time and reason. **(A)** Post discontinuation survival (PDS) of patients according to time of duration on BTKi treatment; **(B)** PDS of patients according to the cause of discontinuation. NS, no significance.

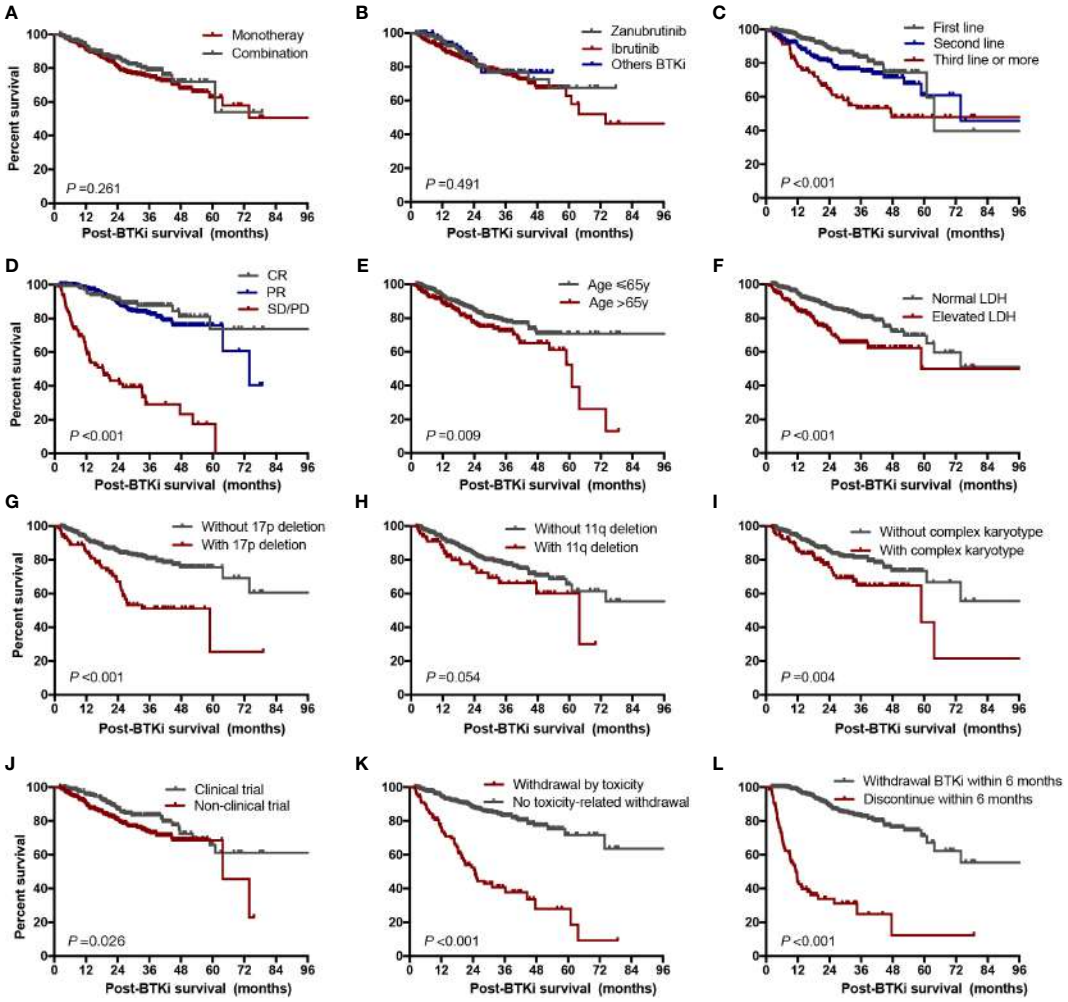

FIGURE 7
Post-BTKi survival according to prognostic factors. Overall survival after start of BTKi treatment stratified by treatment regimen **(A)**, selectivity of BTKi **(B)**, line of therapy **(C)**, depth of response **(D)**, age **(E)**, LDH level **(F)**, 17p deletion status **(G)**, 11q deletion status **(H)**, karyotype status **(I)**, clinical trial participation **(J)**, reason of discontinuation **(K)**, and duration time on BTKi **(L)**. LDH, lactate dehydrogenase.

TABLE 2 Multivariate regression models evaluating risk factors for overall survival after start of BTKi treatment.

Variable	HR (95% CI)	*P* value
Category of disease (MCL vs. CLL/WMLPL)	1.8 (1.1-3.1)	**0.021**
Age (>65 vs. ≤65)	1.6 (1.0-2.4)	**0.045**
LDH (Elevated vs. Normal)	1.4 (0.9-2.2)	0.145
Complex karyotype (Yes vs. No)	1.2 (0.8-1.9)	0.369
17p deletion (Yes vs. No)	2.7 (1.7-4.3)	**0.001**
Line of therapy (Non-first line vs. First line)	2.3 (1.5-3.6)	**0.001**
Best response of BTKi (CR vs. PR/SD/PD)	0.6 (0.3-1.2)	0.167
Clinical trial participation (Yes vs. No)	0.7 (0.4-1.2)	0.177
Withdrawal BTKi by toxicity (Yes vs. No)	2.4 (1.4-3.9)	**0.001**
Withdrawal BTKi within 6 months (Yes vs. No)	6.4 (4.0-10.3)	**0.001**

Significant P values were marked in bold. BTKi, Bruton tyrosine kinase inhibitors; CLL, chronic lymphocytic leukemia; WM/LPL, Waldenstrom macroglobulinemia/lymphoplasmacytic lymphoma; MCL, Mantle cell lymphoma HR, Hazard ratio. LDH, Lactate dehydrogenase; CR, Complete response; PR, Partial response; SD, Stable disease; PD, progressive disease.

4 Discussion

This study outlines the impact of BTKi exposure duration and reasons for discontinuation on survival in patients with BLPD. To the best of our knowledge, this series is the most comprehensive report so far on BTKi-treated patients in a clinical setting in China. Our observations showed a post-BTKi FFS of 70.3 months for untreated CLL and 40.6 months for R/R CLL. This is slightly shorter than that previously reported in clinical trials where the median progression-free survival (PFS) was not reach in untreated CLL [RESONATE-2 cohort (14)] and was 44.5 months in R/R CLL [RESONATE cohort (4)]. Nevertheless, the outcome in our cohort align with the results of the real-world analysis from other countries, such as US, UK, Denmark (15–17) (Supplementary Table 2). These findings suggest that outcomes in real-world clinical practice may be less favorable when compared to those from clinical trial patients. In our cohort, 30 percent of patients participated the BTKi-related non-blind clinical trials. The preference of first or second-generation BTKi was different, while more patients took ibrutinib in commercial use, and more patients took zanubrutinib and other BTKi in clinical trial (Supplementary Table 1). It is interesting to find that adherence time on BTKi treatment and post-BTKi FFS was similar in the two groups. But patients in clinical trial showed superior post-BTKi survival than those in commercial usage (Figure 7J, median survival 63.8 months vs. not reach, P=0.026). However, this outcome difference no longer existed in the following multivariate analysis. This superior survival in clinical trials could, in part, be attributed to a more specialized supervision and a lower incidence of comorbid patients. Although random clinical trials remain to be gold standard for evidence-based medicine, real-world evidence is crucial to bridging knowledge gaps and guide decision-making in regular clinical practice.

In spite of the outcome in our study were comparable with other real-world studies, the reasons for discontinuation were quite different in our setting. With a median follow-up of 28.8 months, the overall discontinuation of BTKi was 42.8%, which is consistent with both real-world and clinical trial studies. However, the most common reason for discontinuation in our cohort was disease progression in both first-line group and R/R group. This contrasts with clinical trial and other real-world studies where the majority of patients discontinue were due to toxic toxicity (18–20). In the long-term follow-up data of RESONATE-2 study, 41% discontinued ibrutinib treatment; of these, 21% discontinued by AE, and only 6% discontinued by progression (14). Similarly, in a real-world US setting, 41% of CLL patients (n=616) discontinued ibrutinib, and toxicity was the most common reason for discontinuation in all settings, accounting for 63.1% of discontinuations in front-line use and 50.2% in R/R use (15). This disparity could be attributed to the relatively poorer compliance of Chinese patients, resulting in dose reductions and temporary discontinuation more commonly occurred. Therefore, fewer intolerance-related discontinuations and more progression were observed in our cohort. On the other hand, most discontinuation events due to toxicity occurred within the first year on BTKi. But the cumulative incidence of progression went up year by year. Thus, the discrepancy could also be explained by a longer follow-up time in our cohort. In addition, this inter-study difference may in part be due to the biological and genotype variations between Chinese and Western BLPD patients.

Patients exhibited rapid disease progression following the discontinuation of BTKi treatment. We found patients who discontinued BTKi due to toxicity had comparably dismal outcome to those discontinued due to progression, in line with a prior real-world study (16). However, this was in contrast with prior studies reporting inferior OS for patients with progressing on ibrutinib compared with patients discontinuing due to AEs (15, 21). This inter-study discrepancy may partially be explained by improved later-line treatment options with other targeted agents such as venetoclax upon progression on BTKi. In addition, we identified the initial 6 months of BTKi treatment as critical, but the majority of discontinuations due to AEs appear in this time period. This could partially elucidate the exceedingly poor outcome in patients who discontinued due to toxicity. As a consequence, we need to precisely select patients who can tolerate and derive the most benefit from BTKi treatment. To this end, we conducted one of the most comprehensive studies to identify the independent factors in predict of post-BTKi survival. A multivariate cox model was developed taking account of demographic data, clinical characteristics, cytogenetic abnormalities, treatment patterns, treatment response, as well as reasons and timing for discontinuation. As expected, age>65 and 17p deletion were independent predictors of inferior survival following BTKi treatment. Similar findings regarding del(17p) patients were reported in a 3-year follow-up multicenter study (22), as well in the RESONATE-17 study (23). Interestingly, discontinuation due to toxicity and discontinuation within 6 months on BTKi remained predictive markers for survival when other prognostic markers were considered. Regardless of the disease subtype, number of therapy lines or presence of 17p deletion, patients with non-relapse discontinuation or early discontinuation of BTKi experienced significantly dismal outcome. These findings emphasized the importance of maintaining high adherence to BTKi. Complications, physical fitness status as well as financial barriers needs to be fully considered before initiation of BTKi treatment.

It is noteworthy that this study explored the effects of BTKi exposure duration and discontinuation reasons on survival in patients with BLPD. BTKi has become the routine clinical practice for untreated and R/R CLL and WM/LPL. While BTKi monotherapy was not one of the front-line treatments recommended by the NCCN guidelines for untreated MCL (24), it is important to note that frequent use is observed in studies leveraging real-world data. Even though the relatively high proportion of front-line BTKi usage in our MCL cohort, patients with MCL still showed markedly dismal outcome than those with CLL or WM/LPL. Therefore, in order to reduce the influence of disease variation on our conclusion, we included the category of disease into the multivariate analysis. We found the impact of the timing and reason of BTKi discontinuation on survival remained significant after adjusting for disease category.

In addition to evaluating real-world adherence to BTKi, we also discussed the impact of the selection of different BTKi. Currently,

ibrutinib is most commonly used BTKi. However, ibrutinib is associated with AE attributed to off-target effects in at least 20% of the patients, such as hemorrhage, atrial fibrillation, ventricular arrhythmias, and hypertension (25, 26). More recently, the next generation of BTKi such as Zanubrutinib and orelabrutinib have become available in clinical practice in China. The phase 3 ALPINE study showed a superior ORR and improved PFS in patients with R/R CLL treated with zanubrutinib compared with ibrutinib (27). In real-world investigation, we found patients with zanubrutinib had noticeably fewer toxicities and a lower risk of drug withdrawal than those on ibrutinib. However, the median adherence time on BTKi treatment was similar, and we did not obverse a significant difference in post-BTKi FFS or OS between the two groups. We arrived at the same conclusion when we only took account of patients with CLL. This might partially be explained by the higher proportion of R/R disease in those who took zanubrutinib than those with ibrutinib (43.0% vs. 38.6%), but the difference is not significant (P=0.318). Nevertheless, it's important to note that the retrospective nature of the study might introduce selection bias. In addition, it is difficult to collect data on dose adherence data in real-world practice. We only focused on the impact of discontinuation, while the influence of dose reduction was not addressed. Due to these limitations, the conclusion should be interpreted cautiously.

We have demonstrated the real-world treatment patterns of BTKi. Prior clinical trials have reported promising efficacy with BTKi combination therapy (28–30). However, in this study, the combination therapy group demonstrated better CR rates but similar ORR rate and relatively higher rate of toxicity. As a consequence, we observed no difference of survival outcome between the combination and monotherapy groups. It is important to carefully consider the patient tolerance and drug discontinuation before making decision of BTKi combination regimen.

5 Conclusions

In conclusion, despite higher rates of discontinuation than anticipated, our study demonstrated that BTKi is an effective and well-tolerated treatment for long-term use in Chinese patient population. Early discontinuation and withdrawal BTKi by toxicity were confirmed to be predictors of post-BTK survival, independent of response depth, lines of therapy and baseline cytogenetics including 17p deletion. Further investigation is necessary to identify patients at high risk of poor adherence, and better guide optimal individualized treatment decisions.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SY and LQ designed the study and provided leadership. YYa analyzed the data and wrote the manuscript. RL, SY, TW, YYu, QW, YYa, WS, WH, DZ, and GA managed patients and collected samples. YH, YL, and WX performed clinical data annotation. LQ, SY, and JW were responsible for checking diagnosis. All authors reviewed the manuscript and provided final approval for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1184395/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Patient attrition flowchart. BLPD, B-cell lymphoproliferative diseases; CLL, chronic lymphocytic leukemia; WM/LPL, Waldenstrom macroglobulinemia/lymphoplasmacytic lymphoma; MCL, Mantle cell lymphoma.

SUPPLEMENTARY FIGURE 2
Time on BTKi treatment stratified by treatment regimen: BTKi monotherapy or BTKi combined with other regimens.

SUPPLEMENTARY FIGURE 3
Post-BTKi survival according to disease subtypes and treatment statuses. Post-BTKi survival stratified by reason of discontinuation in CLL patients **(A)**, MCL patients **(B)**, WM/LPL patients **(C)**, treatment-naïve patients **(G)** and relapsed/refractory patients (I); Post-BTKi survival stratified by duration time on BTKi in CLL patients **(D)**, MCL patients **(E)**, WM/LPL patients **(F)**, treatment-naïve patients **(H)** and relapsed/refractory patients **(J)**.

SUPPLEMENTARY FIGURE 4
Post-BTKi failure-free survival (FFS) according to prognostic factors. FFS after start of BTKi treatment stratified by age **(A)**, clinical trial participation **(B)**, LDH level **(C)**, karyotype status **(D)**, 17p deletion status **(E)**, 11q deletion status **(F)**, treatment regimen **(G)**, depth of response **(H)**, line of therapy **(I)**. LDH, lactate dehydrogenase.

SUPPLEMENTARY FIGURE 5
Time on BTKi treatment **(A)**, post-BTKi failure-free survival **(B)** and post-BTKi overall survival **(C)** stratified by the selectivity of BTKi in patients with CLL.

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Safety and efficacy of transarterial chemoembolization combined with tyrosine kinase inhibitors and camrelizumab in the treatment of patients with advanced unresectable hepatocellular carcinoma

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Objective: This study was aimed to evaluate the efficacy and safety of transarterial chemoembolization combined with tyrosine kinase inhibitors and camrelizumab in the treatment of unresectable hepatocellular carcinoma and to explore a new therapeutic strategy for the treatment of advanced HCC.

Patients and methods: A total of 87 patients aged 18-75 years with at least one measurable lesion per Response Evaluation Criteria in Solid Tumors (version 1.1) were included in the study. TACE was administered as needed, and camrelizumab and TKI medication were initiated within two weeks and one week after TACE, respectively. The primary endpoints were progression-free survival and objective response rate.

Results: The 87 patients in this trial were last evaluated on September 28, 2022, and 35.8% were still receiving treatment at the data cutoff. A total of 34 patients (39.1%) died, and the median OS was not reached. The median PFS was 10.5 months (95% CI: 7.8-13.1). The ORR rate was 71.3% (62/87), and the DCR rate was 89.7% (78/87) per mRECIST. According to RECIST version 1.1, the ORR rate was 35.6% (31/87), and the DCR rate was 87.4% (76/87). Ten patients (11.5%) successfully underwent conversion therapy and all achieved R0 resection. Two patients achieved a complete pathological response, four achieved a major pathological response, and four had a partial response. All treatment-related adverse events were tolerated. No serious adverse events were observed, and no treatment-related deaths occurred.

Conclusions: TACE combined with TKI and camrelizumab was safe and effective in treating advanced HCC. Triple therapy may benefit patients with large tumor burden and portal vein cancer thrombus and is expected to provide a new treatment strategy for advanced HCC.

Clinical Trial Registration: ClinicalTrials.gov, identifier ChiCTR2000039508

KEYWORDS

camrelizumab, transarterial chemoembolization, tyrosine kinase inhibitors, unresectable hepatocellular carcinoma, therapeutic evaluation

1 Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide, especially in China, where HCC cases alone account for more than half of the estimated total (1). Most HCC patients have an insidious onset, and about 50% are in the middle and late stages of diagnosis, missing the opportunity for surgical excision. Transarterial chemoembolization (TACE) is a widely accepted treatment for patients with unresectable HCC. However, not all patients are beneficial, and the number of patients with disease progression increases with the number of TACE treatments, with unsatisfactory long-term survival outcomes (2, 3). In recent years, with the application of targeted drugs for liver cancer and the rise of immunotherapy, systematic therapy has made a breakthrough in the treatment of liver cancer. Systemic therapy has been recommended by several guidelines, such as BCLC and Guidelines for the Diagnosis and Treatment of Primary liver Cancer (2022 edition), recommend either a standard treatment for advanced liver cancer or a combination treatment for mid-stage liver cancer (4, 5). Targeted drugs or anti-PD-1 monotherapy have low tumor control rates, high drug resistance rates, and large adverse drug reactions. Therefore, it is urgent to study systematic combination therapy targeting different anticancer mechanisms to reduce the drug resistance rate and improve the efficacy of combination therapy (6). However, ORIENT-32, IMbrave 150, RESCUE, and Keynote-524 studies combined targeted drugs and immunotherapy in the treatment of advanced liver cancer showed a higher ORR rate (20.5%-36%) than single therapy (7, 8). These combination regimens have been recommended by multiple experts as a new first-line treatment option for patients with liver cancer (9).

TACE treatment aggravates hypoxia and immunosuppression of TME, and in addition to local tumor destruction, it has also been shown to have a systemic immune response. For example, PD-1 expression was increased in peripheral mononuclear cells after TACE, and the proportion of CD4+/CD8+ cells was decreased. The main mechanism of ICIs plus TKIs is to improve hypoxia and immunosuppressive tumor microenvironment (TME) by normalizing tumor blood vessels. Therefore, TACE in combination with TKIs and ICIs may theoretically have the potential to further improve the efficacy of uHCC (10, 11). In addition, TACE is the main treatment for advanced liver cancer, but the use of TACE alone has great limitations (12). Local treatment can effectively achieve satisfactory local control; However, it does not always translate into long-term survival benefits. On the other hand, the key to improving long-term prognosis is systemic treatment, but unsatisfactory local control effect will damage the long-term survival advantage of patients. The combination of anti-angiogenic drugs and immune checkpoint inhibitors may overcome the deficiency of TACE and increase efficacy (13). The purpose of this study was to explore the efficacy and safety of three combined treatment modalities.

2 Materials and methods

2.1 Study design and patients

The prospective study was conducted at five centers in Shandong Province. The study was approved by the Ethics Review Board, and all patients provided written informed consent. All procedures carried out in studies involving human participants complied with the ethical standards of institutional and national research councils, the 1964 Declaration of Helsinki and its subsequent amendments. Eligible patients were aged 18-75 years with unresectable advanced HCC confirmed by histopathology, or diagnostic imaging (CT or MRI) with at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The performance status of the Eastern Cooperative Tumor Group (ECOG) was 0 or 1, and the Child-Pugh classification was A to B. Patients with the following conditions were excluded: 1) existing immunodeficiency disease or a history of organ transplantation; 2) allergic to camrelizumab active ingredients or excipients; 3) those who participated in other clinical trials; 4) severe cardiopulmonary and coagulation insufficiency, which cannot receive combined therapy with TACE plus targeted drugs and camrelizumab; 5) history of other malignant tumors.

2.2 Procedures

During the treatment of TACE, the Seldinger method was used for percutaneous femoral artery catheterization with catheter placed in the abdominal trunk and the common hepatic artery for DSA imaging. The imaging images were collected, including the arterial, parenchymal, and venous phases. In some patients, the superior mesenteric artery, right renal artery, and right phrenic artery were performed to find the blood supply of tumor collateral when necessary. The microcatheter was used to superselect tumor supplying arteries, and all tumor supplying arteries should be superselected successively according to the results of arteriography. The chemotherapy drugs included epirubicin hydrochloride 40-80 mg and oxaliplatin 100 mg. One part of the chemotherapy drugs was injected through the microcatheter, and then the other part was mixed with epirubicin and hydroxycamtocrin and iodide to form an emulsion. At the same time, granular embolization agents (polyvinyl alcohol granules and gelatin sponge granules) were injected alternately for combined embolization. After embolization was complete, angiography was performed again to determine whether the tumor was completely embolized. If there was a local deficiency, embolization was continued until angiography showed complete embolization. Large tumors and multiple tumors in the left and right lobes were treated with TACE twice to prevent liver and kidney failure.

Oral TKI medication should be started within one week after the syndrome was relieved after TACE therapy. The three available TKIs were sorafenib (400 mg, twice a day), regorafenib (80-160 mg, once daily, for 21 days, 7 days off), and lenvatinib. For lenvatinib, patients with a body mass of ≥ 60 kg received an initial dose of 12 mg once a day, and patients with a body mass of < 60 kg received an initial dose of 8 mg once a day. Discontinue TKI medication 3 days before and after TACE treatment. When the patient had a large adverse reaction, the dose of the TKI drug was reduced or taken orally every other day. When adverse drug reactions cannot be tolerated, the medication was discontinued.

Camrelizumab (200 mg intravenously) was administered within two weeks after the syndrome was relieved after TACE treatment and repeated every 3 weeks. If an immunotherapy-related serious adverse event (irSAE) occurred, camrelizumab was discontinued, and immunosuppressants were administered depending on the severity of the complication and the affected organ.

Tumor imaging was conducted at baseline, week 6, week 12, week 18, week 24, and then every 9 weeks thereafter, by contrast-enhanced computed tomography and magnetic resonance imaging. The response was assessed on the basis of the Response Evaluation Criteria in Solid Tumors version 1.1 and Modified solid tumor Evaluation Criteria.

Early combination therapy was defined as treatment with camrelizumab and TKI prior to the first TACE treatment. Late-stage combination therapy was defined as treatment with camrelizumab and TKI after the first TACE treatment. The timing of the use of camrelizumab in combination with TKI was determined by the physician and patient. Excision was performed after a successful descent with sufficient future residual liver (FLR). If researchers observed evidence of clinical benefit, patients may continue the combination therapy after disease progression or be treated with monotherapy.

2.3 Outcomes

Efficacy outcomes included complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD), disease control rate (DCR), progression-free survival (PFS), and overall survival (OS). Efficacy was assessed according to Modified solid tumor Evaluation Criteria (mRECIST) and RECIST version 1.1. DCR was defined as the sum of CR, PR, and SD. PFS referred to the time between the initiation of combination therapy and tumor progression or death from any cause. OS referred to the time from the start of combination therapy to the last follow-up or the time of death from any cause.

Adverse events (AEs) were evaluated using the National Cancer Institute Common Terminology Criteria for adverse events version 5.0. Post-embolization syndrome after TACE treatment referred to a series of clinical symptoms such as nausea, vomiting, abdominal pain, fever, and decreased appetite caused by ischemic necrosis of tumor tissue after TACE treatment.

2.4 Statistical analysis

All results were statistically analyzed by SPSS 26.0 (SPSS, Chicago, IL, USA). The categorical variable is shown as frequency (percentage) and compared using the Chi-square test or Fisher's exact test when appropriate. The Kaplan-Meier method was used to estimate the median OS and PFS. A two-sided p-value< 0.05 was considered statistically significant.

3 Results

3.1 Patient characteristics

A total of 87 patients were enrolled between August 2020 and November 2021 (Table 1). All patients received TKI+TACE+ camrelizumab combined therapy. The median age was 56 years (range 34-75), and 81 (93.1%) were male. There were 28 patients (32.2%) with an ECOG performance status 1, 38 (58.6%) with Child-Pugh grade A, 47 (54.0%) with extrahepatic metastases, and 65 (74.7%) with portal vein cancer thrombus. Sixty-nine patients (79.3%) had BCLC stage C, and 75 (86.2%) had hepatitis B infection. Thirty-one (35.6%) patients received sorafenib, 54 (62.1%) patients received lenvatinib, and two patients received regorafenib.

3.2 Efficacy

As of September 28, 2022, the median duration of follow-up was 13.6 (0.83-24.9) months. A total of 34 patients (39.1%) died, and the median OS was not reached. The OS rates of 6, 12 and 18 months

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TABLE 1 Baseline characteristics.

Characteristics	TACE plus TKI plus camrelizumab (n=87)
Age	
Median, years (range)	56 (34~75)
<65	70 (80.5%)
≥65	17 (19.5%)
Sex, n (%)	
Male	81 (93.1%)
Female	6 (6.9%)
Median tumor diameter, cm	8.2 (4.8~17.9)
ECOG performance status, n (%)	
0	46 (52.9%)
1	28 (32.2%)
NE	13 (14.9%)
Child-Pugh Class, n (%)	
A	51 (58.6%)
B	29 (33.3%)
NE	7 (8.1%)
CNLC, n (%)	
I	5 (5.7%)
II	14 (16.1%)
III	46 (52.9%)
NE	22 (25.3%)
Portal vein tumor thrombus, n (%)	
Vp0-2	40 (46.0%)
Vp3-4	25 (28.7%)
HBV infection, n (%)	
Yes	75 (86.2%)
No	12 (13.8%)
Extrahepatic metastasis, n (%)	
Yes	43 (49.4%)
No	44 (50.6%)
Number of previous TACE, n (%)	
0	45 (51.7%)
1-2	39 (44.8%)
≥3	3 (3.5%)
AFP (ng/mL), n (%)	
≥400	36 (41.4%)
<400	51 (58.6%)
TKI type, n (%)	
Sorafenib	31 (35.6%)
Lenvatinib	54 (62.1%)
Regorafenib	2 (2.3%)

NE, Not evaluable; CNLC, China liver cancer staging.

were 84.6%, 73.4% and 55.2%, respectively (Supplementary Figure 1). The median PFS was 10.5 months (95% CI: 7.9-13.1) (Figure 1). According to mRECIST, the ORR rate was 71.3% (62/87) and the DCR rate was 89.7% (78/87), including 16 patients with CR, 46 patients with PR, 16 patients with SD, and six patients with PD. According to RECIST1.1, the ORR rate was 35.6% (31/87) and the DCR rate was 87.4% (76/87); there were 31 patients with PR, 45 with SD, and eight with PD, and none with CR (Table 2). Compared to the baseline, the target lesion burden decreased in 78 (89.7%) patients (Figure 2). Ten of the patients (11.5%) successfully underwent conversion therapy. All patients achieved R0 resection. There were no Clavien-Dindo III or higher complications. Common postoperative complications such as pain and elevated aminotransferase were effectively controlled after symptomatic management. Postoperative pathology reports included two complete pathological responses (cPR), four pathological responses (MPR), and four partial responses (pPR) (Figure 3).

Figures 4 , 5 show the analysis of ORR and PFS in different subgroups. Poisson regression with robust error variance calculated treatment effectiveness and 95% confidence intervals. The ORR and PFS showed consistent benefits in subgroups based on ECOG score, HBV infection, baseline alpha-fetoprotein level, combined TKI, and the number of TACE treatments. In addition, the combination of triple therapy with lenvatinib was more beneficial than that with sorafenib, and there was a trend toward benefit among the TACE-naïve group as compared with the previous TACE-treated group. Late-stage combination therapy significantly improved PFS than early combination therapy (Supplementary Table 1).

3.3 Safety

After enrollment, the median number of interventional therapy was two times (1-9 times), and the cumulative total number of interventional therapy was 203 times. The median treatment period for camrelizumab was 6 cycles (range 1-25). The adverse reactions during the observation period are shown in Table 3. A total of 87 patients (100%) who received combination therapy developed at least one treatment-related AE. The most common AEs were hypoproteinemia (80 cases), elevated lactate dehydrogenase (70 cases), elevated glutamic oxalacetic transaminase (69 cases), elevated bilirubin (68 cases), abdominal pain (54 cases), nausea (29 cases), and RCCEP (23 cases). The incidence of grade 3-4 adverse reactions was 67.8%, and no treatment-related deaths

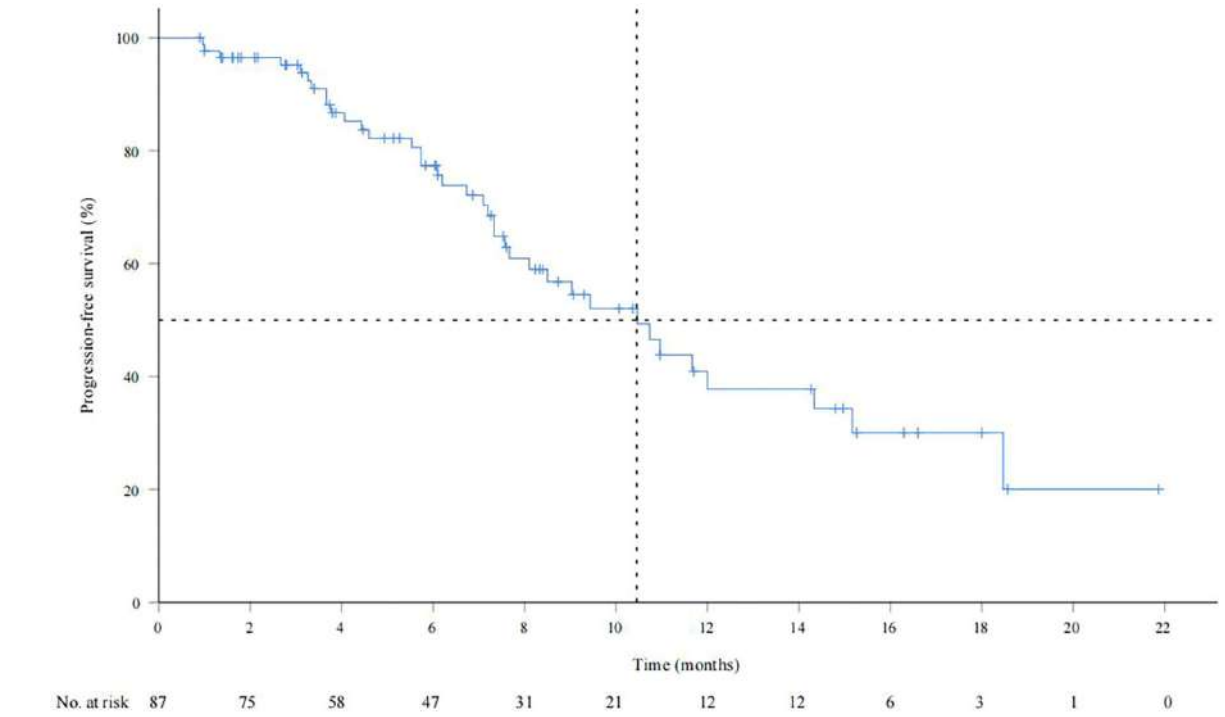

FIGURE 1
Progression-free survival in the all patients by the Kaplan Meier method per RECIST v1.1.

occurred. After three cycles of camrelizumab combined with lenvatinib, one patient developed liver failure, which was cured and stopped targeted therapy and immunotherapy after prednisolone and hepatoprotective therapy. Related AE symptoms or signs were relieved or eliminated after symptomatic treatment, dose reduction, or interruption of medication.

4 Discussion

TACE is currently the standard first-line treatment for mid-stage HCC recommended by various guidelines around the world (4, 14). It is also a common modality of palliative treatment for advanced HCC. However, mid-stage and late-stage HCC has a large tumor burden and is characterized by intratumoral heterogeneity, often accompanied by portal vein cancer thrombus and arteriovenous fistula, which often require multiple TACE treatments in a short period of time. After TACE treatment, the tumor often makes it difficult to achieve pathological complete necrosis (15). In addition, for large liver cancer or massive liver cancer, repeated TACE will cause serious damage to liver function. Still, after these treatments fail, there is a lack of corresponding remedial measures (16). Based on this situation, it is necessary to explore effective treatment programs based on TACE (17).

TABLE 2 Response to combined therapy.

Tumor response	RECIST1.1 n (%)	mRECIST n (%)
CR	0	16 (18.4%)
PR	31 (35.6%)	46 (52.9%)
SD	45 (51.7%)	16 (18.4%)
PD	8 (6.1%)	6 (6.9%)
NE	3 (3.4%)	3 (3.4%)
ORR(CR+PR)	31 (35.6%)	62 (71.3%)
DCR(CR+PR+SD)	76 (87.4%)	78 (89.7%)

CR, Complete response; PR, Partial response; SD, Stable disease; PD, Progressive disease; NE, Not evaluable; ORR, Objective response rate; DCR, Disease control rate.

In recent years, immunotherapy has achieved the obvious curative effect in the field of liver cancer (18), while TACE combined immunotherapy has strong rationality in theory, which can directly kill or inhibit the growth of tumor cells, destroy the release of tumor antigen substances by tumor cells, enhance the immune effect, and thus improve the curative effect and prolong the survival of patients. In addition, targeted combination immunotherapy can synergically enhance the efficacy of TACE, and TKI combined immunotherapy will help eliminate the factors of tumor recurrence caused by tumor angiogenesis after TACE (19). Although PD-1 immunotherapy has shown promising efficacy in the treatment of HCC, its efficacy is still less than 20% when used alone. IMbrave 150 and RESCUE studies confirmed that target-free therapy showed good efficacy and safety in first-line or second-line therapy for advanced HCC, providing a new treatment option for unresectable HCC (6, 7, 20). In these clinical studies, most patients received local therapy, including TACE (21), but there were few studies on TACE combined with anti-angiogenic therapy and anti-PD-1 antibody.

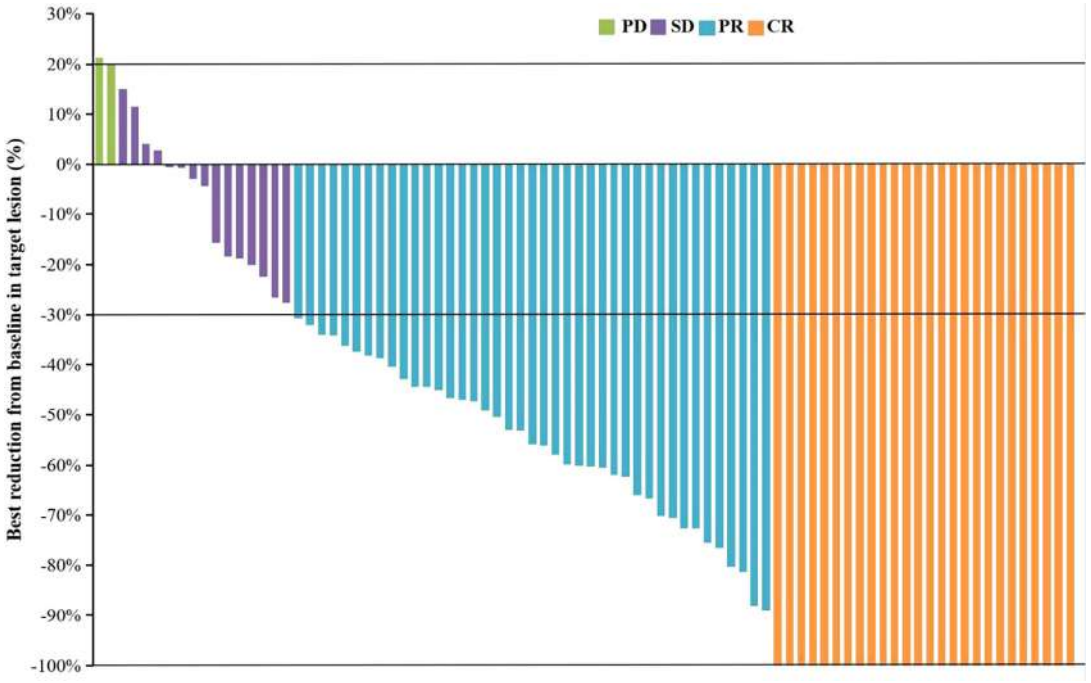

FIGURE 2
The best change from baseline in the sum of the target lesion diameter per patient. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

This study evaluated the efficacy and safety of TACE + TKI + PD-1 antibody in the treatment of unresectable HCC and explored the factors influencing its prognosis. The median OS was not reached, and the median PFS was 10.5 months (95% CI 7.8-13.1), the ORR was 35.6% and the DCR was 87.4% (RECIST version 1.1), higher than the previous PD-1 inhibitors or TKI monotherapy, and higher than RESCUE study results (19). Apatinib combined with camrelizumab for first-line and second-line treatment of unresectable HCC had PFS of 5.7 and 5.5 months, respectively, and the ORR of first-line treatment in the phase 2 RESCUE study was 34.3%. In the IMbrave150 trial, 48% of patients in the atezolizumab plus bevacizumab group received prior topical therapy, while more than 60% of patients in the RESCUE trial received interventional therapy. This study showed that TACE combined with TKI and camrelizumab in the treatment of unresectable HCC patients had higher overall survival and tumor response rates, suggesting that TACE combined with targeted therapy and immunotherapy is a promising treatment option. The possible reason is that TACE can cause ischemic tumor necrosis, thus reducing tumor burden, resulting in tumor tissue release of tumor antigen, increased expression of PD-1 and PD-L1, and improved tumor recognition. Anti-vascular endothelial growth factor (VEGF) therapy normalizes the tumor vasculature, reduces additional VEGF-mediated immunosuppression in the tumor and its microenvironment, and promotes T-cell infiltration (22, 23). Therefore, the combination of TACE, TKI, and camrelizumab may produce synergistic antitumor effects and improve clinical outcomes in patients with unresectable liver cancer.

Subgroup analyses of ORR and PFS based on baseline characteristics found that combination with lenvatinib showed better benefits than combination with sorafenib in the triple therapy, which was consistent with the results of the REFLECT study (24). Lenvatinib showed a trend of benefits compared with sorafenib in both alone and combined therapy. In addition, those who did not receive TACE treatment in the past also tended to benefit more than those who did. This may be due to repeated TACE treatments damaging liver function and embolic hypoxia increasing VEGF, which can promote tumor recurrence and metastasis (22). The PFS of late combined target therapy with TACE was longer than early combined therapy, which may be related to the time-consuming process of tumor ischemia-hypoxic necrosis releasing large amounts of antigen and altering the tumor microenvironment after TACE.

Tumor burden and portal vein cancer embolism are factors for poor prognosis of patients with advanced liver cancer. Effective therapeutic approaches must be explored to control tumor progression, reduce tumor volume, and improve patient prognosis. Therefore, transformation therapy has become a research hotspot for unresectable HCC and middle and advanced HCC (25). Although neoadjuvant therapy for colorectal cancer, lung cancer, breast cancer, and other tumors is relatively common, due to the insensitivity of HCC to traditional chemotherapy and radiotherapy, neoadjuvant therapy and transformation therapy have not made breakthrough progress in HCC. In this study, 74.7% of patients had portal vein cancer thrombus, and 28.7% had portal vein main cancer thrombus. In the treatment process of

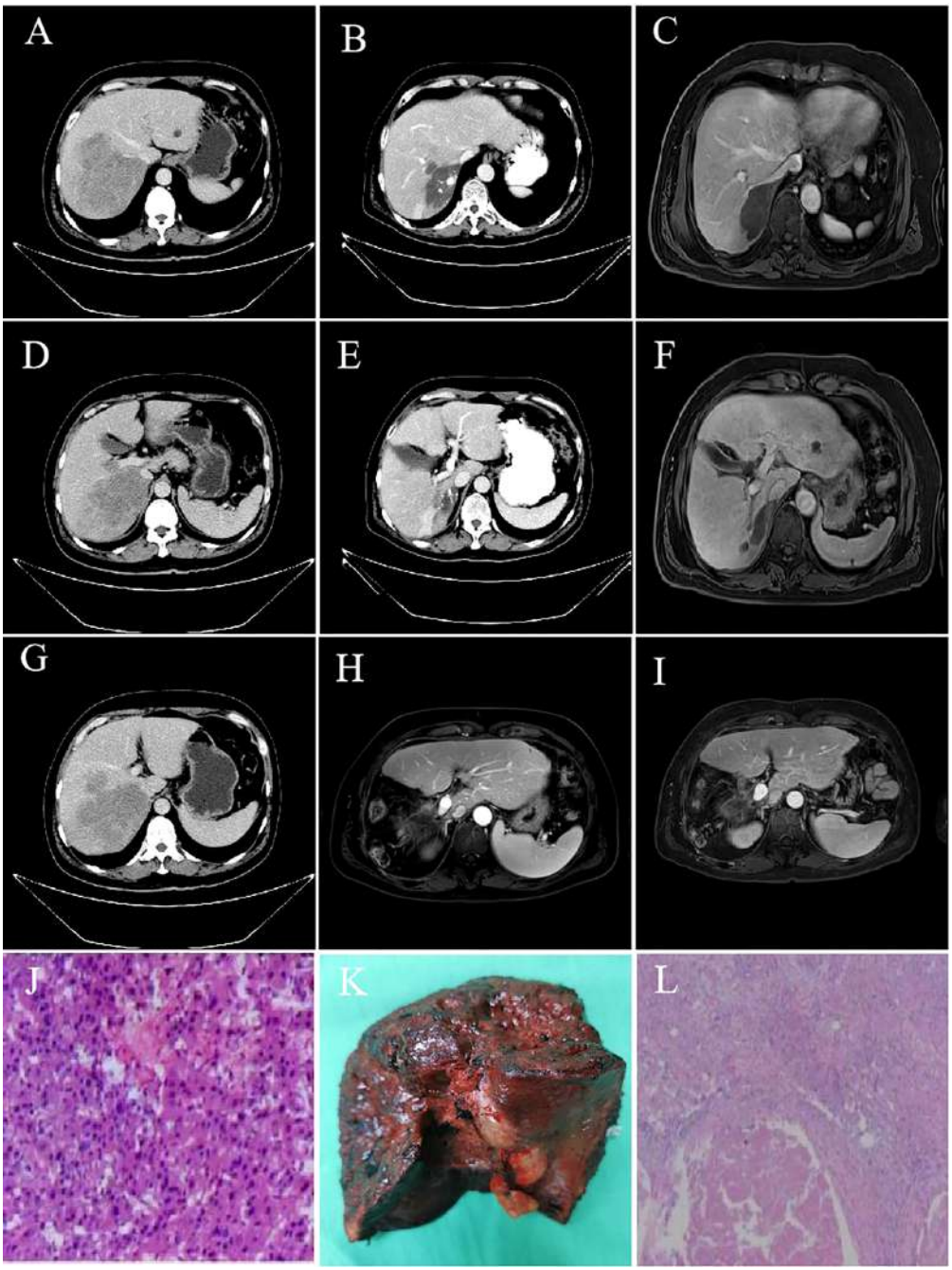

FIGURE 3
Imaging data and postoperative pathology of a 55-year-old male patient with advanced liver cancer after successful conversion therapy. **(A, D, G)** hcc with main carcinoma thrombus of the right portal vein; **(B, C, E, F)**: After 2 TACE combined with camrelizumab and TKI treatment for five cycles, liver lesions shrank, and portal vein cancer thrombus retreated; **(H)**: No intrahepatic recurrence or metastasis one month after resection; **(I)**: No intrahepatic recurrence or metastasis in 20 months after resection; **(J)**: Pathological results of baseline biopsy; **(K)**: Postoperative pathological specimens; **(L)**: The pathological results of the lesion resection after combination therapy showed that the tumor and cancer thrombus were completely necrotic, no cancer cells were detected, inflammatory cell infiltration and interstitial fibrosis reached pathologic complete response.

TACE combined with TKI and PD-1 antibody, ten (11.5%) patients underwent surgery. Notably, two patients achieved pCR, four patients achieved MPR. Eight patients with obvious lipiodide deposition and cancer thrombus retracting to secondary branches, which achieved the purpose of downstaging. None of the patients had serious complications such as liver failure, gastrointestinal bleeding, pulmonary embolism, acute renal failure, severe infection, and bile duct infarction. The results of this study demonstrated the safety and efficacy of TACE combined with targeted therapy and immunotherapy for HCC with portal vein thrombus.

The safety profile of triple therapy was consistent with those of the individual drugs. No new safety signals were observed. But all of these adverse events are generally manageable. Notably, compared

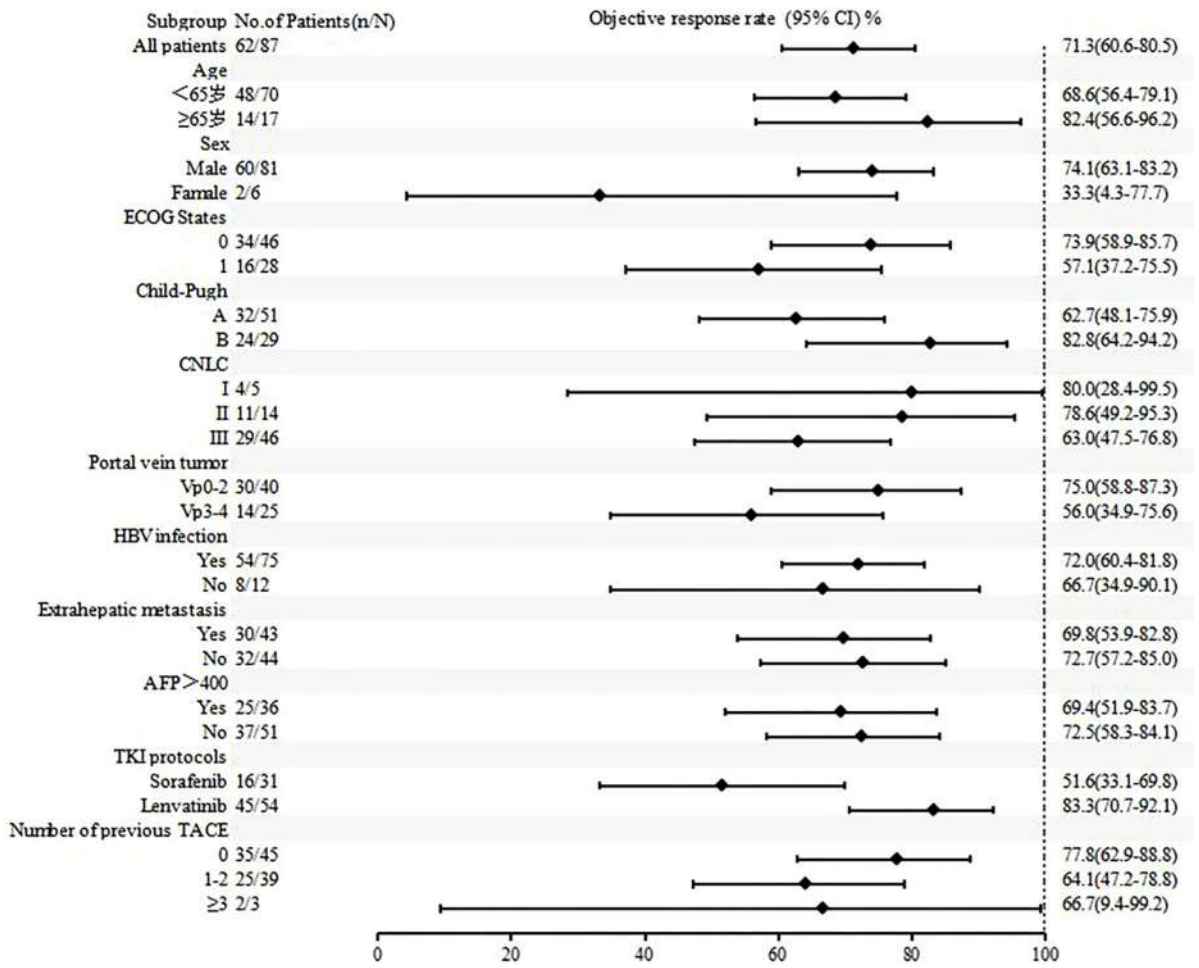

FIGURE 4
Subgroup analysis of objective response rate according to baseline characteristics.

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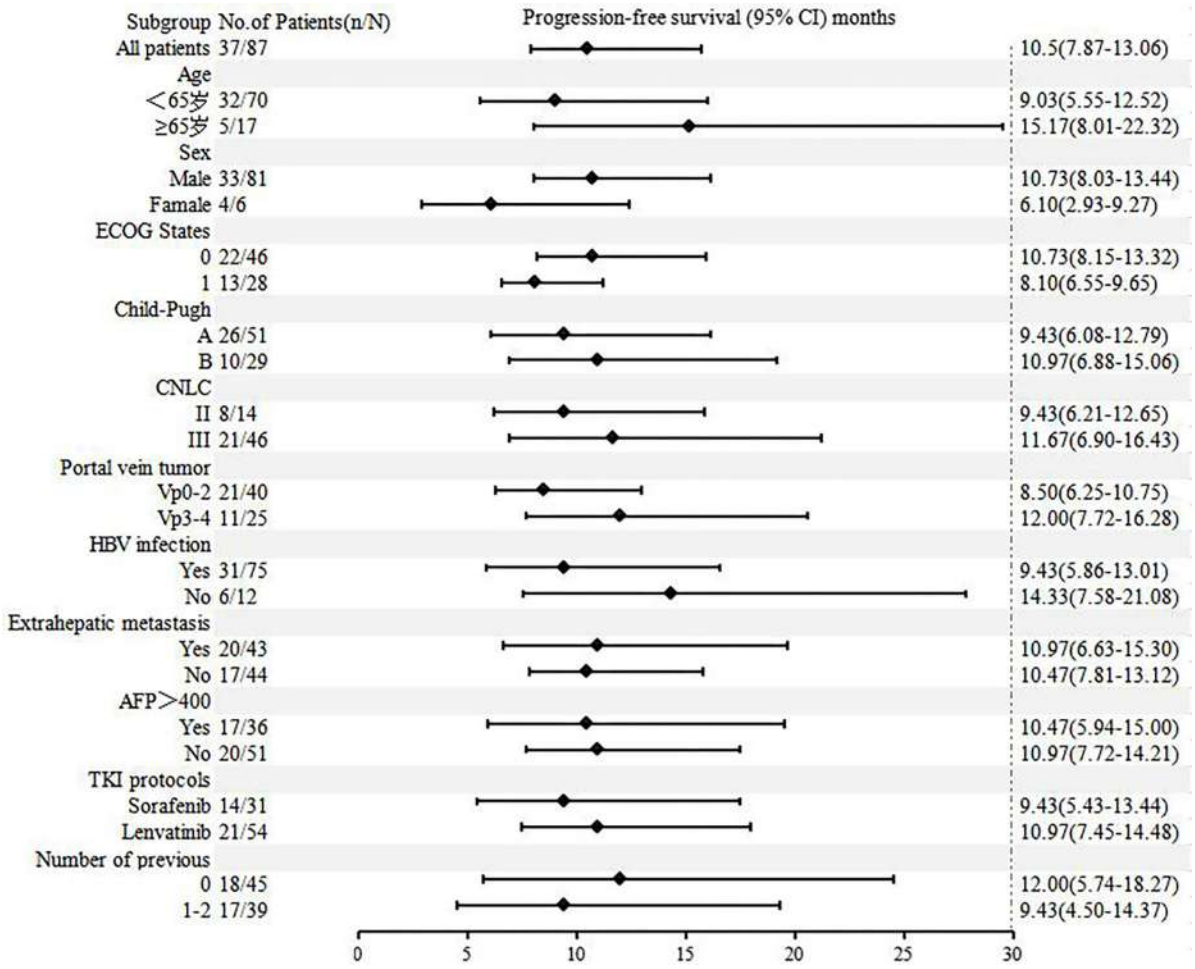

FIGURE 5
Subgroup analysis of progression-free survival according to baseline characteristics.

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TABLE 3 Adverse events.

Adverse events	Any Grade		Grades 3-4	
	N	%	N	%
Hematological toxicities				
Hypoalbuminemia	80	92.0	1	1.1
Binding bilirubin increased	68	78.2	11	12.6
Lymphocyte count decreased	66	75.9	31	35.6
Blood bilirubin increased	64	73.6	9	10.3
Lactate dehydrogenase increased	70	80.5	0	0
Platelet count decreased	65	74.7	15	17.2
Alanine aminotransferase increased	53	60.9	10	11.5
Aspartate aminotransferase increased	69	79.3	14	16.1
Anemia	52	59.8	9	10.3
White blood cell count decreased	43	49.4	7	8.0
Neutrophil count decreased	45	51.7	9	10.3
Nonhematological toxicities				
Reactive cutaneous capillary endothelial proliferation	23	26.4	5	5.7
Nausea	29	33.3	0	0
Pyrexia	21	24.1	0	0
Abdominal pain	54	62.1	8	9.2
Vomiting	13	14.9	0	0

with camrelizumab monotherapy, the incidence of RCCEP was reduced in combination therapy, which may be due to the involvement of VEGF signaling pathways in the mechanism of RCCEP, and other studies reported similar findings (26).

There are several limitations to this study. First, the sample size of this study is relatively small, which may reduce the statistical efficacy. Second, the follow-up time in this study was short, and a longer follow-up time was needed to verify the further OS. Third, there were few cases in the combination timing group, and finding the right combination therapy timing is still necessary.

5 Conclusion

In summary, TACE combined with TKI and camrelizumab has longer progression-free survival benefits and a high tumor control rate with a manageable safety profile in the treatment of advanced liver cancer. Thus, the combination regimen could provide a new treatment option for this patient population.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by China Ethics Committee of Registered Clinical Trial. The patients/participants provided their written informed consent to participate in this study.

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JLS and JL conceived and designed the study. JLS, JL, MK, GY, SW, and ZS enrolled patients and collected the data. HH and YL analyzed the data. All authors participated in data interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors HH and YL were employed by the company Jiangsu Hengrui Medicine.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1188308/full#supplementary-material

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Case Report: Molecular and microenvironment change upon midostaurin treatment in mast cell leukemia at single-cell level

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Mast cell leukemia is a rare and aggressive disease, predominantly with *KIT* D816V mutation. With poor response to conventional poly-chemotherapy, mast cell leukemia responded to the midostaurin treatment with a 50% overall response rate (ORR), but complete remission rate is approximately 0%. Therefore, the potential mechanisms of midostaurin resistance and the exact impacts of midostaurin on both gene expression profile and mast cell leukemia microenvironment *in vivo* are essential for design tailored combination therapy targeting both the tumor cells and the tumor microenvironment. Here we report a 59-year-old male mast cell leukemia patient with *KIT* F522C mutation treated with midostaurin. Single-cell sequencing of peripheral blood and whole exome sequencing (WES) of bone marrow were performed before and 10 months after midostaurin treatment. In accordance with the clinical response, compared to the pretreatment aberration, the decline of mast cells and increase of T-, NK, B-cells in peripheral blood, and the decrease of the *KIT* F522C mutation burden in bone marrow were observed. Meanwhile, the emergence of *RUNX1* mutation, upregulations of genes expression (*RPS27A*, *RPS6*, *UBA52*, *RACK1*) on tumor cells, and increased frequencies of T and NK cells with *TIGIT, CTLA4*, and *LAG3* expression were observed after midostaurin treatment, predicting the disease progression of this patient. As far as we know, this is the first case reporting the clinical, immunological, and molecular changes in mast cell leukemia patients before and after midostaurin treatment, illustrating the *in vivo* mechanisms of midostaurin resistance in mast cell leukemia, providing important clues to develop a sequential option to circumvent tumor progression after targeting oncogene addiction and prolong patients' survival.

KEYWORDS

mast cell leukemia, midostaurin, molecular changes, single-cell RNA sequencing, tumor micoenvironment

1 Introduction

Mast cell leukemia is a rare but aggressive form of systemic mastocytosis (SM) characterized by the expansion of neoplastic mast cells in bone marrow (BM) and peripheral blood (1). Pathogenic mutation of *KIT* D816V is found in 80% of SM; *TET2, SRSF2, ASXL1, RUNX1, JAK2*, and *RAS* are also frequently mutated and related to poor prognosis of mast cell leukemia patients (1, 2). No curative therapy is yet available for mast cell leukemia other than allogeneic hematopoietic stem cell transplantation; a median survival time in mast cell leukemia patients is less than 12 months when treated with chemotherapy or *KIT* inhibitors (2). Midostaurin is a small molecule inhibitor of multiple tyrosine kinase receptors and has been approved by the FDA and EMA as a *KIT* inhibitor in treating mast cell leukemia, with an overall remission rate of 50%, a complete remission (CR) rate of 0%, and a median progression-free survival (PFS) of 11.3 months (3). Reasons why mast cell leukemia responded to midostaurin but displayed a low CR rate and the underlying mechanisms behind relapse *in vivo* after midostaurin treatment were not clearly specified. In our study, we performed whole exome sequencing (WES) of bone marrow and single-cell RNA sequencing of peripheral blood on a mast cell leukemia patient before and after midostaurin treatment to illustrate the *in vivo* mechanism of midostaurin on mast cell leukemia.

2 Case presentation

In our study, we reported a 59-year-old male who experienced recurrent diarrhea, severe weight loss, urticaria, anemia and weakness. He came to our hospital in June 2018 and blood tests showed white blood cells (WBCs) 18.4×10^9/L ($3.97\text{-}9.15\times10^9$/L), red blood cells 1.64×10^{12}/L ($4.09\text{-}5.74\times10^{12}$/L), hemoglobin 8g/dl (13.1-17.2 g/dl), platelets 142×10^9/L ($85\text{-}303\times10^9$/L), and alkaline phosphatase (AKP) 196 U/L (38-126 U/L). BM examinations indicated 84% abnormal mast cells with irregular sizes and positive toluidine blue staining. Peripheral blood aspirate demonstrated neoplastic mast cells with immature nuclear chromatin, abundant tightly packed cytoplasmic granules and high nuclear to cytoplasmic (N:C) ratio (Figure 1A). Flow cytometric analysis was performed on BM sample before treatment to observe mast cells in circulation and used as evidence for diagnosis according to the diagnose criteria of mast cell leukemia (2). As shown in Supplementary Figure 1, a significant population was observed in bone marrow sample showing extremely bright expression of CD117 and positive CD45 with moderate FSC and SSC (red color). Gating on CD117 bright expression cells, the population expressed CD13, CD33, CD203c and CD2 (partially), but not lineage specific markers such as cytoplasmic MPO, CD3, CD79a or the remaining markers. The very bright expression of CD117 combining the positive of CD203c indicated the mast cell origin. The partial expression of CD2 was considered as a common feature of abnormal mast cells. We further stained CD25 and obtained negative result. WES of BM cells revealed *KIT* F522C mutation and *SETD2* mutation, but negative for *TET2*, *ASXL1*, *BCR-ABL*, *JAK2*, *FLT3*, and *NPM1* mutations (Figure 1B). According to the dose modification instructions of midostaurin (4), the patient was treated with 100 mg/d midostaurin due to anemia and severe fatigue, and achieved a major response 10 months after treatment, with mast cells decreasing to 24% in BM. Meanwhile, blood smears showed mast cells with condensed nuclear chromatin, less granules in cytoplasm and lower N:C ratio (Figure 1A). Upon midostaurin treatment, compared to the pretreatment gene mutation profiles, variant allele frequency (VAF) was also significantly decreased in genes involved with the *RAS-MAPK* pathway, such as *KIT* and *ERBB3*. However, *RUNX1* mutation, relating to poor prognosis of mast cell leukemia patients, was only detected after midostaurin treatment, which might indicate subsequent midostaurin resistance. (Figure 1B). Gene set enrichment analysis (GSEA) showed the top 5 downregulated pathways, including *PI3K/AKT* signaling in cancer, regulation of cell division, *MAPK* family signaling cascade, cell response to growth factor stimulus, and regulation of cell cycle process (Figure 1C), in which *PI3K/AKT* and *MAPK* signaling pathway are the downstream of *KIT* signaling and are associated with mast cell leukemia progression (5).

Before treatment, single-cell RNA sequencing revealed that mast cells were the major fraction (81.40%) in peripheral blood mononuclear cells (PBMC) (Figures 1D–F). After 10 months of midostaurin treatment, the fraction of mast cells in peripheral blood decreased significantly (from 81.40% to 25.10%), while immune cells increased, including T cells (from 6.79% to 41.67%), Natural killer (NK) cells (from 8.07% to 22.16%) and B cells (from 2.18% to 8.72%) (Figure 1G). The top 5 downregulated pathways on mast cells included positive regulation of blood vessel endothelial migration, vascular endothelial growth factor receptor signaling, IL-4 and IL-13 signaling, *NF-KB* signaling regulation, and cytokine production pathways regulation (Figure 1H). The expression level of genes such as *S100A4*, *LGALS1*, *TPSB2*, and *S100A6* were decreased. Instead, the expression level of *RPS27A, RPS6*, *UBA52*, and *RACK1*, involved in tyrosine kinase inhibitors (TKI) resistance, were increased after midostaurin treatment (Figures 1I, J).

Regarding the impact of midostaurin on mast cell leukemia microenvironment, pathways upregulated on T cells mainly included antigen presenting, T cell cytotoxicity, interferon γ signaling, T cell receptor signaling, regulation of immune response, and T cell-mediated immunity (Figure 2A). Among NK cells, antigen presentation, regulation of cell killing, natural killer cells mediated cytotoxicity, leukocyte mediated cytotoxicity, regulation of cell surface receptor, immune response, and cytokine production pathways were upregulated (Figure 2B). However, upon further classifying T and NK cells into 4 clusters, we found that most immune checkpoints such as *TIGIT, CTLA4, LAG3*, and *HAVCR2* (also named *TIM3*) were highly expressed on cluster 1 and the cell ratios of cluster 1 in both T and NK cells were significantly increased after midostaurin treatment ($P<0.05$, Figures 2C, D). Further GSEA revealed that as compared to other clusters, the downregulated pathways on cluster 1 cells, including adaptive immune system, T cell activation, antigen processing and presentation pathways on cluster 1 of T cell (Supplementary Figure 2A), as well as vesicle-mediated transport, TCR, endosome

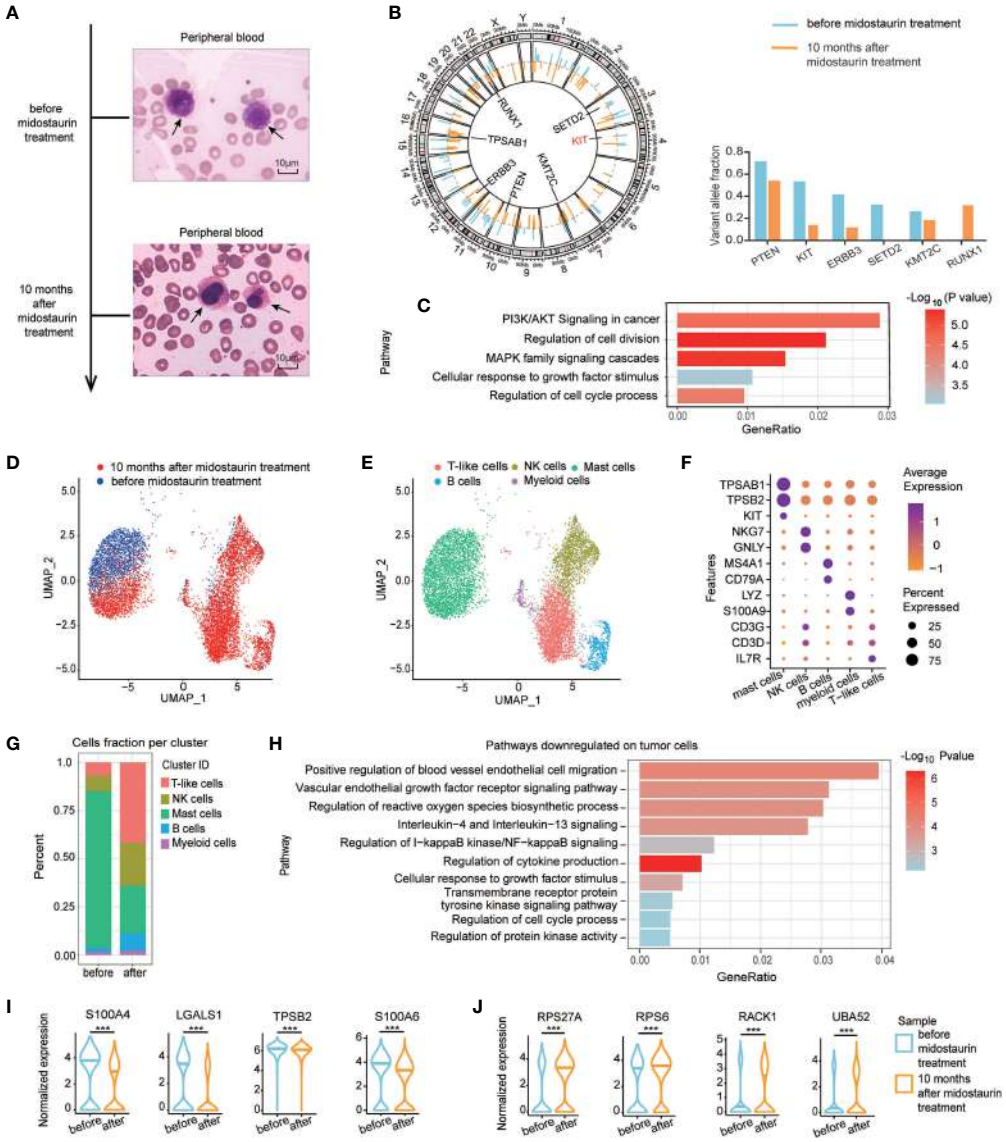

FIGURE 1
The morphological characteristics, mutation profiles, and single-cell analysis of tumor cells in the patient before and after midostaurin treatment. **(A)** Peripheral blood aspirate under Wright's stain, demonstrating neoplastic mast cells with immature nuclear chromatin, abundant tightly packed cytoplasmic granules, high nuclear to cytoplasmic (N:C) ratio before treatment (top) and mast cells with condensed nuclear chromatin, less granules in cytoplasm and lower N:C ratio after 10 months of midostaurin treatment (bottom), bar=10μM. **(B)** Circos plot of single-nucleotide variations based on WES before and after midostaurin treatment. The outer lines colored in blue represent mutations detected before treatment. The inner lines colored in orange represent mutations detected after treatment. **(C)** Gene set enrichment analysis of genes decreased with mutation burden after treatment. **(D)** UMAP of single-cell RNA sequencing experiments of patient PBMCs before and after midostaurin treatment. Each cell represents a cell. Blue and red represent cells collected before or after 10 months of midostaurin treatment. **(E)** UMAP of single-cell RNA sequencing experiments as in **(D)** Annotated cell types are distinguished by colors. **(F)** Dot plot of cell-type-specific marker genes used to annotate the clusters in single-cell RNA sequencing experiments. **(G)** Fraction of different cell types before and after midostaurin treatment. **(H)** Pathways downregulated on tumor mast cells after midostaurin treatment. Normalized gene expression of downregulated genes **(I)**, and upregulated genes **(J)** on tumor cells after midostaurin treatment. ***, represented significant difference of P<0.001.

to lysosome transport pathways on cluster 1 of NK cell (Supplementary Figure 2B), indicating the impaired functions of antigen presenting and cell cytotoxicity in these T/NK cells.

Finally, the patient had disease progression 15 months after midostaurin treatment, with a percentage of mast cells in BM reaching 77.9% in September 2019; the patient was then treated with dasatinib. Unfortunately, he had no response (without any clinical improvement) to dasatinib, but displayed severe pleural effusion. Due to the poor response to dasatinib, the patient received low dose chemotherapy with azacitidine plus homoharringtonine and arsenic trioxide, but showed no response to these treatments. Eventually, the patient died of disease progression in 2020, with severe pleural effusion and ascites, with an OS of 24 months.

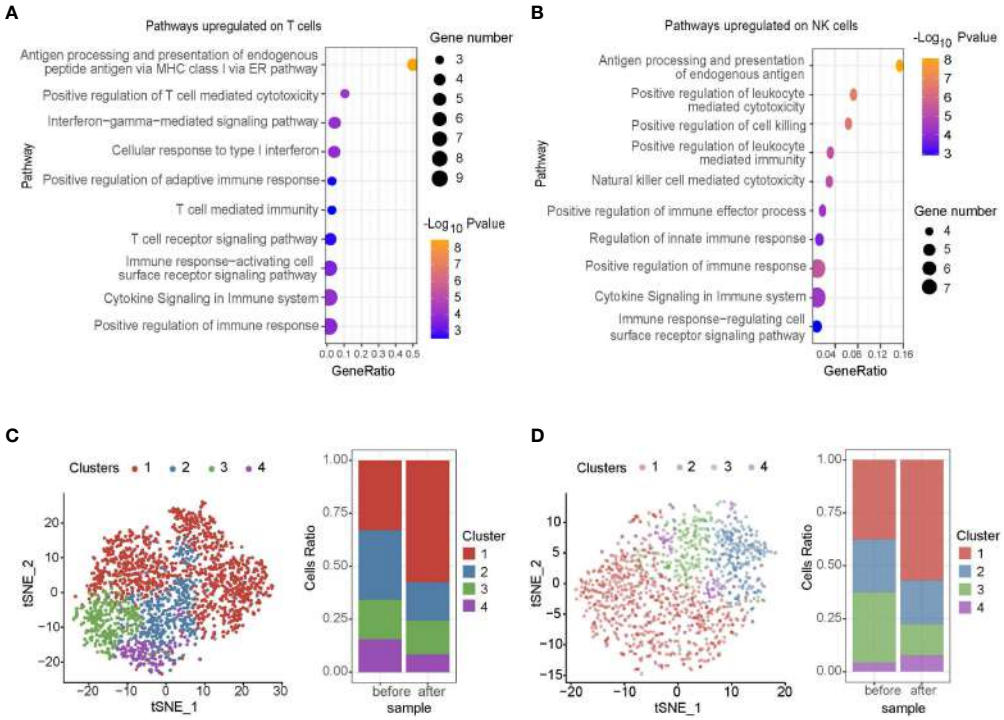

FIGURE 2
Single-cell RNA sequencing of immune cells in patient before and 10 months after midostaurin treatment. Pathways upregulated on T cells **(A)** and NK cells **(B)** after midostaurin treatment. T cell clusters and fraction of clusters **(C)**, NK cell clusters and fraction of clusters **(D)** before and after midostaurin treatment.

3 Discussion

Midostaurin has significantly improved the life quality of mast cell leukemia patients with *KIT* D816V mutation. Few reports focused on its efficacy in *KIT* F522C mutated patients (3). Our patient with *KIT* F522C responded well to midostaurin as the first-line therapy and achieved 15 months of progression-free survival. *KIT* gain-of-function mutation triggers the activation of several downstream signaling, including *MAPK, JAK-STAT*, and *PI3K* pathways which regulates the proliferation, survival, and antiapoptotic function in mast cells (5). Consistent with the clinical response, we found the downregulation of *PI3K/AKT* and *MAPK* signaling pathways following midostaurin treatment.

However, during the response to midostaurin, emergent TKI resistances were observed not only in tumor cells but also in immune cells. In terms of tumor mast cells, cytokine pathways such as IL-4 and IL-13 signaling pathways, were downregulated after midostaurin treatment, thus indicating mast cells' inactivation (6). Vascular endothelial-derived growth factor (VEGF) signaling pathway involved in activating blood vessel endothelial migration could also be attenuated through midostaurin treatment (7). These alterations in signaling pathways on tumor cells indicated that midostaurin inhibits tumor activation and metastasis. On the other hand, the upregulation of genes, such as *RPS27A, RPS6, UBA52, and RACK1* on tumor cells, involved in TKI resistance (8–11), as well as *RUNX1* mutation after midostaurin treatment, all these observations might partially explain the mast cell leukemia progression during midostaurin maintenance and afterward, the resistance to dasatinib, another TKI which has synergetic effect with midostaurin on mast cells (12). Consistent with our observation, in acute myeloid leukemia, midostaurin resistance was mainly attributed to the emergence of new mutations, including either mutation on different locus of *KIT*, or mutation on a new gene contributing to the activation of downstream signaling pathways (13). In our case, residual tumor cells with *KIT* F522C mutation after midostaurin treatment or the emergent *RUNX1* mutation-containing cells might contribute to the resistance to midostaurin. Indeed, *RUNX1* mutation was described as a risk factor in mast cell leukemia patients, and as a transcriptional regulator, *RUNX1* could activate the enhancer of *c-KIT*, trigger the transcriptional regulation of *c-KIT* to promote the proliferation of malignant cells (14). In addition to *RUNX1*, increased expression of *RPS27A, RPS6, UBA52, and RACK1* on mast cells might also contribute to midostaurin resistance. These emergent/secondary aberrations before and after midostaurin treatment thus suggested multiple mechanisms underlying the relapse of the mast cell leukemia patient. These aberrations could also be served as potential targets in further studies. For example, knockdown of *RPS27A* could arrest cell growth in TKI-resistant leukemia cell lines (15). *PI3K* pathway inhibitors could effectively dephosphorylate *RPS6* in imatinib-resistant cell lines (9); *RACK1* overexpression upregulated protein kinase C (PKC) activity. Therefore, PKC inhibitors also induced cell apoptosis in chemoresistant leukemia cell lines (16). Additionally, novel *KIT* inhibitor avapritinib, which was licensed by the FDA since 2021 and by the EMA since 2022, has distinct resistance profiles with

midostaurin (17), might be an option for midostaurin-resistant patients.

Besides, tumor microenvironment had been improved, with the decrease of mast leukemia cells and increase of T and NK cells, indicating an activated antitumor condition in the patients after midostaurin treatment. In addition, single-cell RNA sequencing revealed an activation in the immune response of T cells, including upregulation of antigen presenting, T cell cytotoxicity and T cell-mediated immunity pathways (18). NK cells mediated cytotoxicity, positive regulation of cell killing, and regulation of cytokine production pathway were also activated on NK cells after treatment (18). Meanwhile, the frequencies of T and NK cells with *TIGIT, CTLA4*, and *LAG3* expression were significantly increased after midostaurin treatment, displaying the resistance mechanisms of microenvironment during the exposure to midostaurin. Consistent with our observations, $TIGIT^+$ T cells or $TIGIT^+$ NK cells had impaired cell function and decreased cytokine secretion, which provided a tumor-supportive microenvironment (19, 20). Because upregulation of these immune checkpoints in peripheral blood were correlated with disease relapse in leukemia (21), an increase of dysfunctional T (e.g., $TIGIT^+$ T) and NK (e.g., $TIGIT^+$ NK) cell clusters might contribute to the relapse of this patient. In the future, therapeutically blockading of TIGIT (e.g., anti-TIGIT antibody) might be introduced to combined therapy in mast cell leukemia to enhance the cytotoxicity of T and NK cells (22).

4 Conclusion

In this case, we analyzed the genetic and tumor microenvironment changes in the mast cell leukemia patient after midostaurin treatment, in order to illustrate the *in vivo* mechanisms of aquired resistance to midostaurin. Although the pathogenic gene mutation *KIT* was repressed by midostaurin when the patient achieved major response, we inferred that the arising of *RUNX1* mutation, upregulating genes expression (*RPS27A, RPS6, UBA52, RACK1*) on tumor cells and the immune suppressive tumor microenvironment with increased dysfunctional T and NK cells could contribute to the subsequent midostaurin resistance and disease progression in the patient. Our observations explain the reasons why mast cell leukemia responded to midostaurin but displayed a low CR rate (0%) and short median PFS (11.3 months) (4), indicating the underlying mechanisms behind relapse *in vivo* after midostaurin treatment. Furthermore, understanding of these mechanisms can be used to better monitor treatment response and the selection of resistant subclones, providing important clues to develop a sequential option to circumvent mast cell leukemia progression upon midostaurin treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Shanghai Ruijin Hospital Ethics Board. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

W-LZ and LW supervised the study. M-KL, X-QW, L-LC, L-QF collected clinical data and made the figures. M-KL, FL, Y-TD, HF, HL, LJ, X-JS carried out bioinformatic analysis and W-LZ, LW, M-KL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1210909/full#supplementary-material

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Comparative safety of tyrosine kinase inhibitors in the treatment of metastatic renal cell carcinoma: a systematic review and network meta-analysis

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Objective: This study aimed to compare the safety profile of tyrosine kinase inhibitors (TKIs) approved for use as monotherapy or combination therapy for the first-line treatment of adult patients with metastatic clear cell renal cell carcinoma (RCC).

Methods: A systematic review with frequentist network meta-analysis (NMA) was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. We included randomized controlled trials (RCTs) investigating the use of: cabozantinib, pazopanib, sorafenib, sunitinib, tivozanib, cabozantinib + nivolumab, lenvatinib + pembrolizumab, axitinib + avelumab, and axitinib + pembrolizumab in previously untreated adult patients with metastatic clear cell RCC. Eligible studies were identified by two reviewers in MEDLINE (via PubMed), EMBASE, and Cochrane Library. The risk of bias for RCTs was assessed using the Cochrane Collaboration tool. The P score was used to determine the treatment ranking. The mean probability of an event along with the relative measures of the NMA was considered with the treatment rankings.

Results: A total of 13 RCTs were included in the systematic review and NMA. Sorafenib and tivozanib used as monotherapy were the best treatment options. Sorafenib achieved the highest P score for treatment discontinuation due to adverse events (AEs), fatigue, nausea, vomiting of any grade, and hypertension of any grade or grade ≥3. Tivozanib achieved the highest P score for AEs, grade ≥3 AEs, dose modifications due to AEs, and grade ≥3 diarrhea. Sunitinib was the best treatment option in terms of diarrhea and dysphonia of any grade, while cabozantinib, pazopanib, and axitinib + pembrolizumab–in terms of grade ≥3 fatigue, nausea, and vomiting. TKIs used in combination were shown to have a poorer safety profile than those used as monotherapy. Lenvatinib + pembrolizumab was considered the worst option in terms of any AEs, grade ≥3 AEs, treatment discontinuation due to AEs, dose modifications due to AEs, fatigue of any grade, nausea, vomiting, and grade ≥3 nausea. Axitinib + avelumab was the worst treatment option in terms of dysphonia, grade ≥3 diarrhea, and hypertension, while cabozantinib + nivolumab was the worst option in terms of grade ≥3 vomiting. Interestingly, among the other safety endpoints, cabozantinib monotherapy had the lowest P score for diarrhea and hypertension of any grade.

Conclusion: The general safety profile, including common AEs, is better when TKIs are used as monotherapy vs. in combination with immunological agents. To confirm these findings, further research is needed, including large RCTs.

KEYWORDS

renal cell carcinoma, safety, tyrosine kinase inhibitors, meta-analysis, systematic review

1 Introduction

Renal malignancies are relatively rare, with renal cell carcinoma (RCC) being the most common, accounting for approximately 90% of all cases (Hsieh et al., 2017). The incidence of kidney cancer peaks between the sixth and eighth decade of life and is estimated at 74,000 new cases annually in the United States (Siegel et al., 2019). There are several subtypes of RCC classified on the basis of microscopic examination of a tumor specimen. The most common subtypes include clear cell RCC (75%), papillary RCC (10%), and chromophobic RCC (5%) (Hsieh et al., 2017; Padala et al., 2020). Clear cell RCC is the most serious diagnosis, as this subtype is linked with the presence of distant metastases and the highest grade of histological malignancy at diagnosis (Hsieh et al., 2017; Protzel et al., 2012).

The prognosis of patients with RCC depends on the clinical stage of cancer. The 5-year survival rate is 80%–90% for patients with stage I cancer; 50%–70%, with stage II; 20%–30%, with stage III; and about 5%, with stage IV (Siegel et al., 2019; Padala et al., 2020). Most patients present with localized disease (stage I or II) that can be treated surgically; however, up to 20%–30% of patients who undergo surgical resection may relapse and develop metastases (Tyson and Chang, 2017). Moreover, about 25% of patients with RCC have locally advanced or metastatic disease at diagnosis, and in approximately 20%–40% of patients, localized primary tumors will metastasize (Osawa et al., 2019). Therefore, it is particularly important to choose an appropriate therapeutic option that would allow to improve survival and the quality of life of patients with advanced kidney cancer.

Treatment depends on the stage of cancer at diagnosis. For patients in early stages (I or II), the most common treatment options are surgical tumor excision and partial or complete nephrectomy. The standard therapeutic strategy in advanced kidney cancer has changed with the introduction of molecularly targeted drugs that selectively inhibit tumor growth without affecting the growth of other rapidly dividing cells. Targeted therapy for kidney cancer includes three groups of drugs: tyrosine kinase inhibitors (TKIs), mTOR serine-threonine kinase inhibitors, and anti–vascular endothelial growth factor (VEGF) monoclonal antibody (Hsieh et al., 2017). The new molecularly targeted drugs have vastly improved the prognosis of patients with advanced kidney cancer, with a significant increase in the median overall survival (Thomson et al., 2023).

In patients with RCC, changes in the von Hippel–Lindau gene, VHL, cause the activation of angiogenic factors such as an increase in VEGF levels. Thus, TKIs, which prevent cell division and growth of new blood vessels, seem to be the most effective therapeutic option (Thomson et al., 2023; Pal et al., 2012). The drugs precisely target the genetic mechanisms based on oncogenesis and proliferation of renal cancer cells (Roberto et al., 2021). According to the latest data from the National Cancer Institute, the following TKIs are currently approved for use by the Food and Drug Administration (FDA): sunitinib, sorafenib, pazopanib, tivozanib, lenvatinib, axitinib, and cabozantinib (cancer.gov). A network meta-analysis (NMA) showed no differences in the effectiveness of TKIs used as monotherapy (Manz et al., 2020). TKIs were reported to be highly effective in terms of improving the median progression-free survival and overall survival (Mihály et al., 2012; Motzer et al., 2006). The objective response rate ranged from 20% to 35% (Hutson et al., 2010; Wang et al., 2020). Studies conducted in recent years also provided the basis for approving TKI use in combination therapy for metastatic RCC. Hahn et al. (2019) reported that cabozantinib treatment, combination therapy with avelumab and axitinib, and combination therapy with pembrolizumab and axitinib have comparable efficacy and are the preferred treatment option for most patients with metastatic RCC (Hahn et al., 2019).

As each drug, especially anticancer drug, has a certain toxicity profile, it is often necessary to modify treatment to prevent the high rate of side effects and to control for side effects so that adequate therapy can be continued (Oh et al., 2014). However, to our knowledge, there have been no systematic reviews that would assess the safety profile of TKIs in a more comprehensive way by focusing on the risk of individual AEs. In addition, as new TKIs have been approved for use in the last few years, an update of the current knowledge is needed. We assumed that if individual TKIs have similar effectiveness, an in-depth assessment of the safety profile might help clinicians in decision-making on the best and safest therapy for individual patients.

To fill in the existing gaps in knowledge and evidence, we decided to compare the safety profile of TKIs used in adult patients with metastatic clear cell RCC. We conducted a systematic review with an NMA with the aim to perform a comprehensive safety assessment of selected TKIs approved for this indication.

2 Materials and methods

2.1 General principles

The systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Hutton et al., 2015; Page et al., 2021) and guidelines for conducting and interpreting the NMA developed by the International Society for Pharmacoeconomics and Outcomes Research Task Force (Jansen et al., 2014) and Cipriani et al. (2013). The protocol of systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO;

TABLE 1 Detailed inclusion and exclusion criteria for systematic review and meta-analysis.

	Inclusion criteria	Exclusion criteria
Population	Adult patients (or majority of patients) with metastatic clear cell RCC (or with clear cell component) not previously treated systemically (trials with ≥70% of patients previously untreated were eligible; the local treatment such surgery or radiotherapy were allowed)	Pediatric patients with RCC, patients previously treated, trials with no information about line of therapy, trials with patients with other than clear cell RCC
Intervention and comparators	Registered TKIs in monotherapy or in combination therapy (details about dosing provided in Supplementary Table S3): tivozanib, sunitinib, sorafenib, pazopanib, cabozantinib + nivolumab, lenvatinib + pembrolizumab, axitinib + avelumab, axitinib + pembrolizumab compared to each other or with placebo or with other therapy registered by EMA or FDA for first line treatment of RCC	Interventions not of interest (e.g., not approved for metastatic RCC); trials without direct comparison of safety of any of the mentioned interventions to any other included TKI or placebo or other therapy registered by EMA or FDA for first line treatment of RCC
Outcomes	Adverse events, grade ≥3 adverse events, discontinuation because adverse events, dose modification due to adverse events, individual adverse events (all grades and grade ≥3): fatigue, diarrhea, nausea, vomiting, hypertension, dysphonia	Trials or additional articles for included trials not reported defined outcomes
Study types	Randomized controlled trials	Non-randomized controlled, trials, observational studies, case reports, reviews, additional analysis of included trials, additional references for included trials without safety data or without newer safety data than reported in main publication, cross-sectional studies
Treatment period	Duration of treatment: until disease progression or as long as clinical benefit is observed or until unacceptable toxicity occurs	Trials in which the length of the treatment period was predetermined/restricted, regardless of progression, toxicity, or treatment benefit
Publication type	Full text articles, data from clinical trials registers were allowed to use	Abstracts, posters, editorials, letters
Language	English	Languages other than English

EMA, European Medicines Agency; FDA, Food and Drug Administration; TKIs, tyrosine kinase inhibitors; RCC, renal cell carcinoma.

registration number, CRD42022375275) (PROSPERO database, 2022).

2.2 Data sources and search

A comprehensive search of the three main databases: MEDLINE (via PubMed), EMBASE, and Cochrane Library, was conducted in November 2022. During the search, keywords related to the analyzed population and interventions were used, identified in medical subject heading (MeSH) terms or Emtree, combined with Boolean logical operators. The detailed search strategy is described in Supplementary Table S1. In addition, the trial registration database https://clinicaltrials.gov/ (the detailed search strategy is described in Supplementary Table S2), the reference lists of the most recent systematic reviews on TKI use in metastatic RCC, and the reference lists of the included studies were hand searched. Only articles written in English were included.

2.3 Inclusion criteria and trial section process

Full-text publications of prospective randomized controlled trials (RCTs) published in English, conducted in a group of adult patients with a clinical diagnosis of metastatic clear cell RCC, treated with TKIs as monotherapy or combination therapy, were included. The following TKI-based therapies approved by the European Medicines Agency (EMA) and/or the Food and Drug Administration (FDA) included: tivozanib, sunitinib, sorafenib, pazopanib, cabozantinib + nivolumab, lenvatinib + pembrolizumab, axitinib + avelumab, and axitinib + pembrolizumab compared with one another, with placebo, or with other therapy registered by the EMA or FDA for the first-line treatment of RCC. The safety outcomes of interest were as follows: 1) AEs (all grades and grade ≥3); 2) treatment discontinuation due to AEs; 3) dose modification due to AEs; and 4) individual AEs (all grades and grade ≥3) that are most commonly reported in the summary of products characteristics (SmPC Fortivda®, SmPC Sutent®, SmPC Votrient®, SmPC Inlyta®, SmPC Cabometyx®). These individual AEs included fatigue, diarrhea, nausea, vomiting, hypertension, and dysphonia. If no appropriate data were available in a full-text publication, information from clinical trial registries was allowed. The most recent available data were considered. Detailed inclusion and exclusion criteria for the systematic review and meta-analysis are described in Table 1 and Supplementary Table S3.

The screening and selection of studies were carried out in accordance with the PRISMA guidelines (Hutton et al., 2015; Page et al., 2021) by two independent reviewers (KŚ, KK). First, the titles and abstracts of studies identified during the search were assessed, and a list of studies that initially met the inclusion criteria was prepared. Then, the full texts of the remaining articles were examined to determine whether they contained relevant information, considering all the inclusion and exclusion criteria for the analysis. The degree of compatibility between the reviewers

was high (estimated at 97.5%). Conflicts in study selection at this stage were resolved by consensus and consultation with a third reviewer (PK), referring to the original article. At the end of the selection process, the final list of included trials was prepared.

2.4 Data extraction and quality assessment

Two independent reviewers (KŚ, KK) extracted data from included trials, using a predefined data extraction form. The following information was extracted to assess the homogeneity of trials: design (methodology), treatment regimens, size of the study arms, duration of treatment/exposure to the drug, and detailed patient characteristics including the stage and histological type of RCC, age, sex, performance status, previous surgery and/or radiation therapy, and prognosis according to Memorial Sloan-Kettering Cancer Center (MSKCC) criteria. The Cochrane risk-of-bias tool 2 (RoB2) for randomized trials (Higgins et al., 2019) was used to assess the bias of eligible RCTs. This tool allows an evaluation of the following domains: randomization process, deviations from intended intervention, missing data outcome, measurement of the outcome and selection of the reported results. The domain-based evaluation allows the assignment of the following ratings to each domain: low risk of bias ("+"), high risk of bias ("–"), or unclear risk of bias ("?"). The robvis tool (https://www.riskofbias.info/welcome/robvis-visualization-tool) was used to graphically present the results of the risk-of-bias assessment for individual trials.

2.5 Data analysis and synthesis

The NMA was conducted using the netmeta R software package (Rücker, 2012), which incorporates the graph-theoretic method of an NMA (vertices, treatments; edges, randomized comparisons) and provides a point estimate from the network along with 95% confidence intervals (CIs). This frequentist method is an alternative to a standard NMA conducted within the Bayesian framework (Neupane et al., 2014). In the NMA, we used consistency and random effects models with adjustment for multi-arm studies. All eligible treatments and their regimens with different doses or dosing intervals from the identified studies were included in the network, and each treatment in each dose regimen constituted one node (vertex in a graph).

All comparisons evaluated in the trials, including suboptimal and experimental dose regimens and treatments not assessed in the systematic review, were included in the NMA and presented in Supplementary Material. However, only the treatments of interest in their licensed dose regimens were presented (Supplementary Table S3). These treatments included: oral tivozanib (1.5 mg once daily for 3 weeks, followed by 1 week off), oral sunitinib (50 mg once daily for 4 weeks, followed by 2 weeks off), oral sorafenib (400 mg twice daily), oral pazopanib (800 mg once daily), oral cabozantinib (40 mg once daily) + intravenous nivolumab (240 mg once every 2 weeks), oral lenvatinib (20 mg once daily) + intravenous pembrolizumab (200 mg once every 3 weeks), oral axitinib (5 mg twice daily) + intravenous avelumab (10 mg per kilogram of bod weight every 2 weeks), and oral axitinib (5 mg twice daily) + intravenous pembrolizumab (200 mg once every 3 weeks).

The networks were created for each outcome with a similar definition in all trials. The heterogeneity of evidence was assessed using the Cochran's Q test, I^2 statistic, and tau (i.e., the square-root of between-study variance). The consistency of the network was assessed using a design-based decomposition of Cochran's Q, the splitting approach, and comparison with direct evidence (Freeman et al., 2019). The funnel plot for "small-study effects" was used to assess publication bias.

The P score, a frequentist equivalent of the surface under the cumulative ranking curve, was used to determine the treatment ranking. A higher P score indicates better treatment safety (i.e., lower risk of AEs) (Rücker and Schwarzer, 2015). The treatment ranking alone should be interpreted with caution, because it provides information only about the probability that a treatment is the best while not directly incorporating the effect size of the difference between treatments. The mean probability of an event along with the relative measures of the NMA should be taking into account with the treatment rankings (Manz et al., 2020; Bhatnagar et al., 2014). The mean probability of an event for each treatment was calculated using the odds ratio from the NMA and the mean probability for sunitinib. The latter was obtained from the meta-analysis of the sunitinib arm from all trials included in the NMA, using the random effects model based on the Freeman-Tukey (double arcsine) transformed proportion.

3 Results

3.1 Search results and included studies

During the database search, a total of 2,574 possibly relevant references were screened, of which 2,372 were excluded after screening the titles and abstracts (Figure 1). After careful consideration of 117 articles assessed in the full-text review, 85 were excluded (Supplementary Table S4). Finally, 13 trials, described in 32 references (7,125 patients randomized), were included in the review and meta-analysis. The methodology of included trials is characterized in Table 2, and the baseline characteristics of patients are provided in Supplementary Table S5.

3.1.1 Homogeneity of included trials and risk-of-bias assessment

Most studies were multicenter randomized phase III trials, except phase II NCT00117637 (Escudier et al., 2009; NCT00117637, 1176), the Alliance A031203 CABOSUN (Choueiri et al., 2017; Choueiri et al., 2018; NCT01835158, 1835), and TemPa (Tannir et al., 2020). Ten trials had a parallel design, while the remaining three trials [SWITCH (Eichelberg et al., 2015; NCT00732914, 2914), SWITCH II (Retz et al., 2019; NCT01613846, 1613) and CROSS-J–RCC (Tomita et al., 2017; Tomita et al., 2020; NCT01481870, 1481)] had a sequential cross-over design, that is, after disease progression during the first-line randomized treatment, patients received the therapy used in the other group. In line with the inclusion criteria for systematic review and meta-analysis, only data for the first-line setting were used in the case of studies with a sequential cross-over design. All included trials were open label; however, in some studies [NCT00098657/NCT00083889 (Motzer

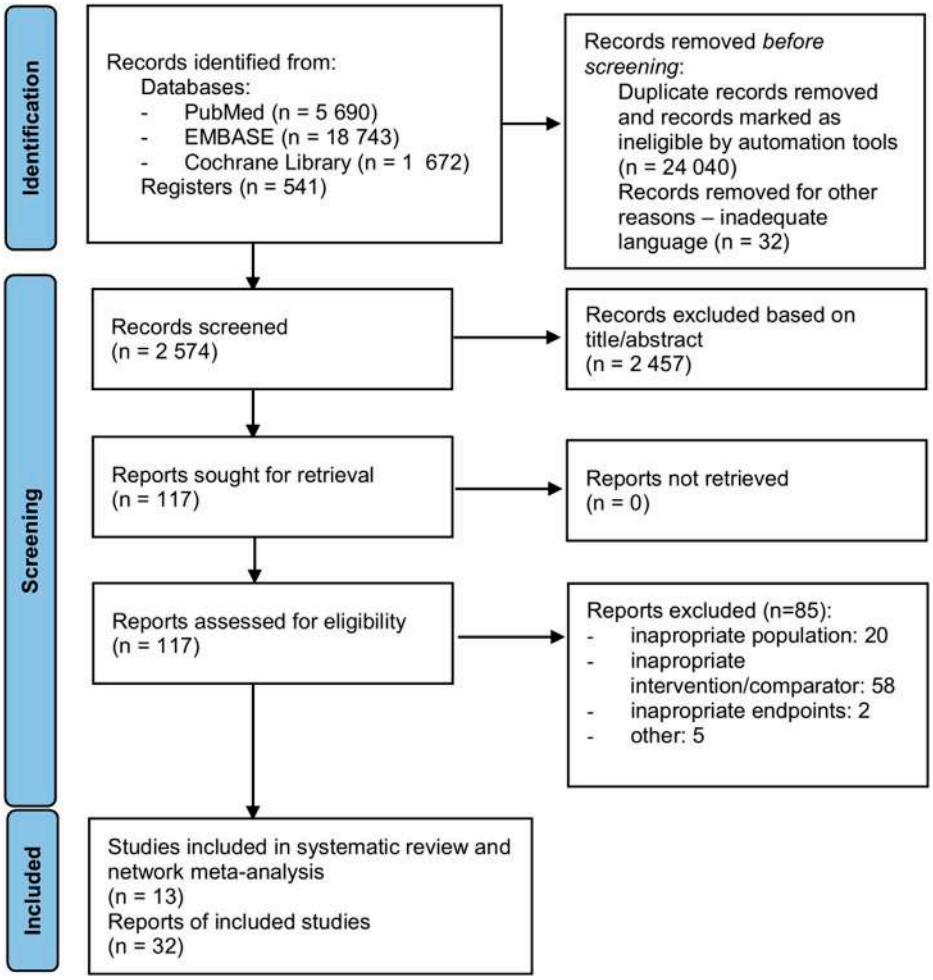

FIGURE 1
Search flow diagram.

et al., 2007; Motzer et al., 2009; NCT00098657, 8657; NCT00083889, 3889), TIVO (Motzer et al., 2013a; NCT01030783, 1030), TemPa (Tannir et al., 2020), CheckMate 9ER (Motzer et al., 2022a; Choueiri et al., 2021; NCT03141177, 1411), KEYNOTE-426 (Rini et al., 2019; Powles et al., 2020; NCT02853331, 2853), and JAVELIN Renal 101 (Rini et al., 2022; Motzer et al., 2019; NCT02684006, 2684)], only the analysis of the primary endpoint (survival outcomes such as progression-free survival) was assessed by a blinded investigator or a blinded radiology review. This means that in all studies, both patients and physicians/medical staff involved in the safety evaluation were unblinded, resulting in a high risk-of-bias assessment in the Cochrane risk-of-bias tool 2 (Figure 2).

According to the inclusion criteria and baseline characteristics, participants in all studies were patients with advanced or metastatic (most patients) RCC with clear cell histology or with a clear cell component. The most common metastatic sites were the lungs, lymph nodes, and bones. In most trials, all patients were systemic therapy naïve, except the TIVO trial, where >70% of patients were systemic therapy naïve. The median age of patients in included trials ranged from 59 to 68 years, and patients had generally good performance status (Eastern Cooperative Oncology Group [ECOG] score of 0–1). Most patients had favorable or intermediate prognosis according to MSKCC criteria, except the TemPa trial, where about 70% of participants had poor prognosis (Tannir et al., 2020). The most common TKI therapy was sunitinib monotherapy. In four trials [CheckMate 9ER (Motzer et al., 2022a; Choueiri et al., 2021; NCT03141177, 1411), KEYNOTE-426 (Rini et al., 2019; Powles et al. 2020; NCT02853331, 2853), CLEAR (Motzer et al., 2021; NCT02811861, 2811) and JAVELIN Renal 101 (Rini et al., 2022; Motzer et al., 2019; NCT02684006, 2684)], TKIs were used in combination with anti-PD-1 monoclonal antibodies (nivolumab, pembrolizumab, or avelumab) (Supplementary Table S5). The therapy was continued until disease progression or unacceptable toxicity occurred, which was in line with the recommendations in the summary of product characteristics.

3.2 NMA results

Thirteen trials had sufficient homogeneity to be included in the NMA (Supplementary Figure S1; Supplementary Tables S5, S6). Not all predefined endpoints were reported in each trial, and for grade ≥3 dysphonia, it was impossible to conduct an NMA. The

TABLE 2 Methodology of trials included in systematic review and network meta-analysis.

Study	Methodology	Comparison and the number of randomized patients	Median duration of treatment (range)
NCT00098657, NCT00083889 Motzer et al. (2007), Motzer et al. (2009), NCT00083889, (3889), NCT00098657, (8657)	RTC, partially-blinded (blinded only for primary efficacy endpoint analysis), phase III, multicenter, parallel groups	Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 375) vs.	11.0 months (<1–41 months) in the sunitinib group and 4.0 months (range <1–40 months) in the interferon alpha-2a group
		Interferon alpha-2a subcutaneously at a dose of 9 MU thrice a week (N = 375)	
NCT00117637 Escudier et al. (2009), NCT00117637 (1176)	RCT, open, phase II, multicenter, parallel groups	Sorafenib orally at a dose of 400 mg twice daily (*N* = 97) vs.	6.0 months (0.2–13.8) in the sorafenib and 5.5 months (0.4–7.5) in inferferon alpha-2a group
		Interferon alfa-2a subcutaneously at a dose of 9 MU thrice a week (N = 92)	
TIVO Motzer et al. (2013a), NCT01030783, (1030)	RCT, open (only independent radiology review blinded), phase III, multicenter, parallel groups	Sorafenib orally at a dose of 400 mg twice daily (*N* = 257) vs.	12.0 months in tivozanib and 9.5 months in sorafenib group
		Tivozanib orally at a dose of 1.5 mg once daily every day for 3 weeks followed by 1 week off (*N* = 260)	
Alliance A031203 CABOSUN Choueiri et al. (2017), Choueiri et al. (2018), NCT01835158 (1835)	RCT, open, phase II, multicenter, parallel groups	Sunitinib orally at a dose of 50 mg for 4 weeks, followed by 2 weeks without treatment (*N* = 78) vs.	6.5 months (IQR 2.8–16.5) in the cabozantinib and 3.1 months (IQR 2.0–8.2) in sunitinib group
		Cabozantinib orally at a dose of 60 mg once daily (*N* = 79)	
COMPARZ Motzer et al. (2013b), NCT00720941 (2094)	RCT, open, phase III, multicenter, parallel group	Pazopanib orally at a dose of 800 mg once daily (*N* = 557) vs.	8.0 months (0–40.0) in the pazopanib and 7.6 months (0–38.0) in sunitinib group
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 553)	
SWITCH[a] Eichelberg et al. (2015), NCT00732914 (2914)	RCT, open, phase III, multicenter, cross-over (but results for first line-treatment provided)	Sorafenib orally at a dose of 400 mg twice daily (*N* = 182) vs.	During first-line treatment: 37.5 weeks (SD = 37.4) in sorafenib group and 43.9 weeks (SD = 44.3) in sunitinib group
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 183)	
SWITCH II[a] Retz et al. (2019), NCT01613846 (1613)	RCT, open, phase III, multicenter, cross-over (but results for first line-treatment provided)	Sorafenib orally at a dose of 400 mg twice daily (*N* = 189) vs.	During first-line treatment: 3.9 months (0.0–42.2) for sorafenib and 5.7 months (0.3–43.3) for a pazopanib group
		Pazopanib orally at a dose of 800 mg once daily (*N* = 188)	
CROSS-J -RCC[a] Tomita et al. (2017), Tomita et al. (2020), NCT01481870 (1481)	RCT, open, phase III, multicenter, cross-over (but results for first line-treatment provided)	Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 60) vs.	During first-line treatment: 6.7 months (0.1–45.3) for sunitinib and 6.1 months (0.3–46.1) for sorafenib group
		Sorafenib orally at a dose of 400 mg twice daily (*N* = 64)	
TemPa Tannir et al. (2020)	RCT, open (only response to treatment assessed by blind investigator), phase II, parallel group	Temsirolimus intravenously at a dose of 25 mg twice a week (*N* = 35) vs.	Not reported, but median PFS for temsirolimus group was 2.7 months and 5.2 months for pazopanib group
		Pazopanib orally at a dose of 800 mg one daily (*N* = 34)	
CheckMate 9ER Motzer et al. (2022a), Choueiri et al. (2021), NCT03141177, (1411)	RCT, open (blinded only for primary efficacy endpoint analysis), phase III, multicenter, parallel group	Nivolumab intravenously at a dose of 240 mg once every 2 weeks + cabozantinib orally at a dose of 40 mg once daily (*N* = 323) vs.	14.3 months (0.2–27.3) for nivolumab + cabozantinib and 9.2 months (0.8–27.6) for sunitinib
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 328)	Total median observation time of 18.1 months
KEYNOTE-426 Rini et al. (2019), Powles et al. (2020), NCT02853331 (2853)	RCT, open (blinded only for primary efficacy endpoint analysis), phase III, multicenter, parallel group	Pembrolizumab intravenously at a dose of 200 mg once every 3 weeks + axitinib orally at a dose of 5 mg twice daily (*N* = 432) vs.	The median duration of any treatment was 10.4 months (0.03–21.2) in the pembrolizumab + axitinib group and 7.8 months (0.07–20.5) in the sunitinib group

(Continued on following page)

TABLE 2 (*Continued*) Methodology of trials included in systematic review and network meta-analysis.

Study	Methodology	Comparison and the number of randomized patients	Median duration of treatment (range)
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 429)	Total median observation time of 30.6 (23.4–38.4) months
CLEAR Motzer et al. (2021), NCT02811861 (2811)	RCT, open, phase III, multicenter, parallel group	Lenvatinib orally at a dose of 20 mg once daily + pembrolizumab intravenously at a dose of 200 mg once every 3 weeks (*N* = 355) vs.	17.0 months (0.1–39.1) in the lenvatinib + pembrolizumab, 11.0 months (0.1–40.0) in the lenvatinib + everolimus group, and 7.8 months (0.1–37.0) in the sunitinib group. Median observation period for the total survival of 26.6 months
		Lenvatinib orally at a dose of 18 mg once daily + everolimus orally at a dose of 5 mg once daily (*N* = 357) vs.	
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 357)	
JAVELIN Renal 101 Rini et al. (2022), Motzer et al. (2019), NCT02684006 (2684)	RCT, open (blinded only for primary efficacy endpoint analysis), phase III, multicenter, parallel group	Avelumab intravenously at a dose of 10 mg per kilogram of body weight every 2 weeks + axitinib orally at a dose of 5 mg twice daily (*N* = 442) vs.	8.6 months (0.5–25.3) in patients who received avelumab, 9.0 months (0.02–24.9) in patients who received axitinib, and 7.3 months (0.2–23.0) in the sunitinib group
		Sunitinib orally at a dose of 50 mg once daily for 4 weeks, followed by 2 weeks without treatment (*N* = 444)	During the first indirect analysis, the minimum observation period was 6 months

[a]After disease progression, treatment was changed to an alternative drug. Only first-line data were used in the meta-analysis; RCT, randomized clinical trial; IQR, interquartile range; SD, standard deviation.

final number of trials for each endpoint is presented in Supplementary Table S7. The input data for NMA for the overall safety profile were presented in the Supplementary Table S8; for the detailed safety profile results - selected adverse events in all grades in Supplementary Table S9 and for selected grade ≥3 adverse events in Supplementary Table S10.

3.3 Rakings of TKIs

The P score–based ranking of TKIs (and interventions used as comparators in included trials) is presented in Table 3 and Supplementary Table S11 (all therapies from included trials).

The results indicated that individual drugs and combination therapies rank differently depending on the safety endpoint. The TKIs sorafenib and tivozanib were shown to be the best treatment options: sorafenib had the highest P score in terms of treatment discontinuation due to AEs, fatigue, nausea, vomiting, and hypertension of any grade, and grade ≥3 hypertension. Tivozanib had the highest P score in terms of any AEs, grade ≥3 AEs, dose modifications due to AEs, and grade ≥3 diarrhea. The best treatment option in terms of diarrhea and dysphonia was sunitinib, while cabozantinib, pazopanib, and axitinib + pembrolizumab were ranked as the best options in terms of fatigue, nausea, and vomiting (all grade ≥3).

Generally, TKIs in combination with other drugs were found to have a poorer safety profile than TKI monotherapies. Lenvatinib + pembrolizumab was ranked as the worst option in terms of any AEs, grade ≥3 AEs, treatment discontinuation due to AEs, dose modifications due to AEs, fatigue, nausea, and vomiting of any grade, and grade ≥3 nausea. Axitinib + avelumab was the worst option in terms of dysphonia of any grade, grade ≥3 diarrhea, and grade ≥3 hypertension, while cabozantinib + nivolumab was the worst option in terms of grade ≥3 vomiting. Interesingly, considering the remaining safety endpoints, cabozantinib monotherapy showed the lowest P score for diarrhea and hypertension of any grade.

3.3.1 General safety profile

The general safety profile was assessed in terms of any AEs, grade ≥3 AEs, treatment discontinuation due to AEs, and dose modifications due to AEs. Most trials reported AEs as treatment-emergent AEs irrespective of their relation to the therapy used. Two trials [NCT00098657/NCT00083889 (Motzer et al., 2007; Motzer et al., 2009; NCT00098657, 8657; NCT00083889, 3889 and NCT00117637 (Escudier et al., 2009; NCT00117637, 1176)] reported individual AEs only as treatment-related AEs, so it was impossible to use these results in the NMA. All included trials generally used a similar definition of treatment discontinuation due to AEs and recorded AEs using the Common Terminology Criteria for Adverse Events, which allowed us to conduct a credible NMA (Supplementary Table S6).

There were no differences between TKIs (monotherapy and combination therapy) in the risk of any AEs, except a reduced risk of AEs for: 1) tivozanib vs. sorafenib ($p = 0.006$), sunitinib ($p = 0.048$), pazopanib ($p = 0.009$), and lenvatinib + pembrolizumab ($p = 0.018$); and 2) axitinib + pembrolizumab vs. lenvatinib + pembrolizumab ($p = 0.032$) (Table 4; Supplementary Figure S2; Supplementary Table S12). The adjusted mean risk of any AEs was generally similar for the treatments, with the highest risk for lenvatinib + pembrolizumab (99.8%; 95% CI: 98.4%, 100.0%), and the lowest risk for tivozanib (95.9%; 95% CI: 84.4%, 98.6%) and cabozantinib (93.8%; 95% CI: 43.4%, 99.5%) (Table 5).

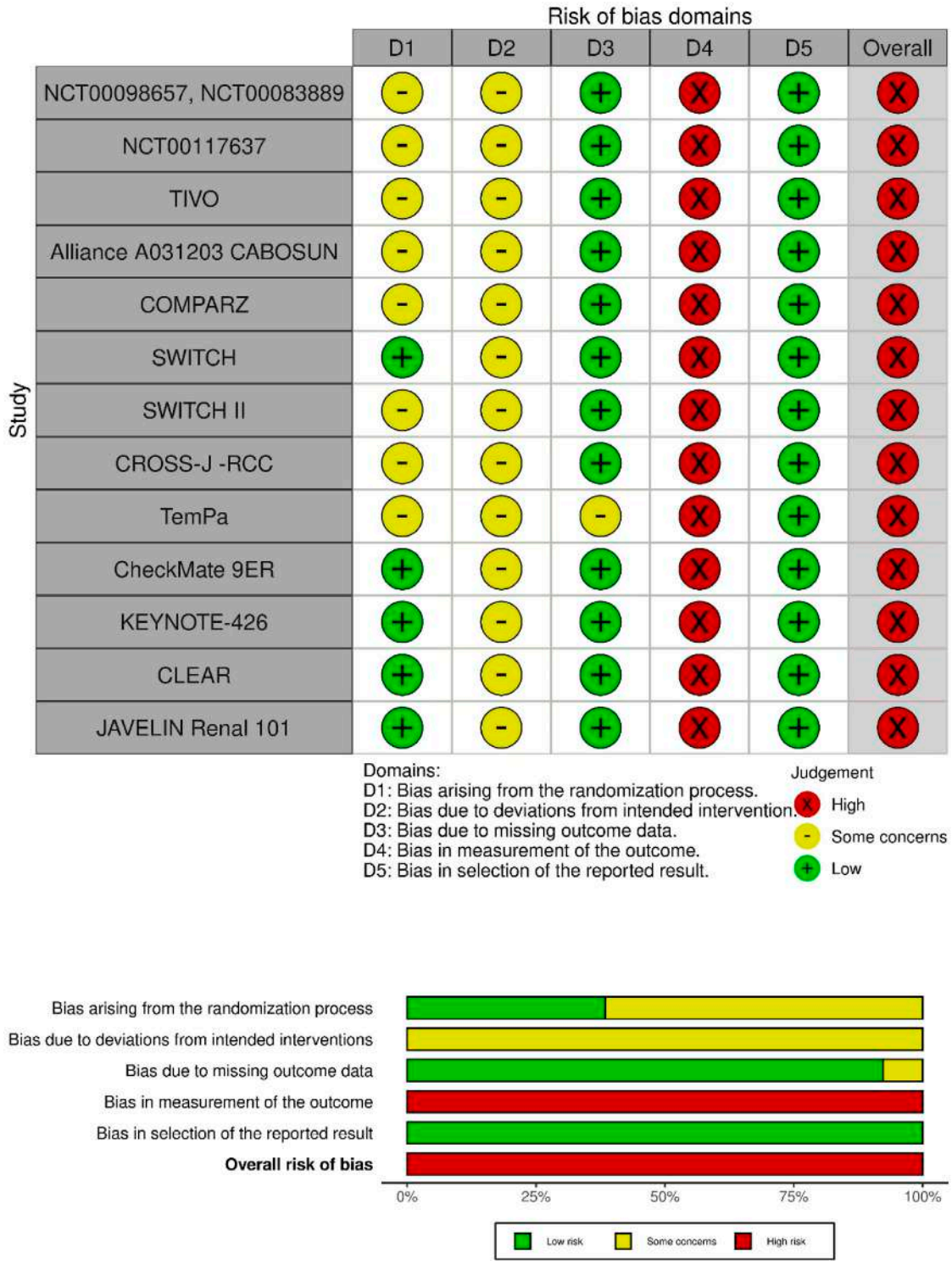

FIGURE 2
Risk-of-bias 2 assessment.

Tivozanib was associated with a lower risk of grade ≥3 AEs compared with sunitinib (p = 0.030), pazopanib (p = 0.019), cabozantinib + nivolumab (p = 0.011), and lenvatinib + pembrolizumab (p < 0.001) (Table 4; Supplementary Figure S2; Supplementary Table S13). A higher risk of grade ≥3 AEs was found for lenvatinib + pembrolizumab compared with most monotherapies (tivozanib, p < 0.001; sorafenib, p = 0.002; sunitinib, p = 0.001; pazopanib, p = 0.012) and axitinib combination therapies (axitinib + avelumab, p = 0.008; axitinib + pembrolizumab, p = 0.011). The adjusted mean risk of grade ≥3 AEs

TABLE 3 Overall ranking of TKIs used as monotherapy and as combination therapy based on the P score for assessed endpoints. Numbers in the parenthesis indicate the therapy position in the ranking.

Intervention	Adverse events	Grade ≥3 adverse events	Discontinuation due to adverse events	Dose change due to adverse events	Fatigue	Diarrhea	Nausea	Vomiting	Hypertension	Dysphonia	Grade ≥3 fatigue	Grade ≥3 diarrhea	Grade ≥3 nausea	Grade ≥3 vomiting	Grade ≥3 hypertension
TIV	0.871 (1)	0.901 (1)	0.662 (4)	0.998 (1)	0.631 (3)	0.757 (2)	0.435 (6)	—	0.494 (5)	—	0.496 (5)	0.801 (1)	0.548 (4)	—	0.463 (4)
SOR	0.606 (4)	0.705 (2)	0.729 (1)	0.647 (3)	0.862 (1)	0.397 (6)	0.844 (1)	0.929 (1)	0.847 (1)	—	0.729 (2)	0.254 (8)	0.569 (3)	0.687 (2)	0.823 (1)
AXI + AVE	0.422 (6)	0.551 (3)	—	0.446 (5)	0.287 (8)	0.338 (7)	0.545 (3)	0.514 (4)	0.171 (7)	0.153 (6)	0.341 (8)	0.183 (9)	0.523 (5)	0.609 (4)	0.231 (9)
SUN	0.527 (5)	0.534 (4)	0.641 (5)	0.412 (6)	0.371 (6)	0.828 (1)	0.317 (8)	0.416 (5)	0.622 (4)	1.000 (1)	0.277 (9)	0.766 (2)	0.484 (7)	0.425 (6)	0.601 (3)
AXI + PEM2	0.817 (3)	0.528 (5)	0.184 (7)	0.727 (2)	0.332 (7)	0.520 (3)	0.511 (5)	0.649 (2)	0.647 (3)	0.346 (4)	0.720 (3)	0.305 (7)	0.492 (6)	0.765 (1)	0.450 (5)
PAZ	0.295 (7)	0.460 (6)	0.726 (2)	0.521 (4)	0.709 (2)	0.500 (4)	0.394 (7)	0.346 (7)	0.329 (6)	—	0.606 (4)	0.656 (4)	0.656 (1)	0.574 (5)	0.388 (6)
CAB	0.857 (2)	0.427 (7)	0.667 (3)	0.249 (7)	0.540 (4)	0.207 (9)	0.580 (2)	0.392 (6)	0.057 (9)	0.383 (3)	0.764 (1)	0.734 (3)	0.595 (2)	0.628 (3)	0.320 (7)
CAB + NIV	0.273 (8)	0.289 (8)	0.533 (6)	—	0.505 (5)	0.275 (8)	0.524 (4)	0.627 (3)	0.704 (2)	0.691 (2)	0.464 (6)	0.449 (5)	0.356 (8)	0.073 (8)	0.603 (2)
LEN + PEM	0.167 (9)	0.080 (9)	0.103 (8)	0.026 (8)	0.199 (9)	0.435 (5)	0.245 (9)	0.156 (8)	0.165 (8)	0.321 (5)	0.342 (7)	0.352 (6)	0.195 (9)	0.171 (7)	0.234 (8)

AXI + AVE, axitinib + avelumab; AXI + PEM2–axitinib + pembrolizumab; CAB, cabozantinib; CAB + NIV, cabozantinib + nivolumab; LEN + PEM, lenvatinib + pembrolizumab; PAZ, pazopanib; SOR, sorafenib; SUN, sunitinib; TIV, tivozanib.

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TABLE 4 Results of a comparative analysis of TKIs used as monotherapy and as combination therapy (at approved doses) in terms of: A–adverse events; B–grade ≥3 adverse events, C–treatment discontinuation due to adverse events, D–dose modifications due to adverse events.

A								
TIV	—	—	0.31 (0.14–0.71)	—	—	—	—	—
1.55 (0.06–42.70)	CAB	—	—	0.15 (0.01–2.93)	—	—	—	—
0.81 (0.09–6.91)	0.52 (0.02–15.21)	AXI + PEM2	—	0.29 (0.06–1.38)	—	—	—	—
0.31 (0.14–0.71)	0.20 (0.01–5.05)	0.39 (0.05–2.83)	SOR	0.80 (0.21–3.03)	—	0.25 (0.03–2.22)	—	—
0.23 (0.05–0.99)	0.15 (0.01–2.93)	0.29 (0.06–1.38)	0.73 (0.22–2.44)	SUN	0.67 (0.11–4.05)	0.49 (0.09–2.70)	0.33 (0.03–3.20)	0.19 (0.02–1.64)
0.16 (0.02–1.56)	0.10 (0.00–3.25)	0.19 (0.02–2.09)	0.49 (0.06–4.27)	0.67 (0.11–4.05)	AXI + AVE	—	—	—
0.10 (0.02–0.56)	0.06 (0.00–1.72)	0.12 (0.01–1.02)	0.31 (0.07–1.46)	0.43 (0.10–1.76)	0.63 (0.06–6.25)	PAZ	—	—
0.08 (0.01–1.13)	0.05 (0.00–2.09)	0.09 (0.01–1.50)	0.24 (0.02–3.16)	0.33 (0.03–3.20)	0.49 (0.03–8.88)	0.78 (0.05–11.27)	CAB + NIV	—
0.04 (0.00–0.59)	0.03 (0.00–1.12)	0.05 (0.00–0.78)	0.14 (0.01–1.64)	0.19 (0.02–1.64)	0.28 (0.02–4.67)	0.45 (0.03–5.89)	0.58 (0.03–13.14)	LEN + PEM
B								
TIV	0.69 (0.48–1.00)	—	—	—	—	—	—	—
0.69 (0.48–1.00)	SOR	—	0.84 (0.56–1.26)	—	0.81 (0.53–1.24)	—	—	—
0.60 (0.34–1.05)	0.86 (0.56–1.32)	AXI + AVE	0.98 (0.73–1.32)	—	—	—	—	—
0.59 (0.36–0.95)	0.85 (0.62–1.16)	0.98 (0.73–1.32)	SUN	1.00 (0.74–1.34)	0.95 (0.73–1.24)	0.89 (0.45–1.75)	0.79 (0.56–1.12)	0.54 (0.38–0.78)
0.59 (0.33–1.03)	0.85 (0.55–1.30)	0.98 (0.65–1.49)	1.00 (0.74–1.34)	AXI + PEM2	—	—	—	—
0.56 (0.34–0.91)	0.81 (0.59–1.11)	0.94 (0.64–1.37)	0.95 (0.75–1.21)	0.95 (0.65–1.40)	PAZ	—	—	—
0.52 (0.23–1.20)	0.75 (0.36–1.59)	0.87 (0.42–1.83)	0.89 (0.45–1.75)	0.89 (0.42–1.86)	0.93 (0.45–1.92)	CAB	—	—
0.46 (0.26–0.84)	0.67 (0.42–1.07)	0.78 (0.49–1.22)	0.79 (0.56–1.12)	0.79 (0.50–1.25)	0.83 (0.54–1.27)	0.89 (0.41–1.91)	CAB + NIV	—
0.32 (0.17–0.58)	0.46 (0.29–0.74)	0.53 (0.34–0.85)	0.54 (0.38–0.78)	0.54 (0.34–0.87)	0.57 (0.37–0.88)	0.61 (0.28–1.32)	0.69 (0.42–1.14)	LEN + PEM
C								
SOR	1.58 (0.55–4.52)	—	0.95 (0.30–3.03)	0.64 (0.28–1.45)	—	—	—	
1.01 (0.46–2.25)	PAZ	—	—	1.25 (0.47–3.37)	—	—	—	
0.95 (0.24–3.82)	0.94 (0.22–4.02)	CAB	—	0.90 (0.26–3.08)	—	—	—	
0.95 (0.30–3.03)	0.94 (0.23–3.84)	1.00 (0.16–6.12)	TIV	—	—	—	—	
0.86 (0.45–1.65)	0.85 (0.39–1.85)	0.90 (0.26–3.08)	0.90 (0.24–3.41)	SUN	0.83 (0.30–2.31)	0.37 (0.13–1.00)	0.28 (0.10–0.78)	
0.71 (0.21–2.40)	0.70 (0.19–2.55)	0.75 (0.15–3.71)	0.75 (0.14–4.02)	0.83 (0.30–2.31)	CAN + NIV	—	—	
0.31 (0.09–1.05)	0.31 (0.09–1.11)	0.33 (0.07–1.62)	0.33 (0.06–1.75)	0.37 (0.13–1.00)	0.44 (0.11–1.87)	AXI + PEM2	—	
0.24 (0.07–0.82)	0.24 (0.07–0.87)	0.26 (0.05–1.26)	0.26 (0.05–1.37)	0.28 (0.10–0.78)	0.34 (0.08–1.46)	0.77 (0.19–3.24)	LEN + PEM	
D								
TIV	—	0.22 (0.11–0.42)	—	—	—	—	—	
0.26 (0.10–0.70)	AXI + PEM2	—	—	—	0.59 (0.33–1.05)	—	—	
0.22 (0.11–0.42)	0.83 (0.40–1.74)	SOR	0.69 (0.36–1.32)	—	0.99 (0.52–1.90)	—	—	
0.18 (0.08–0.41)	0.69 (0.33–1.43)	0.83 (0.50–1.35)	PAZ	—	0.76 (0.44–1.30)	—	—	
0.16 (0.06–0.42)	0.60 (0.27–1.34)	0.72 (0.35–1.48)	0.87 (0.43–1.79)	AXI + AVE	0.98 (0.56–1.71)	—	—	
0.16 (0.07–0.34)	0.59 (0.33–1.05)	0.71 (0.45–1.12)	0.86 (0.55–1.35)	0.98 (0.56–1.71)	SUN	0.62 (0.27–1.41)	0.28 (0.15–0.50)	
0.10 (0.03–0.30)	0.37 (0.13–1.00)	0.44 (0.17–1.12)	0.53 (0.21–1.36)	0.61 (0.23–1.64)	0.62 (0.27–1.41)	CAB	—	
0.04 (0.02–0.12)	0.16 (0.07–0.38)	0.20 (0.09–0.42)	0.24 (0.11–0.50)	0.27 (0.12–0.61)	0.28 (0.15–0.50)	0.45 (0.16–1.23)	LEN + PEM	

Data presented as ORs with 95% CIs. CAB, cabozantinib; PAZ, pazopanib; SOR, sorafenib; SUN, sunitinib; TIV, tivozanib; CAB + NIV, cabozantinib + nivolumab; LEN + PEM, lenvatinib + pembrolizumab; AXI + AVE, axitinib + avelumab; AXI + PEM2, axitnib + pembrolizumab. The results of direct comparisons are presented above the abbreviations of TKIs. On a gray background there is a symbol of appropriate intervention and on a white background statistically significant results are bolded.

was the highest for lenvatinib + pembrolizumab (82.2%; 95% CI: 76.3%, 85.4%), while the lowest risk was noted for tivozanib (59.6%; 95% CI: 47.7%, 67.8%). Axitinib combination therapies were ranked the best among combination therapies; axitinib + avelumab had the lowest adjusted mean risk of grade ≥3 AEs (71.2%; 95% CI: 64.9%, 74.5%) (Table 5).

There were no differences between TKIs (monotherapy and combination therapy) in terms of treatment discontinuation due to AEs, except lenvatinib + pembrolizumab compared with sorafenib (p = 0.022), pazopanib (p = 0.030), and sunitinib (0.015). Interestingly, the adjusted mean risk of treatment discontinuation due to AEs was the highest for tivozanib (20.8%; 95% CI: 6.5%, 44.3%) sorafenib (21.6%; 95% CI: 12.5%, 29.8%), cabozantinib (20.8%; 95% CI: 7.1%, 41.7%), and pazopanib (21.8%; 95% CI: 11.3%, 32.9%), and the lowest for lenvatinib + pembrolizumab (6.3%; 95% CI: 2.4%, 13.0%) (Tables 4, 5; Supplementary Figure S2; Supplementary Table S14). Considering dose modifications due to AEs (irrespective of their relation to treatment), tivozanib reduced the risk of any dose modifications as compared with all other TKI monotherapies and all combination therapies (p < 0.05). On the other hand, lenvatinib + pembrolizumab increased the risk of dose modifications due to AEs compared with tivozanib, sorafenib, pazopanib, sunitinib, axitinib + pembrolizumab, and axitinib + avelumab (p < 0.05). The highest adjusted mean risk of dose modifications due to AEs was noted for lenvatinib + pembrolizumab (72.7%; 95% CI: 59.6%, 78.4%), while the lowest–for tivozanib (10.3%; 95% CI: 5.0%, 16.1%) (Tables 4, 5; Supplementary Figure S2; Supplementary Table S15).

3.3.2 Gastrointestinal adverse events

The individual AEs reported in the references were divided into two categories: gastrointestinal (diarrhea, nausea, and vomiting) and other (fatigue, hypertension, and dysphonia). Data for any AEs and grade ≥3 AEs were presented.

Sunitinib treatment was associated with a lower risk of diarrhea of any grade as compared with sorafenib (p = 0.023), axitinib + avelumab (p = 0.030), cabozantinib + nivolumab (p = 0.018), and cabozantinib (p = 0.048) (Supplementary Figure S3; Supplementary Table S16). The adjusted mean risk of diarrhea of any grade differed between interventions. The mean risk of diarrhea of any grade was the highest for cabozantinib (66.7%; 95% CI: 46.7%, 78.8%) and cabozantinib + nivolumab (63.2%; 95% CI: 49.9%, 71.0%) and the lowest for sunitinib (46.6%; 95% CI: 41.5%, 51.7%) and tivozanib (46.8%; 95% CI: 29.4%, 60.2%) (Table 5).

For grade ≥3 diarrhea, there were no significant differences between TKIs (monotherapy and combination therapy), except the lower risk for tivozanib vs. sorafenib (p = 0.024) and sunitinib vs. lenvatinib + pembrolizumab (p = 0.032), axitinib + pembrolizumab (p = 0.013), sorafenib (p = 0.039), and axitinib + avelumab (p = 0.008) (Supplementary Figure S4; Supplementary Table S17). The adjusted mean risk of grade ≥3 diarrhea was generally low and differed between interventions. The mean risk of grade ≥3 diarrhea was the highest for axitinib + avelumab (11.1%; 95% CI: 5.9%, 14.1%) and the lowest for tivozanib (3.5%; 95% CI: 1.1%, 7.5%) (Table 5).

There were no differences between TKIs in the risk of nausea of any grade except for sorafenib vs. pazopanib (p = 0.011), sunitinib (p = 0.003), and lenvatinib + pembrolizumab (p = 0.023) (Supplementary Figure S3; Supplementary Table S18). The adjusted mean risk of nausea of any grade was similar among interventions, with the highest risk for lenvatinib + pembrolizumab (38.1%; 95% CI: 27.3%, 45.1%) and the lowest risk for sorafenib (22.4%; 95% CI: 15.8%, 26.5%) (Table 5).

There were no significant differences among TKIs used as monotherapy or as combination therapy for grade ≥3 nausea. The mean risk of grade ≥3 nausea was low and was similar among interventions, with the highest risk for lenvatinib + pembrolizumab (6.6%; 95% CI: 0.6%, 33.0%) and the lowest risk for pazopanib (0.8%; 95% CI: 0.1%, 2.8%) (Table 5; Supplementary Figure S4; Supplementary Table S19).

As for vomiting, sorafenib treatment was associated with a lower risk of vomiting of any grade compared with the other interventions such as axitinib + pembrolizumab (p = 0.015), cabozantinib + nivolumab (p = 0.015), axitinib + avelumab (p = 0.009), sunitinib (p = 0.005), cabozantinib (p = 0.010), pazopanib (p = 0.004), and lenvatinib + pembrolizumab (p = 0.002) (Supplementary Figure S3; Supplementary Table S20). In addition, treatment with axitinib + pembrolizumab was associated with a lower risk of vomiting than lenvatinib + pembrolizumab (p = 0.022). Treatment with cabozantinib + nivolumab outperformed that with lenvatinib + pembrolizumab (p = 0.036) (Supplementary Table S20). The mean risk of vomiting of any grade was the highest for lenvatinib + pembrolizumab (27.9%; 95% CI: 21.3%–32.1%) and the lowest for sorafenib (2.9%; 95% CI: 0.6%, 10.8%) (Table 5).

For grade ≥3 vomiting, there were no differences between TKIs (either as monotherapy or as combination therapy), except for a lower risk of vomiting for axitinib + pembrolizumab vs. cabozantinib + nivolumab (p = 0.039) (Supplementary Figure S4; Supplementary Table S21). The mean risk of grade ≥3 vomiting was low and was similar in all interventions, except for cabozantinib + nivolumab (9.2%; 95% CI: 1.2%, 32.7%) and lenvatinib + pembrolizumab (3.8%; 95% CI: 1.4%, 6.1%). The mean risk of grade ≥3 vomiting was the lowest for axitinib + pembrolizumab (0.4%; 95% CI: 0.0%, 2.1%) and for sorafenib (0.5%; 95% CI: 0.0%, 6.6%) (Table 5).

3.3.3 Other individual adverse events

Other AEs (all grades and grade ≥3) included fatigue, hypertension, and dysphonia. There were no differences between TKIs in the risk of fatigue of any grade, except for sorafenib vs. sunitinib (p = 0.001), axitinib + pembrolizumab (p = 0.011), axitinib + avelumab (p = 0.006), and lenvatinib + pembrolizumab (p = 0.003) and for pazopanib vs. sunitinib (p = 0.007), axitinib + avelumab (p = 0.044), and lenvatinib + pembrolizumab (p = 0.022) (Supplementary Figure S3; Supplementary Table S22). The adjusted mean risk of fatigue of any grade was the highest for lenvatinib + pembrolizumab (50.1%; 95% CI: 42.5%, 48.7%) and axitinib + avelumab (48.0%; 95% CI: 41.4%, 45.7%), and the lowest for sorafenib (34.6%; 95% CI: 28.3%, 33.1%) (Table 5).

For grade ≥3 fatigue, there were no significant differences between TKIs (monotherapy and combination therapy) (Supplementary Figure S4; Supplementary Table S23). The mean risk of grade ≥3 fatigue was low and similar in all interventions. The lowest risk was noted for cabozantinib (3.1%; 95% CI: 0.8%, 6.7%), axitinib + pembrolizumab (3.7%; 95% CI: 1.3%, 6.0%), and sorafenib (3.8%; 95% CI: 1.5%, 5.5%) (Table 5).

Treatment with sorafenib was associated with a lower risk of hypertension of any grade as compared with pazopanib (p = 0.001),

TABLE 5 Mean probability of adverse events with 95% CIs in the brackets.

Intervention	Adverse events	Grade ≥3 adverse events	Discontinuation due to adverse events	Dose change due to adverse events	Fatigue	Diarrhea	Nausea	Vomiting	Hypertension	Dysphonia	Grade ≥3 fatigue	Grade ≥3 diarrhea	Grade ≥3 nausea	Grade ≥3 vomiting	Grade ≥3 hypertension
TIV	95.9% (84.4%–98.6%)	59.6% (47.7%–67.8%)	20.8% (6.5%–44.3%)	10.3% (5.0%–16.1%)	40.0% (27.9%–44.4%)	46.8% (29.4%–60.2%)	32.9% (17.7%–47.9%)	n/a	43.7% (30.6%–54.2%)	n/a	5.9% (1.3%–13.9%)	3.5% (1.1%–7.5%)	1.1% (0.0%–23.6%)	n/a	18.5% (7.9%–35.0%)
SOR	98.7% (95.7%–99.4%)	68.1% (60.9%–72.0%)	21.6% (12.5%–29.8%)	34.4% (25.0%–38.6%)	34.6% (28.3%–33.1%)	59.2% (48.4%–64.6%)	22.4% (15.8%–26.5%)	2.9% (0.6%–10.8%)	33.6% (26.2%–38.4%)	n/a	3.8% (1.5%–5.5%)	9.8% (4.9%–13.3%)	1.1% (0.2%–3.9%)	0.5% (0.0%–6.6%)	11.8% (6.8%–18.0%)
CAB	93.8% (43.4%–99.5%)	73.9% (59.0%–83.1%)	20.8% (7.1%–41.7%)	54.4% (34.5%–67.2%)	42.3% (27.1%–50.1%)	66.7% (46.7%–78.8%)	28.9% (15.9%–41.8%)	22.3% (11.8%–34.6%)	63.0% (45.5%–75.0%)	26.6% (7.4%–55.2%)	3.1% (0.8%–6.7%)	4.3% (1.6%–7.7%)	1.0% (0.1%–7.4%)	0.8% (0.1%–4.7%)	22.7% (9.9%–41.3%)
AXI + PEM2	96.6% (85.6%–99.0%)	71.6% (65.3%–74.9%)	8.0% (3.1%–16.0%)	30.4% (19.7%–37.1%)	47.2% (40.4%–45.1%)	56.0% (42.6%–63.9%)	31.4% (22.1%–37.6%)	17.6% (13.0%–20.8%)	39.7% (31.1%–45.3%)	27.1% (17.3%–33.3%)	3.7% (1.3%–6.0%)	9.1% (5.4%–10.2%)	1.6% (0.1%–9.2%)	0.4% (0.0%–2.1%)	19.0% (10.2%–30.0%)
PAZ	99.6% (98.3%–99.9%)	72.6% (67.4%–74.8%)	21.8% (11.3%–32.9%)	38.9% (28.8%–43.2%)	39.2% (34.1%–35.9%)	56.7% (45.7%–62.3%)	34.1% (25.9%–38.3%)	22.6% (18.3%–24.6%)	48.4% (40.7%–52.6%)	n/a	4.9% (2.2%–6.2%)	5.4% (3.7%–5.4%)	0.8% (0.1%–2.8%)	1.1% (0.5%–1.4%)	20.1% (12.1%–29.3%)
SUN	99.0% (98.6%–99.4%)	71.6% (68.9%–74.2%)	19.1% (16.0%–22.5%)	42.5% (36.0%–49.3%)	46.6% (37.8%–55.6%)	46.6% (41.5%–51.7%)	35.5% (31.0%–40.1%)	21.5% (19.0%–24.1%)	40.6% (37.0%–44.1%)	3.6% (2.7%–4.5%)	8.7% (5.1%–13.1%)	4.7% (3.1%–6.5%)	1.6% (0.9%–2.5%)	1.6% (0.9%–2.5%)	16.4% (15.0%–18.0%)
CAB + NIV	99.7% (96.9%–100.0%)	76.1% (69.2%–79.9%)	16.4% (6.6%–30.5%)	n/a	43.8% (36.0%–43.0%)	63.2% (49.4%–71.0%)	31.0% (21.3%–38.0%)	17.9% (12.8%–21.8%)	38.0% (28.7%–44.5%)	17.8% (10.0%–24.2%)	6.4% (2.1%–10.9%)	7.4% (3.8%–9.4%)	3.1% (0.1%–29.0%)	9.2% (1.2%–32.7%)	15.7% (7.8%–26.8%)
AXI + AVE	99.3% (96.1%–99.8%)	71.2% (64.9%–74.5%)	n/a	42.1% (29.5%–49.0%)	48.0% (41.4%–45.7%)	61.2% (48.0%–68.7%)	30.6% (21.6%–36.5%)	20.0% (15.1%–23.1%)	54.4% (44.9%–60.0%)	33.3% (22.0%–39.9%)	8.2% (2.9%–13.1%)	11.1% (5.9%–14.1%)	1.4% (0.1%–7.0%)	0.9% (0.3%–1.8%)	24.7% (13.8%–37.6%)
LEN + PEM	99.8% (98.4%–100.0%)	82.2% (76.3%–85.4%)	6.3% (2.4%–13.0%)	72.7% (59.6%–78.4%)	50.1% (42.5%–48.7%)	58.6% (44.9%–66.7%)	38.1% (27.3%–45.1%)	27.9% (21.3%–32.1%)	54.5% (44.4%–60.7%)	26.9% (17.0%–33.2%)	8.4% (2.9%–13.5%)	8.6% (4.9%–10.0%)	6.6% (0.6%–33.0%)	3.8% (1.4%–6.1%)	24.4% (13.5%–37.5%)

CAB, cabozantinib; PAZ, pazopanib; SOR, sorafenib; SUN, sunitinib; TIV, tivozanib; CAB + NIV, cabozantinib + nivolumab; LEN + PEM, lenvatinib + pembrolizumab; AXI + AVE, axitinib + avelumab; AXI + PEM2–axitinib + pembrolizumab; n/a–not assessable.

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axitinib + avelumab (p = 0.001), lenvatinib + pembrolizumab (p = 0.002), and cabozantinib (p = 0.003). In addition, the following treatments were less likely to cause hypertension of any grade: 1) cabozantinib + nivolumab vs. axitinib + avelumab (p = 0.021), lenvatinib + pembrolimus (p = 0.024), and cabozantinib (p = 0.015); 2) axitinib + pembrolizumab vs. axitinib + avelumab (p = 0.030), lenvatinib + pembrolizumab (p = 0.034), and cabozantinib (p = 0.021); and 3) sunitinib vs. pazopanib (p = 0.047), axitinib + avelumab (p = 0.004), lenvatinib + pembrolizumab (p = 0.006), and cabozantinib (p = 0.012) (Supplementary Figure S3; Supplementary Table S24). The adjusted mean risk of hypertension of any grade differed between interventions. The risk was the highest for cabozantinib (63.0%; 95% CI: 45.5%, 75.0%) and the lowest for sorafenib (33.6%; 95% CI: 45.5%, 75.0%) (Table 5).

There were no significant differences between TKIs (monotherapy and combination therapy) in the risk of grade ≥3 hypertension (Supplementary Figure S4; Supplementary Table S25). The adjusted mean risk of grade ≥3 hypertension was the highest for axitinib + avelumab (24.7%; 95% CI: 13.8%, 37.6%), lenvatinib + pembrolizumab (24.4%; 95% CI: 13.5%, 37.5%), and cabozantinib (22.7%; 95% CI: 9.9%, 41.3%) and the lowest for sorafenib (11.8%; 95% CI: 6.8%, 43.1%) (Table 5).

Treatment with sorafenib was associated with a lower risk of dysphonia of any grade as compared with cabozantinib + nivolumab (p = 0.000), cabozantinib (p = 0.003), axitinib + pembrolizumab (p = 0.000), lenvatinib + pembrolizumab (p = 0.000), and axitinib + avelumab (p = 0.000) (Supplementary Figure S3; Supplementary Table S26). The adjusted mean risk of dysphonia of any grade differed between interventions. The risk was the highest for axitinib + avelumab (33.3%; 95% CI: 22.0%, 39.9%) and the lowest for sunitinib (3.6%; 95% CI: 2.7%, 4.5%) (Table 5).

3.4 Assessment of the networks

There was no heterogeneity in the NMA of vomiting (any grade and grade ≥3) and dysphonia due to network design excluding comparisons other than those with sunitinib. A low overall heterogeneity of the effect sizes was observed in the networks of any AEs (I^2 = 0%, p = 0.764), grade ≥3 AEs (I^2 = 0%, p = 0.383), fatigue (I^2 = 0%, p = 0.655), grade ≥3 diarrhea (I^2 = 0%, p = 0.625), nausea (I^2 = 35.7%, p = 0.211), hypertension (I^2 = 25.1%, p = 0.263), and grade ≥3 fatigue (I^2 = 29.1%, p = 0.244). Moderate heterogeneity was observed in the network of grade ≥3 nausea (I^2 = 49.8%, p = 0.136) and grade ≥3 hypertension (I^2 = 49.8%, p = 0.141), while moderate to substantial heterogeneity was observed in the network of dose modifications due to AEs (I^2 = 59.7%, p = 0.093). Considerable heterogeneity was observed only for the network of treatment discontinuation due to AEs (I^2 = 75.1%, p = 0.007).

There was no significant between-design heterogeneity (inconsistency) in any network, except that of treatment discontinuation due to AEs (p = 0.003; Supplementary Table S27). Therefore, the results of this network should be interpreted with caution.

The evidence for the comparison of pazopanib vs. sorafenib (SWITCH II trial), sorafenib vs. sunitinib (SWITCH, CROSS-J-RCC trials), and pazopanib vs. sunitinib (COMPARZ trial) was the major contributor to the observed heterogeneity in the network of treatment discontinuation due to AEs. A considerable, but not significant, dissagreament between direct and indirect evidence was observed for those comparisons (p-value from 0.202 to 0.242). The relative difference between NMA results and clinical trial results was 56% for pazopanib vs. sorafenib, 35% for sorafenib vs. sunitinib, and 32% for pazopanib vs. sunitinib. A difference of more 10% between NMA results and clinical trial results for those comparisons was also found for the networks of AEs, dose modifications due to AEs, diarrhea, hypertension, grade ≥3 nausea, grade ≥3 fatigue, and grade ≥3 hypertension.

Furthermore, some dissagreement was observed for sorafenib vs. interferon α (NCT00117637) and sunitinib vs. interferon α (NCT00098657/NCT00083889 trial) in the network of dose modifications due to AEs.

Overall, the odds ratios from all networks (direct and indirect evidence combined) were similiar to direct evidence. No publication bias was found in any of the networks, however, there are too few studies to reliably assess this effect.

4 Discussion

In recent years, the number of approved first-line therapies for metastatic clear cell RCC has been gradually increasing. Considering the limited availability of high-quality RCTs allowing direct comparisons, there is still a strong need for a reliable indirect comparison of approved TKIs. Patients with metastatic RCC generally have poor prognosis and limited overall survival. According to clinical guidelines, the selection of therapy in metastatic RCC should be guided by disease stage, risk stratification, comorbidities, and safety profile. Most systematic reviews with NMA published to date (Hahn et al., 2019; Heo et al., 2021; Kartolo et al., 2021) focused primarily on aspects related to efficacy, assessing and comparing individual TKIs in terms of overall survival, progression free-survival, or response to treatment according to RECIST criteria. As for safety, recent NMAs were limited to general safety endpoints such as the overall frequency of AEs, grade ≥3 AEs, or treatment discontinuation due to AEs (Liu et al., 2021). So far, there were no analyses that would compare all approved TKIs (used as monotherapy and in cobmination) with respect to the risk of individual AEs.

In this study, we assessed the most common individual AEs as well as individual AEs of grade ≥3, which may have significant effects on treatment and may require additional therapy. By combining the direct and indirect evidence from 13 RCTs that also assessed TKIs in combination with immunotherapy, we were able to conduct a more comprehensive analysis, and our findings may be useful for clinicians, patients, and healthcare decision makers. Considering TKIs as monotherapy, our NMA showed that sorafenib and tivozanib were the best treatment options: sorafenib ranked highest for treatment discontinuation due to AEs, fatigue of any grade, nausea, vomiting, hypertension (any grade or grade ≥3), while tivozanib had the highest P score for any AEs, dose modifications due to AEs, and grade ≥3 diarrhea. In addition, tivozanib was associated with a significantly lower risk of grade ≥3 AEs compared with sunitinib, pazopanib, cabozantinib +

nivolumab, and lenvatinib + pembrolizumab. As TKIs have antiangiogenic properties, hypertension is recognized as one of the most common side effects of this drug class and a potential marker of treatment effectiveness (Liu et al., 2021). The highest rate of hypertension of any grade and grade ≥3 was noted for cabozantinib, axitinib + avelumab, and lenvatinib + pembrolizumab, and these options were ranked as most effective based on meta-analyses by Nocera et al. (2022) and Liu et al. (2021) (assessing combination therapies) and by Manz et al. (2020) (assessing TKIs used only as monotherapy). The other results obtained in this NMA are also in line with the study by Manz et al. (2020) owed that tivozanib had the most favorable safety profile in terms of grade 3 or 4 AEs and was associated with a significantly lower risk of side effects when compared with other TKIs.

This NMA also showed that TKIs used in combination are less safe than TKIs used as monotherapy. The combination of lenvatinib and pembrolizumab was ranked as the worst option based on the highest mean risk of AEs of any grade, treatment discontinuation due to AEs, dose modifications due to AEs, and grade ≥3 nausea. There was a significantly higher risk of grade ≥3 AEs with the combination of lenvatinib and pembrolizumab compared with most monotherapies and other combination therapies. Combination therapies with axitinib were ranked as the best combination options.

Rizzo et al. (2022) conducted a meta-analysis in which they assessed the occurrence of AEs of any grade and grade ≥3 in studies comparing sunitinib monotherapy and a combination of immunotherapy with a TKI. The relative risk was similar in patients receiving combination therapy and sunitinib monotherapy. However, combination therapy was associated with an increased risk of diarrhea (any grade and grade ≥3), hypothyroidism (any grade or grade ≥3), decreased appetite (grade ≥3), increased aspartate aminotransferase levels (grade ≥3), and increased alanine transaminase levels (any grade). The results of our meta-analysis are consistent with those obtained by Rizzo et al., (2022) and suggest that the risk of treatment emergent AEs should be carefully considered when selecting a combination therapy in patients with metastatic RCC. In an NMA by Nocera et al (2022), based on a ranking quantifying the lowest likelihood of grade ≥3 AEs, sunitinib showed the lowest toxicity (p = 0,74), followed by axitinib + pembrolizumab (p = 0.47), cabozantinib + nivolumab (p = 0.22), and lenvatinib + pembrolizumab (p = 0.06) with the highest probability of grade ≥3 AEs. In an NMA by Quhal et al. (2021), the highest probability of treatment discontinuation related to AEs was shown for lenvatinib in combination with pembrolizumab. This was in contrast to an NMA by Liu et al. (2021), in which the most severe AEs were associated with axitinib in combinaiton with pembrolizumab.

NMA conducted by Manz et al. (2020) showed that cabozantinib, sunitinib, pazopanib, and tivozanib do not differ significantly in terms of efficacy, but tivozanib was associated with a more favorable safety profile in terms of grade ≥3 toxicity, simillary as in our NMA. Therefore, the relative toxicity of these first-line TKIs may play a more significant role than comparisons of efficacy in treatment decisions and planning future clinical trials (Nocera et al., 2022).

Our meta-analysis has some limitations that should be considered when interpreting the results. First, we included only studies on TKIs approved by the European Medicines Agency (EMA) or the Food and Drug Administration (FDA) for use as monotherapy or in combination for the first-line treatment of patients with metastatic RCC. Studies concerning, for example, the use of axitinib as monotherapy in previously untreated patients were excluded, because axitinib is currently approved for use as first-line treatment only in combination with avelumab or pembrolizumab. Second, some of the assessed interventions may differ in terms of efficacy (Heo et al., 2021), because these drugs are usually used until disease progression or unacceptable toxicity occurs, which may result in a different duration of exposure to treatment. On the one hand, a more appropriate measure in this scenario would be a comparison of exposure-adjusted incidence rate of AEs, especially in studies with long-term follow-up (Kartolo et al., 2021), but on the other hand, most of the published studies assessing TKIs reported only the percentages of patients experiencing AEs. To avoid potential diffrences in the duration of exposure to the same intervention between different studies due to different baseline characteristics of patients, inclusion criteria in our review were limited to the stage of the disease (metastatic), the line of treatment (first line) and the histological type of cancer (clear cell). The included studies were generally well balanced. Due to the similar mechanism of action, the differences in the duration of exposure to treatment between TKI monotherapies in the included studies were relatively small. The longest duration of exposure to treatment was observed for studies evaluating the combinations of TKIs with immunotherapy, which may be one of the reasons for the generally worse safety profile of combination therapies vs. monotherapies with TKIs. Another reason is that patients receiving combination therapies are treated with two drugs with different mechanisms of action and overlapping adverse reactions. We included only RCTs because they have the highest credibility. Nevertheless, in included trials, people involved in safety assessment (both patients and physicians) were not blinded (in some studies, only the persons/committee who assess the results for the primary end point, i.e., survival rates, were blinded). This was the main reason why the risk of performance bias was assessed as high. It can be assumed that the risk of bias related to incomplete blinding was similar in all included studies. There was some disagreement between direct and indirect evidence in pazopanib trials, for example, in terms of the rate of grade ≥3 nausea: the CROSS-J-RCC trial reported a rate of 0%, while other trials reported some cases of nausea. Some differences in baseline characteristics between pazopanib trials may cause the heterogeneity of results. The higher rate of grade ≥3 nausea in the TemPa trial may be due to the fact that >50% of patients had an ECOG performance status of 2, while in the remaining studies on pazopanib, <50% of patients had an ECOG performance status of 0. Furthermore, not all included trials reported the assessed safety outcomes. The results from clinical trials registries could not be used, because they report these endpoints in a different way: only serious AEs or nonserious AEs. Furthermore, the results presented in registries are not official results, and, by definition, they have lower reliability than data from full-text publications. Sometimes, individual but rare AEs are not reported because of the threshold used in a publication (e.g., only AEs that occurred in at least 10% or 20% of patients in either group). Therefore, it was impossible to conduct an NMA in terms of grade ≥3 dysphonia.

According to the latest clinical ASCO, ESMO and NCCN guidelines for the treatment of metastatic clear cell RCC, the TKIs still play an important role in the first-line setting (Rathmell et al., 2022; European Society for Medical Oncology,

2022; Motzer et al., 2022b). Patients with favorable-risk disease who require systemic therapy may be offered an immunotherapy with an immune checkpoint inhibitor in combination with a vascular endothelial growth factor receptor (VEGFR TKI); patients with intermediate or poor risk should be offered a doublet regimen (immune checkpoint inhibitor in combination with a VEGFR TKI or TKIs as monotherapy). For selected patients, monotherapy with either an immune checkpoint inhibitor or a VEGFR TKI may be offered depending on comorbidities and general health (Rathmell et al., 2022; Motzer et al., 2022b).

In summary, when choosing the appropriate therapy for individual patients, clinicians should consider the overall safety profile of TKIs as well as the prevalence of the most common AEs (particularly specific AEs), rather than looking at efficacy. Despite several limitations, this systematic review with NMA is the first original study to provide new data on the relative safety of various TKIs, focusing on the AEs (all grades and grade ≥3), treatment discontinuation due to AEs, dose modification due to AEs, and the risk of specific AEs that are most commonly listed in the summary of products characteristics (i.e., fatigue, diarrhea, nausea, vomiting, hypertension, and dysphonia). Since this approach has not been used in previous systematic reviews, our study provides the most up-to-date results in terms of an in-depth comparative safety analysis of TKIs used alone or in combination. Our findings underscore the importance of considering monotherapy with TKIs as the preferred way to achieve improved safety outcomes, especially when compared with combination therapy based on immune drugs. The results may help clinicians and patients choose the best treatment option from a wide range of available TKIs. Moreover, they may serve as guidance for healthcare policymakers in developing reimbursement policies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

KK, PK, and KŚ conceived the conception and design of the study; KK and KŚ performed systematic review and the data extraction; PH conducted network meta-analysis, validated the models, and visualized the results; KK, KŚ, and PH drafted the manuscript; PK critically revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1223929/full#supplementary-material

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Aumolertinib: effective treatment for asymptomatic pulmonary giant cell carcinoma with *EGFR* L858R mutation - a case report

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Aumolertinib, as a novel third-generation epidermal growth factor receptor tyrosine kinase inhibitor (*EGFR*-TKI), has been widely employed as a first-line treatment for advanced non-small cell lung cancer (NSCLC) patients with *EGFR* mutation. However, reports regarding the benefit of using aumolertinib as a monotherapy in pulmonary giant cell carcinoma are relatively scarce. In this report, we present a pulmonary giant cell carcinoma case harboring the *EGFR* Leu858Arg (L858R) mutation, with the patient at stage cT2bN3M1c IVB. Through the use of autolearning as a single agent, we effectively controlled the progression of pulmonary giant cell carcinoma, achieving a 6-month progression-free survival during the treatment course. Notably, the patient's tumor not only ceased its growth but also continued to shrink, highlighting a significant therapeutic effect. This case reveals the effectiveness of aumolertinib as a monotherapy in controlling disease progression. The finding underscores the therapeutic advantage of aumolertinib in this particular subgroup of patients, offering a novel treatment option for pulmonary giant cell carcinoma.

KEYWORDS

aumolertinib, PGCC, EGFR mutation, L858R, treatment

Introduction

Pulmonary giant cell carcinoma, a rare subtype within non-small cell lung cancer, accounts for only 0.11% of cases (1). Due to its complex pathological features, rapid clinical progression, poor prognosis, and frequent early metastasis, surgical treatment options are limited. Advanced non-small cell lung cancer treatment has undergone significant transformation in recent decades. EGFR-TKIs have become the first-line treatment for EGFR-positive patients. However, within the realm of pulmonary giant cell carcinoma, there remains a scarcity of reports on *EGFR* mutations and the solitary utilization of a drug like aumolertinib.

In this study, we present a pulmonary giant cell carcinoma case with the *EGFR* exon 21 L858R mutation. Treatment with aumolertinib led to significant reductions in tumor size and lymph node enlargement in the patient. Furthermore, we conducted a comprehensive literature review, exploring the advantages of aumolertinib in treating pulmonary giant cell carcinoma, while also investigating prevailing mechanisms of resistance and potential therapeutic strategies. This case not only contributes to the clinical understanding of pulmonary giant cell carcinoma but also provides valuable insights into the application of third-generation *EGFR*-TKIs for managing this uncommon subset of lung cancer.

Case presentation

A 60-year-old female patient with no smoking history or family history of cancer presented in February 2023 with a 4-day history of left upper limb pain. Subsequent shoulder joint X-ray revealed a shadow in the left upper lobe of the lung (Figure 1A). Despite the absence of typical respiratory symptoms such as chest pain, chronic cough, fever, or shortness of breath, contrast-enhanced chest computed tomography (CT) disclosed a solid lesion within the left upper lobe measuring 5.0 x 3.7 cm, characterized by clear and lobulated margins. Furthermore, an enlarged right diaphragmatic lymph node was observed (Figure 1A). Fiberoptic bronchoscopy demonstrated patent left main bronchus, lobar bronchi, and segmental bronchi, with no evidence of new growth or mucosal erosion. Analysis of bronchoalveolar lavage fluid indicated a slight presence of neutrophils and macrophages, alongside a significant amount of epithelial cells. A bone scan revealed elevated basal metabolic activity, particularly in the right pubic ramus, suggesting potential metastasis. Subsequent CT-guided biopsy of the left upper lung revealed atypical giant cells within fibrous tissue (Figure 1A). The cranial magnetic resonance imaging (MRI) examination did not reveal any evidence of intracranial metastases (Figure 1B). Immunohistochemical analysis further confirmed its pulmonary origin, with positive markers including CK(+), CK7(+), NaspinA (+), TTF-1(+), Ki67(+, 5%), p53(+, 10%), CK5/6 (–), P40 (–), and VIM (–) (Figures 2A–I).To assess the possibility of distant metastasis, the patient underwent positron emission tomography-computed tomography (PET-CT), which indicated elevated glucose metabolism in the left upper lobe tumor. Additionally, lymph nodes near the aortic arch and left pulmonary hilum, as well as adjacent to the aorta and left portal vein, demonstrated potential signs of tumor metastasis. Notably, the possibility of bone metastasis was also considered, with the left humerus, bilateral pubic bones, and right ischium being potentially affected. Upon comprehensive evaluation

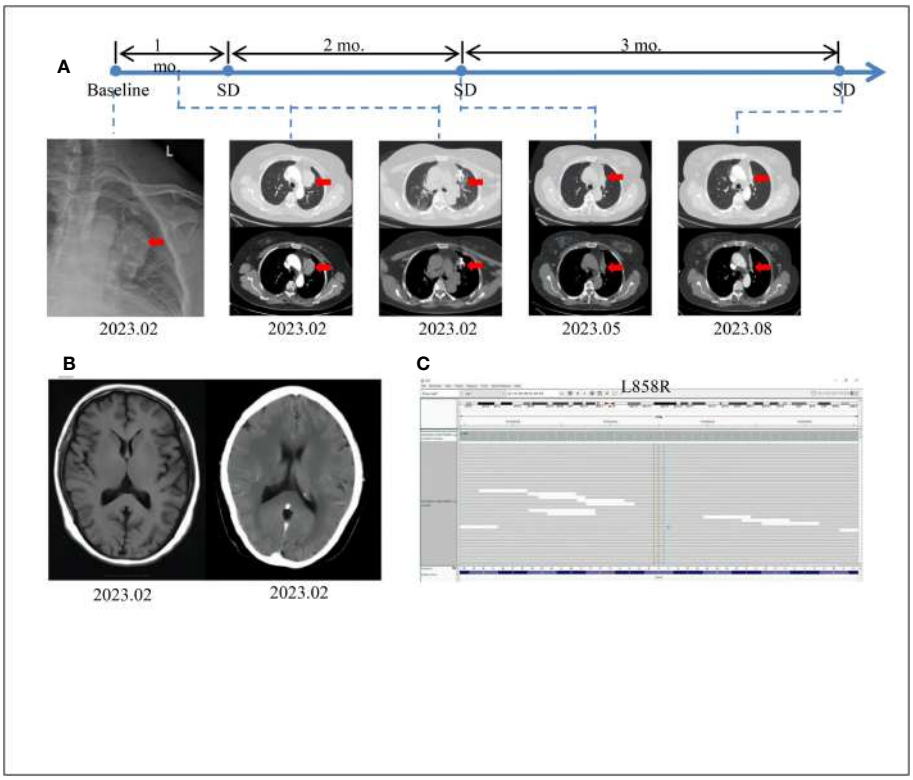

FIGURE 1
Tumor progression of the patient before and after treatment. **(A)** The timeline of therapies and tumor progression are indicated (Top). CT images revealed lesions in upper left lung. The tumor is indicated by red arrows. PFS, progression-free survival;SD, stable disease; PD, progressive disease; NGS, next-generation sequencing; mo., months. **(B)** Brain MRI scans revealed no metastasis at February 2023. **(C)** The EGFR exon21 c.T2573G p.L858R mutation was visualized by IGV software.

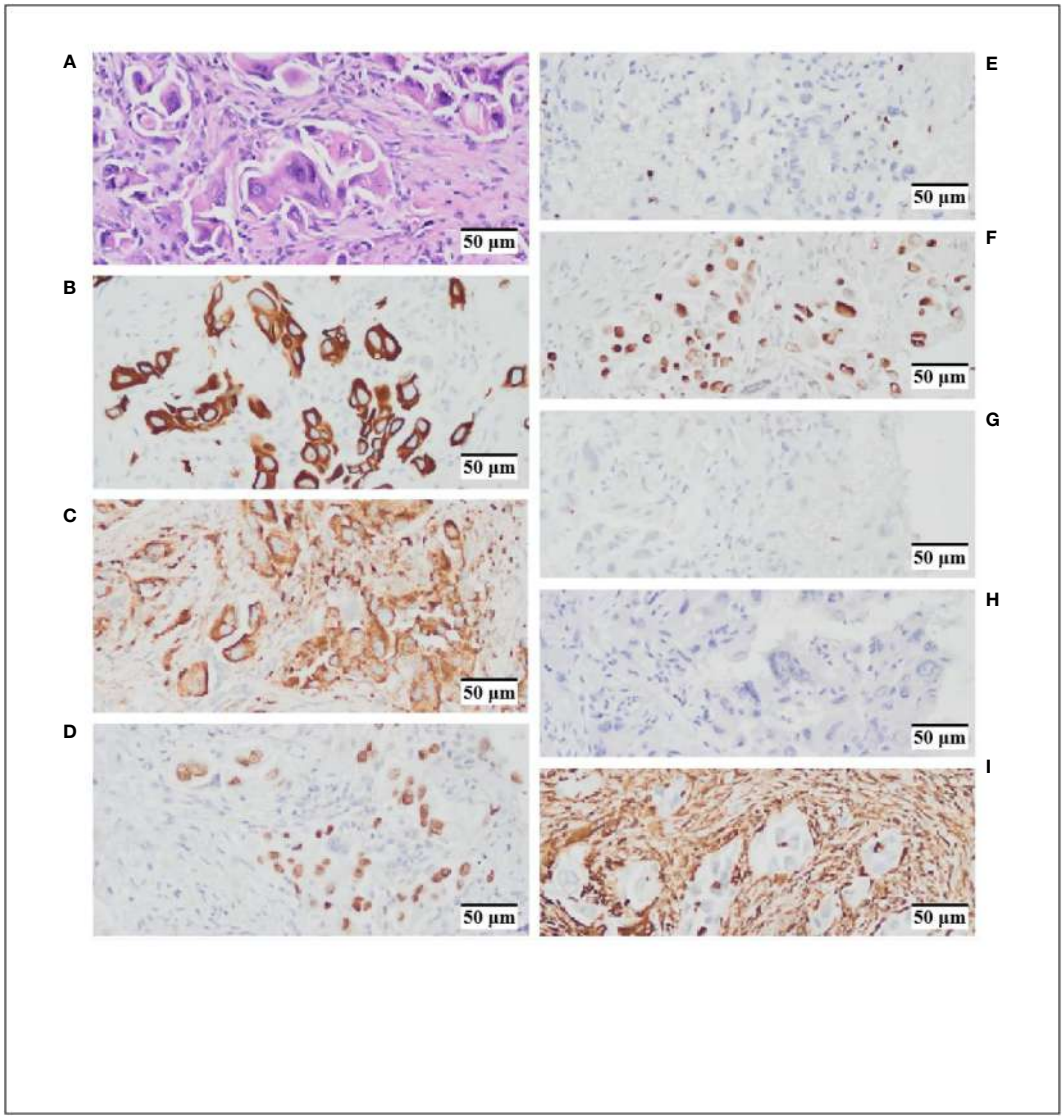

FIGURE 2
Hematoxylin and Eosin (HEx200) staining and immunohistochemistry (IHCx200) results. **(A)** HE staining indicates cytoplasmic acidophilia and nuclear basophilia, with evident intracellular cell proliferation features. **(B)** CK-7 (+). **(C)**. NaspinA (+). **(D)** TTF-1 (+). **(E)** Ki67 (+, 5%). **(F)** p53 (+, 10%). **(G)** CK5/6 (–) **(H)**. P40 (–) **(I)** VIM (–).

of these clinical and investigative findings, the diagnosis of stage IVB pulmonary giant cell carcinoma (cT2bN3M1b according to the TNM staging system) was confirmed. By the recommendations of the National Comprehensive Cancer Center, chemotherapy intervention was initiated. The patient underwent one cycle of chemotherapy, including pemetrexed and carboplatin, which resulted in the emergence of nausea and vomiting symptoms. To alleviate these adverse effects, intravenous administration of granisetron hydrochloride was employed, leading to symptomatic relief and subsequent discharge.

Considering the patient's strong aversion to chemotherapy-related adverse effects, a decision was made to explore more effective therapeutic options. Consequently, next-generation sequencing (NGS) was performed to assess genes related to lung cancer. The results revealed a clinically significant *EGFR* p.L858R-positive mutation (Figure 1C), accounting for an overall mutation rate of 5.68%. Following consideration of guidance from FNA, NMPA, NCCN, ASSO guidelines, and public databases, multiple treatment options were identified, including osimertinib, gefitinib, rociletinib, and aumolertinib. Considering the potent anti-resistance capabilities and lower side-effect profile of aumolertinib, the third-generation *EGFR*-TKI aumolertinib was chosen, with a daily dose of 110mg. Encouragingly, a follow-up chest CT after three months of aumolertinib treatment showed a significant reduction in the size of the tumor in the left upper lobe (4.4 x 1.9 cm) and the lymph node in the right cardiogenic angle (Figure 1A). Upon a follow-up examination in August, the tumor had decreased to 3.8x1.3cm, and the associated lymph nodes had also decreased in size (Figure 1A). In accordance with Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1), the patient's condition

was classified as stable disease (SD). Given the patient's favorable tolerability to aumolertinib and stable disease status, the decision was made to continue aumolertinib treatment. Presently, the patient remains on ongoing treatment with regular monitoring.

Discussion

Pulmonary giant cell carcinoma, as a type of lung sarcomatoid carcinoma, is primarily characterized by a combination of multinucleated giant cells and neutrophils within an inflammatory milieu. Under high magnification, cells exhibit abundant cytoplasm, eosinophilia, smooth nuclear membranes, and small basophilic nucleoli. Additionally, conspicuous intracytoplasmic inclusions are observed, characterized by increased intracellular cellularity (Figure 2A). Immunohistochemical analysis reveals positivity for CK7(+), NaspinA(+), and TTF-1(+), implies a potential tendency toward glandular epithelial differentiation within the tumor. The positive expression of Ki67(+, 5%) and p53(+, 10%) suggests a certain degree of malignancy. In this case, we have identified a patient harboring a positive *EGFR* p.L858R mutation, coupled with the tumor's propensity for glandular epithelial differentiation in the context of pulmonary giant cell carcinoma. This implies a potential benefit from *EGFR*-TKI treatment for the patient.

aumolertinib, a domestically developed third-generation TKI, gained domestic approval for marketing on March 18, 2020. Compared to the first-generation (gefitinib, erlotinib) and second-generation (afatinib) *EGFR*-TKIs, aumolertinib demonstrates enhanced stability and irreversible covalent binding with the ATP-binding domain of *EGFR*, efficiently inhibiting activating mutations (such as 19del and L858R) and resistance mutations (such as T790M), while displaying limited activity against wild-type (WT) *EGFR*. In contrast to osimertinib, aumolertinib introduces a cyclopropyl group to enhance stability (2, 3), allowing it to flexibly bind to the pocket of *EGFR*-T790M mutant protein, thereby increasing its affinity to T790M (2). Furthermore, it improves blood-brain barrier penetration in advanced NSCLC patients, suppressing brain and spinal cord metastases. In the Phase II clinical trial APOLLO, aumolertinib exhibits significant advantages owing to its unique anti-tumor properties. The primary endpoint, overall response rate (ORR), reaches 68.9%, and the secondary endpoint, median progression-free survival (PFS), extends to 12.4 months. Notably, patients with L858R mutations and exon 19 deletions achieve similar benefits in terms of PFS and overall survival (OS) (4). The design of aumolertinib, incorporating a cyclopropyl group, prevents robust inhibition of WT-*EGFR* metabolites' production, markedly reducing other adverse effects such as diarrhea and rash induced by wild-type *EGFR* inhibition. The most common treatment-related adverse events (TRAEs) ≥10% include creatine phosphokinase (CPK) elevation (20.9%), rash (13.9%), aspartate aminotransferase (AST) elevation (12.3%), white blood cell (WBC) count reduction (12.3%), alanine aminotransferase (ALT) elevation (11.9%), and pruritus (10.7%); 15 patients (6.1%) experience prolonged QT interval, and there are no reports of interstitial lung disease (ILD) (36 cases) (4). Recently, a case of interstitial lung disease induced by aumolertinib was reported (5).

It has been observed that patients with PGCC carrying *EGFR* mutations lack significant and durable clinical responses to *EGFR* inhibitors (6, 7), which may be attributed to tumor resistance. Weng et al. reported two cases of PGCC patients receiving *EGFR*-TKI treatment. The first case demonstrated a favorable response to the treatment with tumor shrinkage, indicating potential benefits from gefitinib in the future. Conversely, the second patient, after receiving icotinib treatment, achieved a PFS of only 4.3 months, experiencing treatment failure with subsequent brain metastasis. *EGFR* mutation was detected in the tumor specimen obtained from the second surgical resection, with the persistent presence of *EGFR* exon 21 L858R gene mutation. The reasons for treatment failure are postulated to include insufficient brain penetration of icotinib to suppress tumor cell growth. Alternatively, the emergence of new *EGFR* mutations leading to treatment failure cannot be excluded. Unfortunately, the patient was not followed up after the second surgery (8). The most common mechanism underlying acquired resistance to first- and second-generation *EGFR*-TKIs is the T790M mutation, occurring in 50%-60% of cases (9). The third-generation aumolertinib can covalently bind to the T790M residue, suppressing the emergence of resistance, and is capable of attaining substantial concentrations in the brain and spinal cord, showcasing therapeutic potential for patients with bone and brain metastases.

Although aumolertinib demonstrates remarkable therapeutic efficacy, the issue of drug resistance should not be overlooked. Six major resistance mechanisms have been identified, including T790M deletion, persistent T790M presence, *EGFR* mutations (C797S, G724S, L718Q), activation of bypass pathways, transformation into small cell lung cancer (SCLC), and enhanced autophagy (10, 11). Regarding aumolertinib resistance mechanisms, they can be broadly categorized into two types: *EGFR*-dependent and *EGFR*-independent mechanisms. However, due to limitations in clinical research on aumolertinib, our understanding of its resistance mechanisms remains incomplete. Nevertheless, drawing insights from studies on osimertinib resistance mechanisms, we postulate that potential *EGFR*-dependent resistance mechanisms for aumolertinib may include the T790M mutation and *EGFR* point mutations. However, robust evidence for these mechanisms is currently lacking in the literature. Meanwhile, *EGFR*-independent resistance mechanisms are prevalent across various TKIs. Reports indicate that aumolertinib can sustainably activate downstream signaling pathways of *EGFR* through alternative pathways, including mTOR, ERK1/2, and STAT3, thereby reducing sensitivity to *EGFR*-TKIs (12). Furthermore, research suggests that certain patients exhibit EML4-ALK fusion mutations after aumolertinib treatment, implying ALK gene rearrangement as another potential mechanism for aumolertinib resistance (13). In conclusion, despite aumolertinib's significant therapeutic potential, the diversity and complexity of its resistance mechanisms necessitate further in-depth research and understanding.

Combining aumolertinib with anti-angiogenesis therapy may enhance the effectiveness against pulmonary giant cell carcinoma.

The rationale behind the combination of aumolertinib and anti-angiogenesis therapy is rooted in the latter's ability to suppress tumor angiogenesis, thereby improving the delivery of *EGFR* TKIs through vascular normalization and enhancing their antitumor effects. As a *VEGFR*2 TKI, apatinib targets the intracellular domain of the receptor and disrupts signal transduction, consequently inhibiting tumor vascular growth. When used as a monotherapy, apatinib's efficacy in advanced pulmonary giant cell carcinoma is not distinct. Li reported a case of palliative apatinib treatment for advanced pulmonary giant cell carcinoma that failed to restrain tumor progression. The patient received a nightly dose of 500mg aumolertinib, and after one month, the tumor had enlarged compared to before, along with an increase in bilateral lung metastases (14). Reliable research indicates that the combination therapy of third-generation *EGFR* TKIs such as osimertinib with immune checkpoint inhibitors (ICIs) or anti-angiogenesis agents only marginally improves median overall survival (mOS) and carries unpredictable safety concerns (15). Presently, there are no reports on the combination of aumolertinib with anti-angiogenesis therapy. Nevertheless, given the highly vascularized histology of pulmonary giant cell carcinoma, the combination of aumolertinib and anti-angiogenesis therapy holds potential within treatment strategies, necessitating further clinical validation.

The combination of aumolertinib with chemotherapy holds the potential to enhance the antitumor effects against PGCC. In the treatment landscape of advanced PGCC, chemotherapy remains an indispensable therapeutic approach. Notably, Fumihiro et al. reported a case of advanced PGCC where prolonged chemotherapy led to a complete remission lasting 15 months (16). Research has shown that the overexpression of ABC transporters contributes to increased drug efflux, a common mechanism of multidrug resistance (17). aumolertinib selectively inhibits the transport function of ABCB1 (MDR1/p-glycoprotein), suppressing drug efflux and restoring the sensitivity of ABCB1-overexpressing cancer cells to drug-induced apoptosis. Additionally, at submicromolar concentrations, aumolertinib effectively reverses ABCB1-mediated human multidrug resistance and maintains drug-induced apoptosis in ABCB1-overexpressing multidrug-resistant cancer cells (18), providing a theoretical basis for combining conventional cytotoxic anticancer drugs with aumolertinib. In a clinical retrospective study, a cohort of 50 patients received single-agent aumolertinib as first-line treatment, while 15 patients underwent combination therapy (pemetrexed administered one week before aumolertinib). The combination therapy group exhibited significantly higher objective response rates (ORR) and disease control rates (DCR) of 93.3% and 100%, respectively, compared to the single-agent aumolertinib group with rates of 64% and 92%. Among the combination therapy recipients, 5 patients with *EGFR* mutations observed notable tumor reduction after 2-3 treatment cycles. Among these, 4 patients transitioned from clinical stage III/IV to postoperative pathological stage I, and 1 patient achieved a complete pathological response from clinical stage IIIB to postoperative pathological stage T0N0M0 (19). In our case, we adopted a strategy of administering pemetrexed before initiating aumolertinib treatment. In the absence of a definitive diagnosis of the lung tumor type, we employed a treatment regimen comprising paclitaxel and carboplatin. After confirming the *EGFR* mutation type two weeks later, the patient continued aumolertinib treatment, without the periodic addition of pemetrexed and aumolertinib. This suggests that periodic use of pemetrexed and aumolertinib could potentially yield greater benefits.

In summary, this is the inaugural report of a rare case of EGFR L858R mutation-positive stage IV pulmonary giant cell carcinoma that has exhibited marked benefits from exclusive utilization of Aumolertinib. As of the present moment, the patient has undergone continuous Aumolertinib treatment for six consecutive months, resulting in substantial tumor regression and an absence of any adverse reactions. These outcomes underscore the remarkable therapeutic potential of Aumolertinib in the treatment of pulmonary giant cell carcinoma. Given the intricate tumorigenic mechanisms, long-term monotherapy with Aumolertinib often precipitates issues of drug resistance. To address this concern, a limited analysis of Aumolertinib resistance mechanisms was undertaken. The scrutiny of relevant literature revealed that the combination of Aumolertinib with concurrent chemotherapy and anti-angiogenic therapies may offer substantial therapeutic advantages. Consequently, we emphasize the distinctive attribute of Aumolertinib as a standalone therapeutic modality for pulmonary giant cell carcinoma, along with its potential when integrated into combination therapies, as pivotal avenues for extending the survival of patients with EGFR-positive pulmonary giant cell carcinoma.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving humans were approved by Affiliated Hospital of Southwest Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The human samples used in this study were acquired from Inpatient routine pathological biopsy and immunohistochemistry. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

WY: Data curation, Resources, Writing – original draft, Writing – review & editing. ZY: Data curation, Resources, Software, Writing – original draft, Writing – review & editing. KW: Resources,

Writing – review & editing. PZ: Writing – review & editing. JP: Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Sequential PET/CT and pathological biomarker crosstalk predict response to PD-1 blockers alone or combined with sunitinib in propensity score-matched cohorts of cancer of unknown primary treatment

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Introduction: The efficacy of immune checkpoint inhibitors (ICIs), including toripalimab and pembrolizumab, has not been confirmed in the treatment of cancer of unknown primary (CUP), which has a very poor prognosis. Combined with anti-angiogenic therapies, ICIs are hypothesized to be effective in prolonging overall survival. The study aims to give evidence on the treatment effects of sunitinib combined with ICIs, find pathological biomarkers associated with changes in volumetric ^{18}F FDG PET/CT parameters, and investigate inner associations among these markers associated with response on PET/CT.

Methods: The study recruited patients receiving combined treatment (ICIs + sunitinib), compared the effects of combined treatment with those of separate treatment and age-matched negative controls, and analyzed propensity score-matched (PSM) pairs. Markers associated with survival were identified, and their inner associations were tested using structural equation modeling.

Results: A total of 292 patients were enrolled in the final analysis, with 53 patients receiving combined treatment. Survival analysis demonstrated significantly prolonged survival in either combined or separate treatment, with the combined arm showing better response when PSM-paired using pre-treatment whole-body PET/CT parameters. The angiogenic markers KDR and VEGF mediate the PD-1 blockade impact on volumetric value changes in positive and negative manners.

Conclusion: The anti-angiogenic agent sunitinib may potentiate PD-1 blockade by diminishing angiogenesis or its downstream effects. The combined separate treatment increased the survival of CUP patients, and the responses could be evaluated using volumetric PET/CT parameters.

KEYWORDS

cancer of unknow primary, PET/CT (18)F-FDG, sunitinb, EFGR, VEGFR

Introduction

Cancer of unknown primary (CUP) is defined as a heterogeneous group of malignancies with the primary site unable to be diagnosed using any current means (1). It has been recognized as an independent disease entity because of its distinct biological behavior, bio-aggressiveness, and pathological signatures (2). Its incidence is not uncommon, accounting for 2% to 5% of yearly incident cancers (3). Although our previous research found encouraging results of sunitinib therapy in CUP management, treatment strategies are still to be determined due to fluctuating therapeutic responses and difficulty of response evaluation (4).

Fortunately, over the last decades, immunotherapies have proved effective in prolonging survival in many solid or hematologic malignancies, shedding new light on the treatment of cancers that traditionally respond poorly to cytotoxic chemotherapy or targeted therapies (5, 6). Among them, immune checkpoint inhibitors (ICIs) by targeting PD-1 or PD-L1 modulate T-cell function and enhance cytotoxicity against tumor cells or deranged immune micro-environment (7). Although CUP has not been shown to respond to immunotherapies in piloting studies, combined drugs of anti-angiogenic agents and immune checkpoint inhibitors were demonstrated to have better effects in a landscape of multi-drug resistant solid tumors, but the regimens have not been studied in CUP patients (8, 9). We, therefore, aimed to test the efficacy of both therapies in CUP patients in either a combined or separate manner.

The second problem in diagnosing or treatment of CUP is biomarker profiling. Previous studies have attempted to find immune and pathological signatures in CUP patients, but they have not provided conclusive evidence on treatment response (8). Indeed, due to the complexity of the host immune system and its interplay with the occult primary lesion, biomarkers cannot be as easy to identify as known primary cancers. Nevertheless, aberrant angiogenesis was one of the main reasons for immune suppression of the T-cell subgroup, and therefore, this work seeks to identify penitential biomarkers associated with treatment response (5).

Different from known primary, the metastatic lesions are usually multiple, and the primary is occult, calling for a novel approach to evaluate treatment response (6). In the prior study of sunitinib therapy of CUP, the efficacy of volumetric bio-signatures of sequential PET/CT scans in drug response prediction has been shown to be independently associated with survival (10). This non-invasive method of evaluating tumor glycolysis combines the tumor volume and metabolic rate and thus has been shown to be superior to traditional measures (10, 11). In this study, to avoid unbalanced potential selection bias, propensity score-matched analyses were applied in comparison to treatment arms (12). Then, potential pathological biomarkers indicating response were analyzed. Finally, the interplay of the biomarkers was investigated using structure equation modeling to identify the indirect effects of biomarkers.

Methods

Patients

The open-labeled study recruited patients diagnosed with cancer of unknown primary who received treatment of sunitinib and immune checkpoint inhibitors at Sun Yat-sen University Cancer Center, Guangzhou, Changzheng Hospital, and had panoramic medical imaging (Panmedic) at the Second Affiliated Hospital of Shantou University, from June 2015 to May 2021. Randomization was based on demographic data and baseline whole-body PET/CT values into combination treatment or separate treatment. Patients not receiving either therapy were included in the study as negative controls. Because the evaluation of the primary site was unavailable using Response Evaluation Criteria in Solid Tumors, the primary goal was to estimate the efficacy of either combined or separate treatment, which was demonstrated using survival prognosis and changes in whole-body PET/CT metabolic signature, and the association between value changes on PET/CT and survival was analyzed. The inclusion criteria, PET/CT imaging, and immunohistochemistry method have been illustrated elsewhere (10, 13) (Supplementary Materials). The study was approved by the institutional review boards and was in accordance with the provisions of the Declaration of Helsinki. All patients provided written and/or oral consent to participation before the study commenced.

The dosage of sunitinib was 50 mg/day given in 6-week cycles, including 4 weeks on treatment followed by 2 weeks off treatment (Schedule 4/2), and dosage was reduced to 37.5 mg/day and subsequently to 25 mg/day on occasions of over grade 3 toxicity. Patients received toripalimab 3 mg/kg once every 2 weeks by intravenous infusion, and the dose was reduced to 2.5 mg/kg in occasions of unbearable toxicity. For the purpose of the study, the

intention-to-treat manner was adopted in the subsequent analysis. The dosage of pembrolizumab was 200 mg every 3 weeks, and the dose was reduced to 130 to 180 mg in occasions of unbearable toxicity, which was defined as any toxicity of greater than grade III or any patient-reported toxicity to stop ICI treatment.

Propensity score-matched analysis

The demographic variables were acquired from the medical records, and the overall condition of the patients was assessed using the Eastern Cooperative Oncology Group Performance Score (ECOG-PS). As there may have been a potential difference in variables not included in the study, patients in each treatment arm were matched by propensity score to reach a 1:1 paired comparison in order to minimize selection bias and confounding variables. Propensity score-matched analysis was carried out by means of a multivariate conditional logistic regression model with a caliper width of 0.05 (14). Factors included in the regression model included demographics, chemotherapy involved, and baseline metabolic activity on whole-body FDG PET/CT scans associated with tumor aggressiveness, including high standard uptake value (HSUV), whole-body metabolic tumor volume (WMTV), and whole-body total lesion glycolysis (WTLG).

Statistics and data assessment

First, the unmatched survival curve of combined or separate treatment was calculated and plotted using the Kaplan–Meier method. Log-rank test was used to test the difference. Second, the propensity score was calculated in each treatment arm to achieve a matched analysis for all treatment arms. Paired Student's t-test was applied to test differences in continuous variables, and the chi-square test was used to test categorical differences. In each treatment arm, univariate and multivariate survival analyses were applied to find independent risk variables associated with survival by means of Cox proportional hazards models. Finally, structural equation modeling (SEM) was performed to examine the direct or indirect effects of immunohistochemistry (IHC) markers on the value fluctuation of PET/CT metabolic biomarkers and survival. Only markers significant in the survival analysis would enter the model to test their significance and regression weights. Pearson's correlation was considered to adjust regression weights if there was more than one variable at the beginning of SEM. The survival analysis was performed on SPSS (Chicago, IL, USA; version 24.0), and SEM was performed on Amos (Chicago, IL, USA; version 24.0).

Results

Baseline characteristics

A total of 299 patients were included in the study at baseline, of whom four patients failed to undergo a second PET/CT scan after treatment discontinuation, and three patients refused to provide information on PET/CT scans. Therefore, a total of 292 patients were enrolled finally (135 men and 157 women), including 43 patients receiving ICIs of toripalimab or pembrolizumab only, 57 patients receiving sunitinib therapy only, 53 patients receiving combined therapy, and 139 patients receiving neither (age-matched negative control). The mean and standard deviation (SD) values of baseline HSUV, WMTV, and WTLG were 18.34 ± 4.57, 56.97 ± 23.70, and 301.03 ± 77.55, respectively. A total of 108 patients were rated using ECOG-PS as 3 and 4, and 184 patients were rated as 1 and 2. The baseline information of all patients and each treatment arm is shown in Supplementary Table 1. The Kaplan–Meier curve demonstration of unmatched survival information is shown in Supplementary Figure 1.

Propensity score-matched comparison

A propensity score-matched comparison was carried out in five paired groups to balance the baseline characteristics shown in Table 1. A total of 43 pairs were matched in combined therapy versus sunitinib therapy (mean score = 0.49 ± 0.12), and baseline characteristics comparison is shown in Table 1. The mean estimated survival time of the combined group was 23.07 months, with 95 confidence intervals (CIs) of 21.02–25.12, which was significantly longer than that of patients receiving sunitinib alone (Figure 1A). At the end of the follow-up PET/CT scan, both therapy arms demonstrated significant improvement in WTLG, WMTV, and HSUV compared with baseline parameters ($p < 0.01$ for all parameters, see Table 1). Changes (Δ) in WMTV and WTLG were significantly different between the combined therapy group and the sunitinib group (Figures 1B, C), but there was no significant difference in ΔHSUV between the two arms (Figure 1D).

A total of 38 pairs were matched in combined therapy versus ICI therapy (mean propensity score = 0.53 ± 0.12), and baseline characteristics comparison is shown in Supplementary Table 3. The mean estimated survival time of the combined group was significantly longer than that of patients receiving ICI alone (Figure 1E). At the end of the follow-up PET/CT scan, both therapy arms demonstrated significant improvement in WTLG, WMTV, and HSUV compared with baseline parameters ($p < 0.01$ for all parameters, Supplementary Table 2). ΔWTLG was significantly different between the combined therapy group and ICI group (Figure 1F), but there was no significant difference in ΔWMTV or ΔHSUV between the two arms (Figures 1G, H).

A total of 51, 54, and 42 pairs were matched in combined therapy, sunitinib therapy, and ICI therapy versus negative control. The mean propensity score of each match was 0.33 ± 0.12, 0.35 ± 0.14, and 0.33 ± 0.10, respectively. Baseline characteristics comparison is shown in Supplementary Tables 2–4. The mean estimated survival time of each treatment arm was significantly longer than that of the control group (Figures 1I–K).

Identification of prognostic biomarkers

Survival analysis using univariate and subsequent multivariate methods was carried out in each treatment arm to identify markers

TABLE 1 Propensity score-matched comparison results of combined therapy and separate therapies.

	ICI + sunitinib group *vs.* sunitinib group (N = 43 pairs)			ICI + sunitinib group *vs.* ICI group (N = 38 pairs)		
Factor	ICI + sunitinib	Sunitinib	p	ICI + sunitinib	ICI	p
Baseline variables						
Sex, male/female	16/27	19/24	0.51	17/21	18/20	0.82
Age, mean (SD)	56.93 (14.14)	57.77 (12.23)	0.77	56.89 (13.77)	56.84 (15.96)	0.99
Pathology type, SCC/adenoCA/UD	13/17/13	10/18/15	0.76	13/15/10	12/13/13	0.75
Chemotherapy, paclitaxel/pt/combined	18/12/13	14/17/12	0.50	11/10/17	11/15/12	0.39
ECOG-PS, 4/3/2/1	3/6/15/19	2/7/17/17	0.92	4/7/17/10	7/10/13/8	0.55
WTLG, mean (SD)	293.90 (72.34)	302.95 (71.91)	0.56	305.09 (74.05)	312.63 (70.86)	0.65
WMTV, mean (SD)	56.40 (23.94)	55.16 (22.05)	0.81	62.00 (25.35)	62.37 (24.84)	0.95
HSUV, mean (SD)	17.28 (4.67)	17.60 (4.55)	0.74	18.32 (4.61)	18.84 (4.65)	0.62
Follow-up PET/CT parameters						
WTLG, mean (SD)	177.34 (77.41)	230.02 (74.86)	<0.01	185.09 (77.87)	238.32 (68.77)	<0.01
ΔWTLG	116.56 (34.45)	72.93 (17.12)	<0.01	120.00 (34.53)	74.32 (15.25)	<0.01
WMTV, mean (SD)	45.07 (22.47)	49.86 (21.97)	0.32	50.05 (24.29)	45.58 (20.56)	0.39
ΔWMTV	11.33 (7.49)	5.30 (10.66)	<0.01	11.95 (6.53)	16.79 (15.96)	0.08
HSUV, mean (SD)	14.44 (3.86)	13.74 (4.01)	0.41	15.13 (3.64)	16.87 (4.03)	0.05
ΔHSUV	2.84 (4.02)	3.86 (4.98)	0.30	3.18 (3.98)	1.97 (4.58)	0.21

SD, standard deviation; ICI, immune checkpoint inhibitor; ECOG-PS, Eastern Cooperative Oncology Group Performance Score; Δ, improvement; WTLG, whole-body total lesion glycolysis; WMTV, whole-body metabolic tumor volume; HSUV, highest standardized uptake value.

associated with survival. In the combined treatment, ΔWTLG (hazard ratio (HR) = 0.96, 95%CI = 0.92–0.99) was the only marker in PET/CT independently associated with longer survival (Supplementary Table 5). In all IHC markers, PD-L1 (HR = 0.18, 95%CI = 0.05–0.64, Supplementary Figure 2A) and KDR (HR = 0.37, 95%CI = 0.10–1.36, Supplementary Figure 2C) were independently associated with significantly longer survival time. VEGF was found to be significantly associated with decreased survival prognosis (HR = 0.93, 95%CI = 0.29–3.02, Supplementary Figure 2B). Two IHC factors, however, were found to be associated with longer survival but lost significance in multivariate analysis (Supplementary Figure 2D, E), in which higher microvascular density was found negatively associated with survival and PDGFR was positively associated with survival.

In the sunitinib treatment arm, ΔWTLG was the only PET/CT biomarker associated with longer survival (HR = 0.98, 95%CI = 0.96–0.99). In all IHC markers, KDR was independently associated with significantly longer survival time (HR = 0.27, 95%CI = 0.12–0.59, Supplementary Figure 2F), and VEGF was independently associated with decreased survival time (HR = 3.63, 95%CI = 1.78–7.42, Supplementary Figure 2G, Supplementary Table 6).

In the ICI treatment arm, ΔWTLG was the only PET/CT biomarker associated with longer survival (HR = 0.96, 95%CI = 0.92–1.00). In all IHC markers, only PD-L1 was associated with longer survival (HR = 0.23, 95%CI = 0.07–0.78, Supplementary Figure 2H, Supplementary Table 7).

PET/CT and pathological biomarkers correlate in structure equation modeling

Pathway analysis using structural equation modeling was carried out to unearth the inner association within the sensitive/resistant biomarkers and their direct or indirect impact on value changes of PET/CT volume-based biomarkers.

In the combined treatment arm (sunitinib combined with toripalimab or pembrolizumab), there was a direct impact of PD-1 blockade on ΔWTLG affecting survival, in which the regression coefficient for PD-L1 expression (β) was 0.32 on the impact of ΔWTLG ($p < 0.01$). Since there were four biomarkers of sunitinib therapy significant in the univariate survival analysis, indirect mediating effects were tested for these biomarkers in the pathway between PD-L1 and ΔWTLG. The final result is shown in Figure 2A. The impact of PD-1 blockade on ΔWTLG was positively mediated by KDR expression (β = 0.53 and β = 0.29, $p < 0.01$ for both) and by VEGF expression (β = –0.31 and β = –0.27, $p < 0.05$ and $p < 0.01$, respectively). There were two variables not significant in the pathway: PDGFR expression and microvascular density (MVD). The direct impact of ΔWTLG on survival was significant (β = 0.92, $p < 0.01$). Levels of ΔWTLG in each IHC expression subgroup are shown in Figure 2B.

In the sunitinib treatment arm, after adjustment by correlation analysis of KDR and VEGF expression (Pearson's r = 0.38, $p < 0.01$), there was a direct impact of KDR expression on ΔWTLG (β = 0.27, $p < 0.05$), and there was also the direct impact of VEGF on ΔWTLG

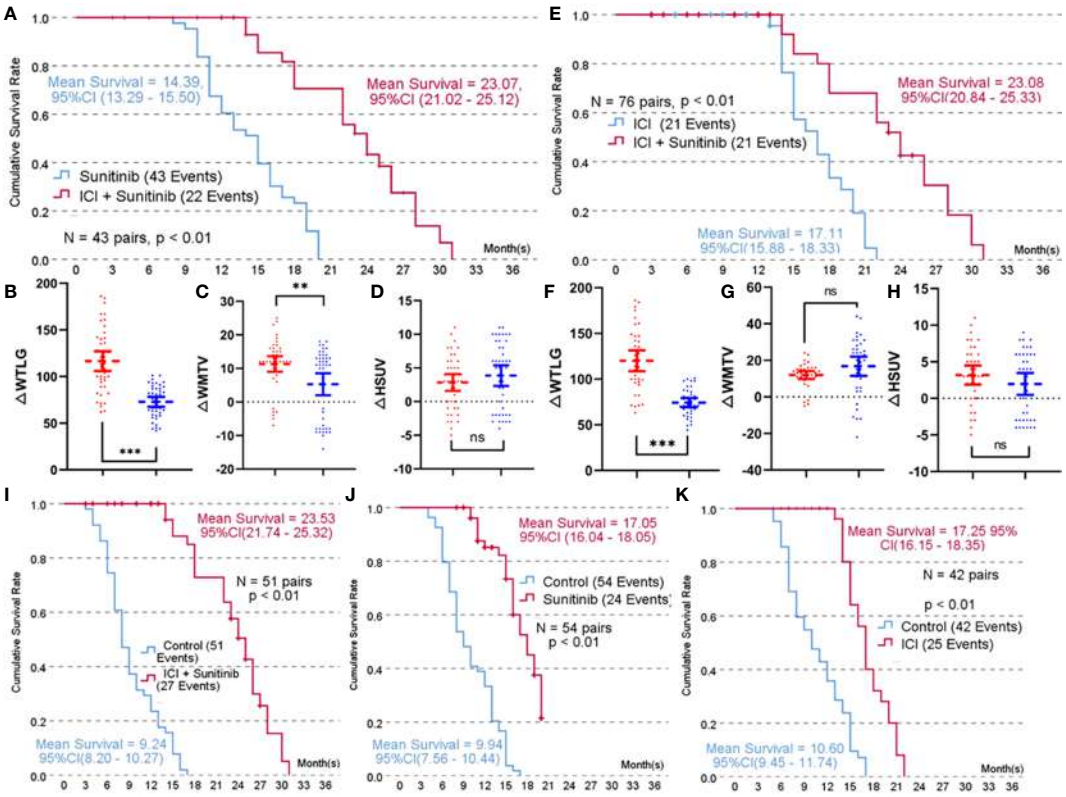

FIGURE 1
Propensity score-matched 1:1 comparison of each treatment arm. **(A)**, Kaplan-Meier survival curve of combined treatment (toripalimab or pembrolizumab + sunitinib) versus sunitinib treatment; **(B)**, Comparison result of improvement in whole-body total lesion glycolysis (△WTLG) in combined treatment versus sunitinib treatment; **(C)**, Comparison result of improvement in whole-body metabolic tumor volume (△WMTV) in combined treatment versus sunitinib treatment; **(D)**, Comparison result of improvement in highest standard uptake value (△HSUV) in combined treatment and sunitinib treatment; **(E)**, Kaplan-Meier survival curve of combined treatment versus immune checkpoint inhibitors (toripalimabor pembrolizumab) treatment; **(F)**, Comparison result of improvement in whole-body total lesion glycolysis (△WTLG) in combined treatment versus immune checkpoint inhibitors; **(G)**, Comparison result of improvement in whole-body metabolic tumor volume (△WMTV) in combined treatment versus immune checkpoint inhibitors; **(H)**, Comparison result of improvement in highest standard uptake value (△HSUV) in combined treatment versus immune checkpoint inhibitors; **(I–K)**, Comparison result of the mean estimated survival time among combined therapy, sunitinib therapy, and ICI therapy.

($\beta = -0.33$, $p < 0.01$). The direct impact of ΔWTLG on survival was significant ($\beta = 0.64$, $p < 0.01$, Figure 2C). Levels of ΔWTLG in each IHC expression subgroup are shown in Figure 2D.

Discussion

This work evaluated the efficacy of PD-1 inhibitors, including toripalimab and pembrolizumab, and sunitinib regimens in the treatment of CUP, analyzed the response-predicting role of sequential volume-based PET/CT scans, and investigated the inner associations of resistant or sensitive biomarkers. First, propensity score-matched cohorts demonstrated the survival prognosis of each treatment arm; second, multivariate analysis showed ΔWTLG to be the independent predictor of drug response and identified pathological markers of each treatment arm; finally, structure equation modeling analyzed the way anti-angiogenesis therapy assisted immune checkpoint blockade to achieve decreased tumor glycolysis in whole-body PET/CT scans. This study was the first to suggest the response of either combined or independent therapeutic efficacy of CUP.

Patients presenting with CUP may have their primary lesions concealed at the beginning or some point of the preclinical disease course for unknown reasons, and the occult primary site presents as an obstacle for precise diagnosis and subsequent management (15). Regardless of the pathogenesis of CUP or grouping methods into genetic subtypes, angiogenesis was aberrant and accelerated in many solid tumors, including CUP, in terms of the basic mechanism behind treatment regimens (16). Unleashed angiogenesis is one of the reasons for nourishing metastatic or primary tumors, and targeting angiogenesis is one of the main strategies in solid tumor treatment (16). Sunitinib, a multi-target receptor tyrosine kinase inhibitor, proved effective in metastatic renal cell carcinomas or gastric stromal cancers (13). The drug proved effective in CUP treatment in our previous work and was reevaluated in the present study, both of which identified VEGFR as the sensitive treatment biomarker and thus supported the anti-angiogenic effect of sunitinib (13, 17). A few other studies also illustrated the beneficial role of sunitinib in tumor immune surveillance combined with PD-L1 inhibitors (18–20).

Notably, since the treatment response of CUP can be difficult to evaluate with traditional measures as known primary tumors in Response Evaluation Criteria in Solid Tumors (RECIST) criteria,

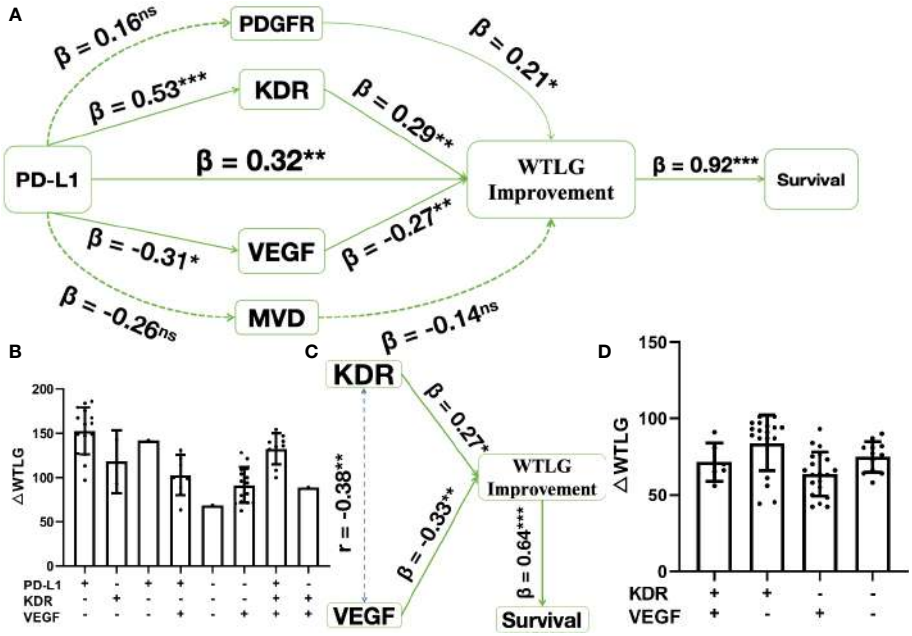

FIGURE 2
Structure equation modeling of biomarkers significant in survival analysis. * p < 0.05, **p < 0.01, ***p < 0.001, ns, insignificant. **(A)**, in combined treatment arm, pathway analysis shows PD-1 blockade has a direct impact on WTLG improvement (△WTLG) affecting survival. This impact is mediated by sunitinib treatment sensitivity, where KDR expression (β = 0.53 and 0.29) positively affects the impact and VEGF expression (β = -0.31 and -0.27) negatively affects the impacts; **(B)**, △WTLG in each subgroups of biomarker expression in combined treatment arm; **(C)**, in sunitinib treatment arm, both KDR and VEGF expression have direct impact on △WTLG affecting survival and there is weak correlation (Pearson r = 0.38) between the two biomarkers; **(D)**, △WTLG in each subgroups of biomarker expression in sunitinib treatment arm.

whole-body scans using PET/CT would be reasonably more actionable in clinical settings in evaluating prognosis or treatment response in CUP management (21). We evaluated the prognostic value of volumetric markers in the control group and found that the WTLG was the only marker associated with survival. Sequential PET/CT corroborated this result by demonstrating ΔWTLG as the only response predictor in the combined or independent therapies. Glycolysis bears more tumor information, as it is the product of tumor SUV and metabolic tumor volumes, and previous research using sequential PET/CT as prognostic markers has demonstrated the response-predicting role of glycolysis (10, 22). Some reports have given solid recommendations that WTLG should be applied in clinical settings as a standard measure of drug response (10, 23, 24). Our previous PANMEDIC report on CUP treatment demonstrated that whole-body glycolysis had more sensitivity and specificity in predicting survival in sunitinib treatment.

As traditional target therapies need appropriate biomarkers or sensitive genes to take clinical effect in certain malignancies, CUPs, being a heterogeneous group of cancers, may be immune to such therapies because concealed primary lesions may have blunted targets due to complex interplay of differential genes, and this also makes vigorous gene testing inapplicable to widespread relevance (25). In the last decade, however, immunotherapies, as represented by ICIs, bypass the genetic targeting in many solid cancers altogether (3, 26). The ICIs aim to rejuvenate exhausted host cytotoxic T cells to exert a potent effect on cancer cells, enabling efficient control of a landscape of solid or hematological malignancies. The effective treatment of CUP in the present study by pembrolizumab or toripalimab alone demonstrates that the immune checkpoint blockade may be effective in reducing progression, thus prolonging patient survival (27). Future randomized controlled trials are encouraged to give more conclusive evidence on CUP treatment.

Despite the fact that ICIs have significantly revolutionized cancer therapies, up to 60% of patients failed to have an adequate response by literature (28). Biomarkers associated with ICI response are difficult to identify, probably because the host immune system is too complex to be represented by independent biomarkers (28). Nevertheless, among the many resistant biomarkers, angiogenesis markers were also found to have crosstalk with T-cell immune function and survival, which has been reported to affect ICI therapy response in previous studies (29).

VEGF, being the "king" of angiogenesis, was found in the study to hinder anti-PD-1 therapeutic effects in the combined therapy group, where the structural equation modeling demonstrated that the VEGF expression levels had a negative impact on the PD-1 blockade response. KDR (VEGFR-2) expression level was found to positively mediate the effect of PD-1 blockade. However, both markers were not significantly associated with treatment response in separate treatment groups, suggesting that sunitinib therapy may diminish the effect of angiogenesis, thus potentiating immune blockade in combination treatment. Also, the indirect pathway by KDR bears more regression coefficients than the direct pathway (β = 0.53 versus β = 0.32), suggesting that the combined treatment needs higher KDR expression than PD-L1 expression to have an impact on WTLG improvement. Higher levels of KDR expression permitted higher anti-angiogenic effects, and thus, PD-1 blockade worked better, and subsequent WTLG improvement was higher. Indeed, the response-

predicting results of both biomarkers of angiogenesis have been validated using the multivariate survival analysis and thus support previous data on the combined treatment of solid tumors that anti-angiogenesis may have a synergistic or permissive effect on PD-1 inhibition (30).

Interestingly, MVD was found to be insignificant in the mediating effects of PD-1 blockade, although, in preclinical settings, endothelial cells mediate decreased cytotoxic T lymphocyte (CTL) infiltration or increased T-cell apoptosis. Studies suggested that microvascular disorganization may not be the main reason for deranged CTL infiltration, and VEGF-associated downstream factors may play more important roles (30). However, the fact that VEGF instead of MVD mediates PD-1 blockade resistance in this study may need further investigation to clarify the mechanism.

This work bears limitations. Although matched comparison by propensity score was performed to determine the survival difference, the sample size is relatively small in each treatment arm, which calls for larger-scale research to be carried out in the future. Also, the research on biomarkers has not been extensive enough to involve genetic signatures, and therefore, future research can evolve into sequencing analysis on the basis of immunohistochemical markers, which could further unravel the inner workings of biomarkers behind combined therapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by The second affiliated hospital of shantou university. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

YW, QH,GZ, JL,QG, YM, XW, JZ contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1191611/full#supplementary-material

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Characterization of prevalent tyrosine kinase inhibitors and their challenges in glioblastoma treatment

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Glioblastoma multiforme (GBM) is a highly aggressive malignant primary tumor in the central nervous system. Despite extensive efforts in radiotherapy, chemotherapy, and neurosurgery, there remains an inadequate level of improvement in treatment outcomes. The development of large-scale genomic and proteomic analysis suggests that GBMs are characterized by transcriptional heterogeneity, which is responsible for therapy resistance. Hence, knowledge about the genetic and epigenetic heterogeneity of GBM is crucial for developing effective treatments for this aggressive form of brain cancer. Tyrosine kinases (TKs) can act as signal transducers, regulate important cellular processes like differentiation, proliferation, apoptosis and metabolism. Therefore, TK inhibitors (TKIs) have been developed to specifically target these kinases. TKIs are categorized into allosteric and non-allosteric inhibitors. Irreversible inhibitors form covalent bonds, which can lead to longer-lasting effects. However, this can also increase the risk of off-target effects and toxicity. The development of TKIs as therapeutics through computer-aided drug design (CADD) and bioinformatic techniques enhance the potential to improve patients' survival rates. Therefore, the continued exploration of TKIs as drug targets is expected to lead to even more effective and specific therapeutics in the future.

KEYWORDS

glioblastoma, tyrosine kinase inhibitors (TKIs), genetic heterogeneity, epigenetic heterogeneity, TKIs resistance, blood-brain barrier, computer-aided drug design (CADD)

1 Introduction

GBM, defined by histopathologic necrosis and endothelial proliferation features, is an aggressive primary brain tumor with a median survival of fewer than 15 months despite surgical resection, radiation, and chemotherapy, in adults (Thang et al., 2023; Wälchli et al., 2023). There are no known risk factors for GBM, and it occurs without warning signs. Furthermore, its incidence increases with age, with white ethnicity being more commonly affected than black ethnicity, and males being more affected than females (Newton et al., 2018; Grochans et al., 2022; Dain and Zhu, 2023). Considering the invasive nature of GBM and resistance to therapies, recurrence is observed after treatments (Sareen et al., 2022). Large-scale genomics and proteomics analysis demonstrated the proteins and pathways

associated with the resistance mechanisms responsible for the recurrence of GBM (Shergalis et al., 2018). One promising avenue for cancer treatment involves the use of tyrosine kinase inhibitors (TKIs) (Bagheri et al., 2022; Li et al., 2023; Zhang et al., 2023).

Genome-wide studies have revealed that cancer initiation, promotion, progression, as well as recurrence are casually associated with kinase mutations (Bhullar et al., 2018). Kinases are enzymes that catalyze the transfer of a γ-phosphate group from ATP to the hydroxyl group of tyrosine, serine, or threonine residues. Around 538 kinases are encoded in the human genome and can activate protein functions to maintain cellular function (Nayak et al., 2022). According to the Cancer Gene Census (CGC), protein kinases are the most prevalent protein family encoded by cancer genes, with 27 out of 291 cancer genes encoding protein kinases (Futreal et al., 2004). The complete set of protein kinases (kinome) has emerged as an appealing target for therapeutic strategies for human malignancies (Fleuren et al., 2016). Several studies reported that the tumor progression and therapy resistance are subsequently related to overexpression and mutation of TKs that activate many critical downstream pathways in GBM (Bolcaen et al., 2021; Peller et al., 2023). In GBM, certain well-characterized mutated TKs are the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor-α (PDGFR-α) (Fleuren et al., 2016; Brar et al., 2022).

Following G-protein-coupled receptors, kinases are the second most targeted proteins for various types of cancer treatment (Jackson et al., 2019). Tyrosine kinase inhibitors (TKIs) are small molecules that selectively inhibit the activity of specific TKs, which are enzymes that play a central role in cell signaling pathways. In GBM, the most common TKs targeted by TKIs are EGFR and VEGFR (Li et al., 2023; Long et al., 2023; Smolenschi et al., 2023). Different TKIs were targeted in the cancer phases I, II, II, III, and IV in clinical trials for 20 years (Huang et al., 2020). Studies are currently underway to identify and validate drug targets; however, many of these targets have failed to demonstrate efficacy in clinical trials, mainly due to several challenges. These challenges include issues such as limited permeability through the blood-brain barrier (BBB), the inherent heterogeneity of GBM, immunosuppressive tumor microenvironment, and the development of resistance to TKIs (Aldaz and Arozarena, 2021; Majd et al., 2021).

GBM is a highly heterogeneous cancer, and different subtypes may have different signaling pathways and molecular profiles. This makes it difficult to identify the most appropriate TKIs for each patient (Olar and Aldape, 2014). Many TKIs have poor BBB penetration, making it difficult to reach therapeutic concentrations in the brain (Bhowmik et al., 2015). GBM cells can develop resistance to TKIs through various mechanisms, including mutations in the targeted TK or activation of alternative signaling pathways (Tilak et al., 2021).

The present work discusses the types of gliomas and the molecular mechanism of TKIs, the physicochemical properties of TKIs required to pass through the BBB, and the characterization of TKI-targeted drugs that have been reported in GBM clinical trials. Finally, the potential of the new generation of TKIs as promising therapeutics will be discussed, including their effectiveness and potential for minimizing off-target effects and toxicity.

2 Gliomas subclassification

Gliomas are classified into four grades according to their aggressiveness and malignancy by WHO (Ratti et al., 2022). The tumors with low proliferative potential are classified into grade I while Grade II gliomas are characterized by infiltrative capacity and low proliferative activity. These tumors tend to progress to grade III, which is known as anaplastic glioma andshows histological evidence of malignancy. Finally, glioblastomas with signs of necrosis and microvascular proliferation are classified in grade IV as the deadly glioma with a median survival of 12–15 months after diagnosis (Molinaro et al., 2019; Delgado-M et al., 2020).

Verhaak et al. (2010) classified GBMs based on multi-dimensional genomic data into four subtypes of abnormalities in PDGFR-α, EGFR, isocitrate dehydrogenase 1 (IDH1), and neurofibromatosis type 1 (NF1) (Verhaak et al., 2010). These subtypes contain proneural, neural, classical, and mesenchymal classes. The enrichment in the oligodendrocytic shows proneural. The association with oligodendrocytic and astrocytic display neural. The murine astrocytic signature is associated with the classical group. The mesenchymal phenotype, Schwann cell markers, and microglial markers exhibit mesenchymal (Verhaak et al., 2010; Jackson et al., 2019). However, the classification of GBMs remains controversial owing to the heterogeneity of tumors.

Traditionally, glioblastoma classification had been based on histological features, though this approach frequently lacked precision. In 2016, the WHO revised glioma classification utilizing molecular parameters to define tumor identities. The most frequent and invasive type of glioma is glioblastoma which is divided to three groups based on the status of the IDH gene. The primary or *de novo* group of glioblastoma contains wild-type IDH, represents 90% of glioblastoma, and is predominantly observed in patients over 55 years old. The progressed from an anaplastic astrocytoma group has mutated IDH and represents 10% of glioblastoma. This group is observed in young patients and its prognosis is easier. The third group is not otherwise specified (NOS) glioblastoma and their status could not be evaluated (Cruz Da Silva et al., 2021). In 2021, the WHO updated glioblastoma classification and introduced new tumor types and subtypes. For the first time, the classification distinctly separates adult- and pediatric-type gliomas, taking into account differences in molecular pathogenesis and prognosis. The 2021 fifth edition of the WHO Classification of Central Nervous System Tumors (WHO CNS5), the significance of laboratory assessments for relevant biomarkers has been heightened for prognostic purposes (Berger et al., 2022). In adults, the classification of diffuse gliomas is streamlined into three types:

1-Astrocytoma, IDH-mutant
2-Oligodendroglioma, IDH-mutant, and 1p/19q-codeleted
3-Glioblastoma, IDH-wildtype (Berger et al., 2022)

In the new update, glioblastomas will now exclusively encompass IDH-wildtype tumors. Mutations in the histone variant 3 (H3) are frequently observed in IDH-wildtype diffuse glioma, especially in pediatric and young adult groups. However, these distinct tumor variants are categorized separately. In IDH-wildtype, H3-wildtype diffuse glioma, the presence of either

microvascular proliferation or necrosis is adequate for diagnosing glioblastoma. However, multiple distinctive molecular characteristics are outlined for IDH-wildtype glioblastoma. These include telomerase reverse transcriptase (TERT) promoter mutation, EGFR amplification, and the combined gain of entire chromosome 7 and loss of entire chromosome 10 (+7/−10). These modifications essentially act as criteria for identifying IDH-wildtype glioblastoma. Consequently, any diffuse glioma containing these alterations, even if it presents as grade II or III based on histopathological assessment, is characterized by poor clinical performance (Horbinski et al., 2022; Whitfield and Huse, 2022).

In the updated classification, diffuse astrocytic tumors with IDH mutations are now collectively categorized as "astrocytoma, IDH-mutant" and are given grades II, III, or IV. The grading system incorporates additional molecular markers, such as the presence of a homozygous deletion of CDKN2A/B, which is linked to a poorer prognosis. Specifically, IDH-mutant astrocytomas displaying these molecular alterations are classified as grade IV, regardless microvascular proliferation or necrosis. This refined differentiation between IDH-wildtype and -mutated astrocytomas represents a noteable improvement. However, it places a substantial responsibility on neuropathology laboratories to conduct thorough molecular testing promptly. This is crucial for identifying the 10% of astrocytomas with noncanonical IDH mutations undetectedable using IDH R132H immunohistochemistry and for recognizing astrocytomas with molecular characteristics resembling glioblastoma (Whitfield and Huse, 2022). In the context of pediatric gliomas, they are categorized into low and high grades. Pediatric-type diffuse low-grade gliomas are further divided into four subtypes, while pediatric-type diffuse high-grade gliomas encompass four subtypes. Certain tumor types, like diffuse low-grade glioma and those with MAPK pathway alterations, indicate potential responsiveness to RAF and MEK inhibitors. Additionally, infant-type hemispheric gliomas often feature fusions that could respond to targeted therapies. These classifications and subtypes are intended to offer a more precise comprehension of gliomas and improve treatment strategies (Wen and Packer, 2021).

Monitoring tumor metabolite 2-hydroxyglutarate (2-HG) during surgery offers crucial information such as tumor classification. The presence of 2-HG servesas a guide for optimal resection, while the absence of 2-HG necessitates monitoring other metabolites or lipids. 2-HG-expressing in the central nervous system (CNS) indicates IDH1 or IDH2 mutations (Veliz et al., 2015). The IDH1 mutation remains a robust molecular marker to distinguish these groups. The IDH enzyme, with five isoforms, catalyzes isocitrate to alpha-ketoglutarate (α-KG) and carbon dioxide (CO_2). Structural alteration due to mutations in IDH1 and IDH2 alter their affinity for isocitrate, leading to the NADPH-dependent reduction of α-KG to 2-HG, resulting in its accumulation in the cells. As an oncometabolite, 2-HG can modify gene expression and inhibit histone demethylation and influence cell differentiation (Turcan et al., 2012; Dang and Su, 2017). Moreover, the primary group can be divided into three subgroups, including 1) metaplastic mesenchymal component of glioblastoma, 2) giant cell glioblastoma, characterized by the presence of multinucleated cells, and 3) epithelioid glioblastoma (Louis et al., 2016).

3 Genetic and epigenetic heterogeneity of GBMs

GBMs exhibit genetic and epigenetic heterogeneity, encompassing variations such as amplifications, mutations, and deletions of genes within a tumor. Where cells acquire mutations that are not present in other cells. This genetic heterogeneity results in diverse cell populations with distinct genetic profiles. Epigenetic modifications including histone modifications, DNA methylation, and non-coding RNA molecules can alter gene expression patterns without modifying the underlying DNA sequence. In GBMs, heterogeneity plays a significant role in the development and progression of the tumor (Zhou et al., 2018; DeCordova et al., 2020; Yabo et al., 2022).

Glioma stem-like cells, also known as glioma-initiating cells, are a subpopulation of glioblastoma cells (Wirsching et al., 2016). These cells may originate from the limited population of adult neural stem and progenitor cells found in specific regions such as the subventricular zone, the dentate gyrus of the hippocampus, and the subcortical white matter. Most glioma-initiating cell progenies exhibit features of astrocytes, and some differentiate into functional endothelial cells and pericytes (Wirsching et al., 2016; Gimple et al., 2022).

The gliomas can arise in the glial tissue of the CNS, with occurrencein the astrocytic, oligodendrocytic, or oligoastrocytic tissues (Pan and Monje, 2022). Recent studies have provided evidence that gliomas arise through direct differentiation from progenitor cells, and this process influences the tumor's response to chemotherapy (Persson et al., 2010). Furthermore, gliomas can be categoriezed based on the degree of invasiveness into two groups: those infiltrating and diffusing into the surrounding brain parenchyma, and frequently recurring after surgical resection, and those with limited growth, manageable through surgical resection (Delgado-M et al., 2020). However, it is important to note that the distinction between glioblastoma classes may not be rigid, with evidence of mosaicism or even class switching observed under the influence of the tumor microenvironment (Veliz et al., 2015).

3.1 Genetic heterogeneity

Some of the most common genetic mutations observed in GBMs include:

I. EGFR amplification and mutation, which can result in increased signaling through the phosphatidylinositol 3-kinase (PI3K) pathway and contribute to tumor growth.
II. Loss of heterozygosity (LOH) in chromosome 10, which can result in the loss of tumor suppressor genes such as phosphatase and tensin homolog (PTEN).
III. Mutations in TP53, a tumor suppressor gene that plays a role in regulating the cell cycle and preventing the formation of tumors.
IV. Mutations in the IDH gene, which are more commonly observed in lower-grade gliomas but can also occur in some cases of GBM.

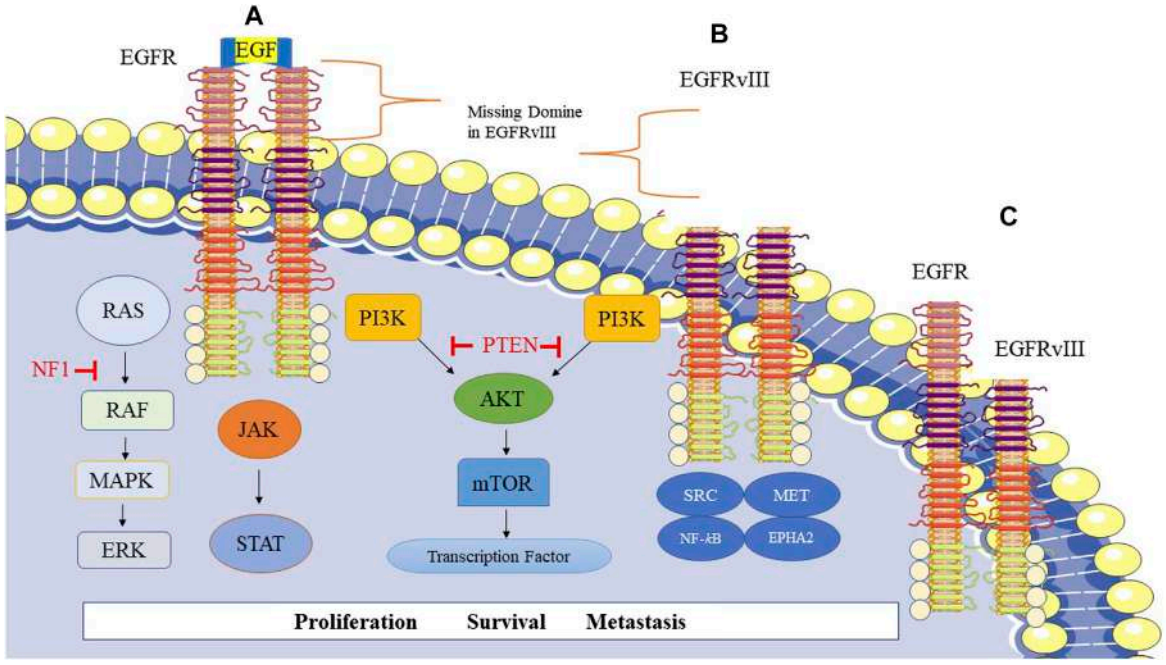

FIGURE 1
Epidermal growth factor receptor (EGFR) signaling and related pathways in GBM. **(A)** Homodimer of EGFR, **(B)** Homodimer of EGFRvIII, EGFRvIII is a genetic variant of EGFR in glioblastoma cells and frequently occurs in GBM. This mutation leads to the missing extracellular domain in EGFRvIII. **(C)** Heterodimer of EGFR-EGFRvIII.

V. Alterations in the retinoblastoma 1 (RB1) gene, which is also involved in regulating the cell cycle.
VI. Mutations in genes involved in the DNA damage response, such as alpha thalassemia/intellectual disability syndrome X-linked (ATRX) (Eskilsson et al., 2018; Hernández Martínez et al., 2022; Verdugo et al., 2022).

Despite sharing identical histology, primary and glioblastoma that originated from a low-grade astrocytoma display distinct differences in their genetic and epigenetic profiles. The primary group is confirmed by amplification and/or mutated EGFR in chromosome 7p, deletion of PTEN, and homozygous deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A-p16^{INK4a}) in chromosome 9p. Moreover, in tumors with no TP53 and TERT mutations, amplification of oncogene mouse double minute 2 (MDM2) is observed. NF1 mutations and homozygous deletion of PI3KR1 are also characteristic of this group (Cancer Genome Atlas Research Network, 2008; Vital et al. (2010). The glioblastoma that originated from a low-grade astrocytoma is characterized by the methylation of the promoter of O6-Methylguanine-DNA-methyltransferase (MGMT) associated with TP53 mutations and partial LOH in chromosomes 10q, 13q, 19q, and 22q (Crespo et al., 2015).

Amplification on chromosome 7, deletion on chromosome 10, amplification or mutation in EGFR, and deletion in the locus of Ink4a/ARF define classical glioblastoma. The mutation or deletion in NF1 and expression of Chitinase-3 like-protein-1 (CHI3L1), hepatocyte growth factor receptor (MET), and genes involved in the tumor necrosis factor (TNF) and nuclear factor of κ-light polypeptide gene enhancer in B-cells (NFκB) pathways display mesenchymal glioblastoma. Mutation in IDH 1 and 2 is associated with the alterations of PDGFR-α and carries the gliomaCpG island methylator phenotype (GCIMP) is known as proneural glioblastoma (lower-grade gliomas GBM) (Zhu and Wong, 2013). Distinguishing tumors with the glioma-GCIMP phenotype from GCIMP-negative tumors usually have wild-type IDH (Veliz et al., 2015). A review by Mellinghoff et al. (2012) focused on the common genetic alterations observed in growth factor signaling pathways in GBM.

3.1.1 Mutation in EGFR

Mutated EGFR type III (EGFRvIII) is frequently found in approximately 50% of glioblastoma tumors that exhibit EGFR amplification. The deletion in exons 2–7 of the EGFR gene (801 base pairs) generates the EGFRvIII protein, which lacks 267 amino acids in the extracellular domain of EGFR (Figure 1). As a result, this mutated protein cannot bind to ligands and produce a constitutive signal (Gan et al., 2013). However, treatment with EGFR TKIs has shown limited success in glioblastoma compared to lung cancer due to changed kinetics of inhibitor binding or the reduced sensitivity of EGFRvIII (Nishikawa et al., 2004; Bonavia et al., 2012; Vivanco et al., 2012). EGFRvIII requires wild-type EGFR to be an oncogene, as it is activated when wild-type EGFR is co-expressed. EGFRvIII induces the production of heparin-binding epidermal growth factor (HBEGF)-like growth factor, which in turn activates the wild-type EGFR. The activated EGFRvIII may homo- or heterodimerize with EGFR (Figures 1B, C), leading to enhanced transactivation of multiple TK receptor families like MET and EPHA2, mediating EGFRvIII oncogenicity. However, ligands

binding to wild-type EGFR can inhibit EGFRvIII and tumor growth (Huang et al., 2007; Veliz et al., 2015).

EGFR/EGFRvIII crosstalk predominantly boosts signal transducer and activator of transcription 3 (STAT3) signaling with less impact on PI3K and mitogen-activated protein kinase (MAPK) signaling pathways. EGFRvIII translocates to the nucleus upon phosphorylation by EGFR, forming a complex with STAT3, resulting in its phosphorylation and activation (Fan et al., 2013).

Francis et al. (2014) demonstrated that multiple EGFR mutational variants exist within glioblastoma tumors, includingEGFRvII and EGFR carboxyl-terminal deletions in the bulk tumor, highlighting the molecular heterogeneity of EGFR alterations in GBM. Therefore, the heterogeneity of glioblastoma is conferred by the plasticity of EGFR amplicons (Francis et al., 2014). The expression of EGFRvII leads to downstream activation of the protein kinase B (AKT) signaling pathway and potentially the STAT3 pathway. Interestingly, EGFR TKI sensitivity is enhanced by EGFRvII. The deletion in exons 2–7 of the EGFR gene generates EGFRvII (Francis et al., 2014; Veliz et al., 2015).

3.1.2 Mutation in PDGFR

PDGF ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) bind to specific receptors on the surface of cells, known as PDGFR (PDGF receptor). PDGFR is a tyrosine kinase receptor, has two isoforms: PDGFR-α and PDGFR-β. Upon binding to PDGF, the dimer of PDGFR-α or PDGFR-β is activated by inducing the receptor dimerization, leading to downstream signaling cascades that trigger cell growth and survival. PDFG and PDFGR are frequently co-expressed in GBM. This co-expression is thought to play a crucial role in the pathogenesis of GBM by promoting the growth and survival of tumor cells. Inhibiting the PDGF/PDGFR signaling pathway has been considered as a promising therapeutic strategy for GBM treatment (Westermark, 2014; Lane et al., 2022). The gene of PDGFR-α is amplified, mutated, or rearranged in GBM. Deletion of exons 8 and 9 in the PDGFR-α gene results in the omission of 243 base pairs and leads to the formation of a constitutively active receptor with tumorigenic ability. Furthermore, a two-base pair deletion in exon 23 can cause truncation of the C-terminal region of the receptor (Mellinghoff et al., 2012; Szerlip et al., 2012).

3.2 Epigenetic heterogeneity

GBM is characterized by significant epigenetic heterogeneity with a profound impact on gene expression and cellular phenotype. The phenotypic heterogeneity in GBM is influenced by multiple factors, including the cell of origin and epigenome (Capper et al., 2018). Chromosomal aberrations, such as copy number alterations, can further affect DNA methylation, leading to the formation of epigenetically dynamic regions. DNA methylation profiles can be used to classify GBMs into distinct subclasses that correlate with transcriptomic subtypes (Noushmehr et al., 2010; Chaligne et al., 2021). Additionally, GBMs exhibit heterogeneous DNA methylation and chromatin accessibility profiles not only in different tumor zones but also at the single-cell level, reflecting the diverse phenotypic states of GBM cells (Yabo et al., 2022).

GBMs have been found to co-opt the core transcriptional networks involved in pluripotency reprogramming, similar to those found in embryonic stem cells. Specifically, GBM cells often express high levels of the transcription factors SRY (sex determining region Y)-box 2 (SOX2) and cellular myelocytomatosis (c-Myc), and lower levels of Octamer-binding transcription factor3/4 (OCT3/4), Nanog, and Kruppel-like factor 4 (KIF4) (Rheinbay et al., 2013). Research studies have shown that genetic activation of pluripotency or neural-specific transcription factors [like brain-specific homeobox/POU domain protein 2 (BRN2), Sox2, spalt-like transcription factor 2 (SALL2), and oligodendrocyte transcription factor 2 (OLIGO2)] can induce tumorigenic cancer stem cell-like states in GBM. This is accomplished through modulation of epigenetic regulators, such as the REST corepressor 2/lysine-specific demethylase 1 (RCOR2/LSD1) histone demethylase and DNA methyl transferase Dnmt1, as well as noncoding RNAs such as HOX transcript antisense RNA (HOTAIR) and metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) (Suvà et al., 2014). Sturm et al. (2012) identified three distinct methylation classes in GBM that correlate with patient survival, highlighting the importance of considering epigenetic subtypes in clinical decision-making. Other studies have also identified epigenetic subtypes of GBM that are associated with patient outcomes. Understanding the epigenetic heterogeneity of GBM could pave the way for developing targeted therapies and personalized medicine approaches (DeCordova et al., 2020; Yabo et al., 2022).

4 Resistance to temozolomide

The standard treatment for GBM involves a multimodal approach, beginning with surgical resection to remove as much of the tumor as possible. Following surgery, patients typically receive radiotherapy in combination with concomitant adjuvant chemotherapy with temozolomide, a DNA alkylating agent. This treatment is sometimes associated with alternating electric fields of intermediate frequency. Based on chemotherapy-induced disorders, combination therapy may decrease side effects, and increase survival rate (Moslemizad et al., 2022). Generally, recurrence occurs within 12 months of diagnosis in 90% of patients (Li X. et al., 2020; Cruz Da Silva et al., 2021). Temozolomide spontaneously turns to 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide, a reactive methylating agent. This agent then degrades to the methyldiazonium cation, which reacts with DNA and produces DNA methyl adducts such as O6-methyl-guanine, N3-methyladenine, and N7-methylguanine. Consequently, DNA strand breaks occur and cannot be repaired by recombination protein A 51 (RAD51)-driven homologous recombination (HR), resulting in cell-cycle arrest and cell death (Veliz et al., 2015).

Methylation from the O6 position of guanine can be removed by O6-methylguanine-DNA methyltransferase (MGMT), resulting in resistance to temozolomide. In addition, the phosphorylation of STAT3 increases in MGMT-overexpressed glioblastoma cells. It appears that STAT3 is necessary for the posttranscriptional elevation of MGMT. MGMT and phosphorylated-STAT3 levels increase in recurrent tumors compared to primary glioblastoma patients (Kohsaka et al., 2012; Bahadur et al., 2019).

Furthermore, resistance to temozolomide is additionally associated with a deficiency in the mismatch repair (MMR) pathway. MMR is unable to repair the original O6-methyl-guanine lesion. Consequently, impaired MMR function for DNA repair causes breaks in the double strand, replication arrest, and cell death. The failure to recognize this position due to impaired MMR leads to continued DNA replication and resistance to the cytotoxic effect of temozolomide (Hegi et al., 2005; Veliz et al., 2015). Overexpression of base excision repair (BER) contributes to resistance to temozolomide. BER cooperates in the removal of damaged or inappropriate DNA bases such as N7-methyl-guanine. Poly ADP-ribose polymerase (PARP) helps BER and repairs single-stranded DNA breaks. Inhibition of PARP activity induces cell death and enhances cytotoxicity by temozolomide (Veliz et al., 2015).

Chronic exposure to alkylating agents, irradiation, and corticosteroids induces mammalian target of rapamycin (mTOR) expression. mTORC2 transcriptionally and post-transcriptionally modulates N-myc downstream-regulated gene 1 (NDRG1) expression through the serum glucocorticoid-induced protein kinase 1 (SGK1). NDRG1 binds and stabilizes MGMT. Therefore, the mTORC2/SGK1/NDRG1 pathway can be a target for future therapy to overcome glioblastoma resistance (Weiler et al., 2014).

5 The kinases signaling pathways

Fleuren et al. (Fleuren et al., 2016) studied the kinome in human cancers, providing crucial information about the dysregulation of the protein kinase superfamily, their role in cancer malignancy, and their sensitivity to anticancer drugs modulated by kinome remodeling (Fleuren et al., 2016). The kinase pathways include receptor and non-receptor TKs activated by phosphorylation in glioblastoma cells. The receptor tyrosine kinases consist of EGFR, erythroblastic oncogene B 2, 3 and 4 (ERBB2, ERBB3 and ERBB4), fibroblast growth factor receptor 3 and 4 (FGFR3 and FGFR4), insulin receptor tyrosine kinase (IRTK), c-rearranged during transfection (c-RET), Insulin-like growth factor 1 receptor (IGF-IR), ephrin type-A receptor 1, 2, 3 and 4 (EPHA1, EPHA2, EPHA3 and EPHA4), macrophage stimulating protein receptor (MSP R), receptor tyrosine kinase like orphan receptor 1 and 2 (ROR1 and ROR2), macrophage colony stimulating factor receptor (M-CSF R), dual leucine zipper kinase (DLK) and tyrosine kinase with immunoglobulin-like and EGF like domains 1 (TIE1). The cytoplasmic non-receptor TKs involve AKT, MAPK, Janus kinase/signal transducers and activators of transcription (JAK-STAT), Wnt/β-catenin, protein kinase A (PKA), cAMP response element-binding protein (CREB), and phospholipase C gamma (PLCɣ) signaling (Joshi et al., 2012). The important signaling pathways that change in glioblastoma include overexpression of EGFR and PDGFR, and activation of Rat sarcoma (RAS), PI3K/PTEN/AKT, RB/CDK N2A-p16^{INK4a}, and TP53/MDM2/MDM4/CDKN2A-p14ARF pathways. Moreover, NOTCH signaling is activated and can be linked to hypoxia, PI3K/AKT/mTOR, and ERK/MAPK pathways in grade IV gliomas that increased malignancy (Huse and Holland, 2010; Banerjee et al., 2021). The whole-exome sequencing data demonstrated that at least one receptor tyrosine kinase (RTK) has altered in almost 67% of glioblastoma overall in 291 patients, alteration is EGFR (57%), PDGFRA (13%), c-MET (1.6%), and FGFR (3.2%), also, 25% and 41% of patients have PI3K mutations and PTEN mutations/deletions, respectively (Wang et al., 2021).

5.1 EGFR

EGFR is a member of the family of four TKs which includes ErbB1 (EGFR, HER1), ErbB2 (Her-2, Neu), ErbB3 (Her-3), and ErbB4 (Her-4) (Wieduwilt and Moasser, 2008). Mutations and amplifications of EGFR (HER1) have been identified in 45%–57% of studied GBM cases, indicating its potential causal role in GBM pathogenesis. EGFRs are known to promote proliferation and are implicated in both the development of glioblastoma and its resistance to treatment (McLendon et al., 2008; Brennan et al., 2013; Zaki et al., 2021). As discussed above, EGFRvIII and EGFRvII, two truncated mutant forms of EGFR, are expressed in GBM.

Interestingly, ErbB2/HER-2 mutation has also been observed in 8%–41% of GBM cases, indicating that other members of this family may also contribute to GBM development. ErbB2/HER2-specific NK cells can be generated through the isolation of NK cells from peripheral blood donors followed by exposure to ErbB2/HER2 protein or peptides *in vitro*. This exposure leads to the expansion and activation of ErbB2/HER2-specific NK cells, which can be infused into patients with GBM. Promising results have been demonstrated with ErbB2/HER2-specific NK cells in preclinical models of GBM, where they were shown to selectively target and kill glioblastoma cells both *in vivo* and *in vitro* (Zhang et al., 2016; Hosseinalizadeh et al., 2022).

5.2 PDGF/PDGFR

The signaling pathway of PDGF/PDGFR is crucial for normal tissue development, but its dysregulation contributes to oncogenesis. The data analyses from the TCGA research network displayed that 10%–13% of the cases studied had amplification of PDGFR-α. The expression of all PDGF ligands (PDGF-A, PDGF-B, PDGF-C, and PDGF-D) and both cell surface receptors, PDGFR-α and PDGFR-β, have been demonstrated in GBM (Pearson and Regad, 2017).

EGFR and PDGFR are RTKs that stimulate signaling pathways. Upon activation, the TK domain of these receptors undergoes autophosphorylation, which leads to the recruitment and activation of PI3K. This, in turn, converts PIP2 to PIP3, which binds to and activates AKT. In the plasma membrane, AKT is phosphorylated at Ser473 and Thr308 by PDK1 and mTORC2, respectively. AKT translocates to the nucleus and activates a cascade of phosphorylation events that ultimately lead to the activation of several proteins involved in angiogenesis, cell growth, and apoptosis, including mTOR and its partner, mTORC1. The tumor suppressor PTEN negatively regulates this pathway by preventing the conversion of PIP2 to PIP3 (Cruz Da Silva et al., 2021). Amplification or activating mutations in EGFR can result in hyperactivation of the PI3K signaling pathway, which promotes tumor growth and survival. In addition, the PI3K pathway can promote lipogenesis through sterol regulatory element-binding protein-1 (SREBP-1) (Veliz et al., 2015).

5.3 VEGF/VEGFR

The malignancy of gliomas progresses through angiogenesis. The VEGF and its receptor (VEGFR) are the principal factors of angiogenesis. VEGF is also known to increase the permeability of blood vessels, which allows fluids, nutrients, and other molecules to pass through the walls of the blood vessels more easily (Shibuya, 2011). The upregulation of VEGF promotes angiogenesis to counteract hypoxia, which is a common feature of GBM tumors (Joensuu et al., 2005). Under hypoxic conditions, hypoxia-inducible transcription factors (HIF-1α and HIF-1β) translocate to the nucleus and bind to the hypoxia-response element (HRE) in the promoter region of the VEGF gene, leading to its activation (Lugano et al., 2020). The binding of HIF-1α in the VEGF promoter enhances the angiogenic mechanisms in brain tumors. Furthermore, PDGF, FGF, angiopoietin-1, angiopoietin-2 (ANG-2), delta-like ligand 4 (DLL4), integrins, interleukin-8 (IL-8), and stromal-derived factor 1 (SDF1) besides VEGF can stimulate the angiogenesis in GBM (Delgado-M et al., 2020).

Treatment with nitrosoureas and bevacizumab is used in recurrence of GBM (Delgado-M et al., 2020). Bevacizumab is the monoclonal antibody against VEGFA and targets angiogenesis and was approved for GBM treatment in 2009. Bevacizumab is added to chemoradiotherapy with temozolomide. Gilbert et al. (Gilbert et al., 2014) showed overall survival did not improve when bevacizumab was used in patients with newly diagnosed glioblastoma. Additionally, in a study by Chinot et al. (Chinot et al., 2014) was demonstrated that bevacizumab addition to radiotherapy and temozolomide did not improve overall survival, Moreover, the use of bevacizumab was associated with a higher rate of adverse effects compared to placebo.

5.4 RAS/MAP/ERK signaling pathway

Many studies have reported that 88% of GBMs have mutations in RAS/MAPK and PI3K/AKT pathways which play the principal role in multiple cellular processes. The RAS/MAP/ERK pathway is a vital signaling pathway that modulates cell growth, differentiation, and survival. Mutations or dysregulation in this pathway can cause abnormal activation and lead to uncontrolled cell proliferation, tumorigenesis, and metastasis in various cancers (Pearson and Regad, 2017).

The pathway is initiated by the activation of RAS proteins, which are localized on the cell membrane. Activation of the RAS/MAPK causes GDP transformation to GTP, RAS undergoes a conformational change that leads to interact with downstream signaling molecules (Regad, 2015). RAS activates rapidly accelerated fibrosarcoma (RAF), which in turn activates MEK and ultimately results in the activation (phosphorylation) of ERK. ERK translocates to the nucleus where it modulates gene expression, thereby regulating various cellular processes such as cell growth, differentiation, and survival (Kolch, 2000; McCubrey et al., 2007). Hyperactivation of this pathway increases growth autonomy and glioblastoma migration (Pearson and Regad, 2017). Additionally, the pathway has also been associated with the development of resistance to chemotherapy and radiation therapy. Therefore, understanding the RAS/MAPK pathway's mechanisms and identifying its aberrations is critical for developing targeted therapies and improving cancer treatment outcomes (McCubrey et al., 2007).

Astrocyte elevated gene-1 (AEG1) as a target of RAS activates multiple signaling pathways such as PI3K-AKT, MAP/ERK, Wnt, and NFκB. In addition, the expression of AEG1 has a negative correlation with the excitatory amino acid transporter 2 (EAAT2). The suppression of EAAT2 results in a reduction of glutamate uptake by glial cells (Berger et al., 2022).

5.5 Other tyrosine kinase pathways

The aberrant activation of NFκB is observed in GBM, making it an attractive target for cancer prevention or treatment (Ghareghomi et al., 2021). This abnormal activation of NFκB is thought to contribute to the development and progression of glioblastoma by promoting cell proliferation, inhibiting cell death, and promoting inflammation. The EGFR pathway activates the transcription factor NFκB (Soubannier and Stifani, 2017). Bredel et al. (Bredel et al., 2011) showed that NFKBIA (nuclear factor of κ-light polypeptide gene enhancer in B-cells inhibitor-α) deletion and EGFR amplification have a similar effect in the pathogenicity of GBM, but their effect is exclusive. NFKBIA is an inhibitor of NFκB and suppresses glioblastoma tumors. Loss of NFKBIA function results in NFκB activation, which contributes to glioblastoma progression. On the other hand, EGFR amplification leads to increased signaling through the PI3K/AKT and MAPK pathways, promoting cell growth and survival (Bredel et al., 2011).

Activation of hepatocyte growth factor receptor (HGFR) also known as c-MET can occur through several mechanisms such as gene amplification, mutation, or ligand binding, and plays a role in cell proliferation, differentiation, and migration. HGF as a ligand binds to c-MET on the surface of tumor cells and can lead to downstream signaling pathways. Dysregulation of the c-MET pathway has been implicated in the pathogenesis of glioblastoma. Overexpression of c-MET has been observed in GBM and is linked to poor prognosis (Kong et al., 2009; Petterson et al., 2015).

The overexpression and amplification of FGFR genes is observed in GBM which causes the activation of FGFR signaling and leads to enhanced tumor growth and invasion. In addition, FGFR signaling has been shown to promote the maintenance of glioblastoma stem cells, which are thought to be responsible for tumor recurrence (Loilome et al., 2009).

The urokinase plasminogen activator (uPA) and its receptor (uPAR) are frequently upregulated in GBM, leading to increased activation of plasminogen and promoting tumor cell migration and invasion. The PI3K/AKT signaling pathway appears to be involved in regulating uPA-induced cell migration, as inhibition of this pathway can downregulate uPA activity. Additionally, uPA can activate matrix metalloproteinases (MMPs), which further contribute to the invasive phenotype of glioblastoma cells (Delgado-M et al., 2020).

Cell motility in glioma cells is associated with Rho-family GTPases, including RhoA, Ras-related C3 botulinum toxin substrate (RAC), and cell division control protein 42 homolog (CDC42), which regulate the actin cytoskeleton. The myosin-actin interactions are promoted through Rho-associated

coiled-coil kinase (ROCK), while the formation of lamellipodia is activated by Rac and the formation of filopodia is activated by CDC42. Dysregulation of Rho-family GTPases is observed in glioblastoma and contributes to increased cell motility, invasiveness, and tumor progression (Delgado-M et al., 2020).

The SRC family tyrosine kinases (SFKs) belong to the broad family of non-receptor tyrosine kinases. SRC can regulate the PI3K/AKT/mTOR axis which suppresses autophagy. SRC activity is overexpressed in GBM. Inhibition of SRC tyrosine kinase can induce autophagy in GBM (Jovanović Stojanov et al., 2022).

In 2008, the TCGA suggested that dysregulation in the RB, p53, and RTK/RAS/PI3K pathways are obligatory events in glioblastoma tumors and can help guide therapeutic decisions. Treatment with cyclin-dependent kinases (CDK) inhibitors can be expected in patients with amplifications of CDK4/CDK6 or inactivating mutations or deletions in CDKN2A or CDKN2C. Furthermore, PI3K or PDK1 inhibitors might be effective for patients with PTEN deletions or activating mutations in PIK3CA or PIK3R, whereas the PI3K pathway that is altered by AKT3 amplification is resistant. Therefore, the design of RTK inhibitors cocktails might be a beneficial strategy to treat the multiple phosphorylated (activated) RTKs in individual glioblastoma specimens (McLendon et al., 2008).

6 Tyrosine kinase inhibitors (TKIs)

The Sokolov et. al. (Sokolov et al., 2021) study found that kinase inhibitors are a versatile class of drug targets in clinical trials for brain cancers, with 87 unique proteins of kinases. These included isoforms of several kinases for the PI3K-AKT-MTOR pathway, Janus kinase (JAK), EGFR, ERBB2, FGFR, anaplastic lymphoma kinase, KIT, cyclin-dependent kinases (CDKs), mitogen-activating protein kinase, tyrosine-protein kinase Lyn, tropomyosin receptor kinase, EPHA2, WEE1 kinase, and many other targets. The meta-analysis showed that anti-EGFR therapies have no impressive effects on the overall survival of patients with GBM (Lee et al., 2020). The inhibitors of angiogenesis have often been combined with other therapies, and a few combinations with bevacizumab have only reached phase III of clinical trials (Sokolov et al., 2021). According to the meta-analysis conducted by Ameratunga *et al.* (Ameratunga et al., 2018), antiangiogenic treatment did not provide any improvement in overall survival for patients with high-grade GBM.

Cabozantinib targets several RTKs, including VEGF/VEGFRs, MET, and AXL. VEGFR and MET are known to promote tumor growth and metastasis by regulating angiogenesis, cell proliferation, cell migration, and epithelial-to-mesenchymal transition. AXL kinase, on the other hand, is implicated in tumor pathogenesis and signaling pathways that promote metastasis. By targeting these RTKs, cabozantinib has shown promise in the treatment of several types of cancer, including renal cell carcinoma, hepatocellular carcinoma, and medullary thyroid cancer (Maroto et al., 2022). Cabozantinib shows *in vivo* efficacy in multiple xenograft models. It has also demonstrated synergistic effects with radiation therapy in glioblastoma cell lines. A phase II clinical trial evaluated the safety and efficacy of cabozantinib in patients with recurrent glioblastoma (Wen et al., 2018).

Sorafenib inhibits RAF, PDGFR, VEGFR, c-KIT, and FLT3. However, this multitarget TKI failed in phase III of the clinical trial (Wilhelm et al., 2008).

Joshi et al. (Joshi et al., 2012) reported that the combination of gefitinib and sunitinib, as well as sunitinib and sorafenib, can inhibit the phosphorylation of MAPK, AKT, and STAT3. The gefitinib and sunitinib combination was found to decrease the phosphorylation of several TKs, including EGFR, FGFR3, ERBB2, MER, TIE2, INSULIN R, rearranged during transfection kinase (C-RET), DLK, TIE1, EPHA1, EPHA4, AKT, MAPK, PKA (CREB), SRC, JAK-STAT, c-JUN, and p53. Therefore, targeting multiple TKs in combination therapy might be an effective approach. However, this combination did not demonstrate any survival benefit in animal models. The authors suggested that targeting multiple targets and improving the drug delivery system should be considered for a successful therapeutic strategy.

Manzano et al. (Manzano et al., 2021a) have demonstrated that patients with GBM who have low C3G expression may not respond to EGFR inhibitors. The downregulation of C3G results in the reduction of EGFR levels. C3G is a guanine nucleotide exchange factor (GEF) for GTPases from the RAS superfamily and can also act through GEF-independent mechanisms. C3G can modulate RTKs such as EGFR, tyrosine kinase receptor A (TRKA), anaplastic lymphoma kinase (ALK), MET, and IRTK, and stimulate proliferation and differentiation in neural cells. It appears that C3G (RAPGEF1) mRNA levels are downregulated during the onset and progression of GBM. However, using C3G as a target for GBM treatment is still not recommended (Manzano et al., 2021a; Manzano et al., 2021b).

Everolimus, an mTOR inhibitor, has received approval for the treatment of subependymal giant cell astrocytoma (SEGA), and is being investigated in combination with other drugs such as temozolomide, lenvatinib (a VEGFR inhibitor), sorafenib, ribociclib (a CDK inhibitor), and dasatinib (a BCR/ABL and SRC inhibitor). Despite the variety of kinase inhibitors available, selumetinib, a mitogen-activated protein kinase 1/2 inhibitor, has successfully passed phase III trials in low-grade glioma and astrocytoma (NCT03871257, NCT04166409) (Sokolov et al., 2021).

Despite advanced knowledge in molecular biology and genetics of GBM due to its heterogeneity, developing an effective therapy is an obstacle. In GBM drug design, permeability and pharmacokinetics should be considered due to the impermeable BBB (Mitusova et al., 2022). For example, gefitinib and erlotinib are EGFR inhibitors that have failed in GBM treatment due to their inability to effectively penetrate the BBB, which limits their concentration in the brain (Pan et al., 2020).

7 Challenges in developing selective TKIs

As mentioned, gefitinib, erlotinib, lapatinib, dacomitinib, and osimertinib are EGFR inhibitors received approval for non–small cell lung cancer (NSCLC) treatment. The results revealed pharmacokinetic failure in GMB therapy is related to BBB penetration of these inhibitors (Wang et al., 2021). Hence, some improvements to this kind of inhibitor are being developed, like the combination of AZD3759, a blood-brain barrier-penetrant EGFR

inhibitor, and WSD0922, a selective EGFR exon 20 insertion mutant inhibitor, which is promising to evaluate the role of EGFR signaling inhibition. Epitinib and AZD3759 are in clinical trials for untreated EGFR-mutant NSCLC with brain metastases and have shown efficacy in patients (Zeng et al., 2015; Zhou et al., 2022). Furthermore, dacomitinib has shown promising results in early-phase clinical trials for patients with recurrent glioblastoma who have EGFR amplification, with or without EGFRvIII. Further clinical trials are required to evaluate its efficacy and safety in a broader patient population (Sepúlveda et al., 2014).

Clinical trials testing anti-angiogenic agents such as bevacizumab, PDGFR, VEGFR, and PKC inhibitors, have not demonstrated significant improvements in overall or progression-free survival compared to standard therapy (Schulte et al., 2021). Rapid resistance development and the potential contribution of factors beyond angiogenesis, such as invasion and immune evasion, may underlie the limited efficacy of these agents in treating glioblastoma (Voutouri et al., 2019).

Indeed, Dasatinib is a multi-kinase inhibitor that targets various kinases, such as SRC, PDGFR, KIT, EPHA2, and BCR-ABL fusion. However, its effectiveness in treating brain tumors is hampered by its inadequate accumulation in the brain. This limitation arises from the activity of P-glycoprotein and related molecules, which actively transport drugs out of the brain, thereby reducing their concentration within the target area (Lassman et al., 2015; Palande et al., 2022). Several clinical trials with EGFR inhibitors have failed because of low CNS penetrance, tumor heterogeneity, and pharmacokinetics properties (Wen et al., 2014; Wen et al., 2020). Despite more than 15% of clinical trials focusing on brain cancer, it is surprising that kinase inhibitors have not achieved treatment success. A significant challenge in developing drugs for brain cancer lies in pharmacokinetic properties, primarily due to BBB, which restricts the passage of molecules exceeding 500 Da, especially those that are lipid-insoluble and polar. Therefore, small molecules are the best candidates for TKIs. However, the desired distribution requires delivery systems. Various delivery systems such as liposomes, polymer nanoparticles, metal nanoparticles, bacterial-derived carriers, and protein nanoparticles have been developed for this purpose (Sokolov et al., 2021). The development of GBM occurs in the interstitial space of the brain, which is separated from the systemic circulation by the BBB. The tumor growth and angiogenesis lead to changes in the function and permeability of the BBB, which can affect the delivery of drugs to the tumor site. The expression of aquaporin proteins, which are involved in water transport across the BBB, can also change during glioblastoma development and contribute to BBB dysfunction (Silantyev et al., 2019).

7.1 The blood-brain barrier (BBB)

The blood-brain barrier is a neuroprotective barrier comprised of a monolayer of endothelial cells, along with ependymal and tanycytic cells. These cells are tightly interconnected by adherens junctions and tight junctions, which effectively restrict the passage of harmful substances into the brain. Occludin, claudin, and junction adhesion molecules are the chief proteins of tight junctions. Serine, threonine, and tyrosine phosphorylation regulate occludin (OCLN). The formation of tight junctions during the acquisition of cell polarity is regulated by junction adhesion molecules. Additionally, zonula occludens and cingulin also help the maintenance and integrity of BBB (Daneman and Prat, 2015; Kadry et al., 2020).

Furthermore, the development, function, and maintenance of the BBB are closely associated with the endothelium and related to nerve terminals, astrocytes, pericytes, CNS-border associated macrophages (BAMs), and a specific myeloid subpopulation. Moreover, the blood-brain tumor barrier (BBTB) also inhibits the entrance of drugs to the tumor bulk. The density of the endothelial cell layer in the BBB is not compromised during alterations at the tumor site; therefore, the function of the BBB remains efficient (Banerjee et al., 2021). The accumulation of small molecules, including potential therapeutics, can be limited by efflux pumps such as P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2), and multidrug resistance-associated proteins (MRP1, 4 and 5, ABCC1, 4 and 5), which are members of the ATP-binding cassette (ABC) transporter superfamily (Sarkar et al., 2018; Mo et al., 2021).

In GBM, the BBB is disrupted due to the infiltration of tumor cells and the secretion of various factors, such as VEGF, that promote angiogenesis and BBB leakage. This disruption can lead to increased permeability of the BBB, allowing for the entry of circulating cells and molecules into the brain. At the same time, the BBB in the peritumoral region may remain intact, creating a BBTB that limits drug delivery to the tumor. Therefore, strategies to target both the BBB and BBTB are being developed to improve drug delivery and treatment efficacy in GBM (Lugano et al., 2020).

The small molecules transport in and out of the brain by active transport, endocytosis, carrier-mediated transport, and passive diffusion (Chowdhury et al., 2021). Several challenges exist for drug transportation in GBM due to neovascular complexity, including effective permeation and drug concentration in brain cells. Efflux pumps recognize and eliminate foreign substances on the brain's luminal side, and ABC transporters can act as obstacles to drug entry into the brain. Furthermore, uptake and efflux transporters can become saturated when exposed to inhibitory signals. Although tumors compromise the structural integrity of the BBB and make it leaky to small molecules at the tumor site, the BBB remains intact at the tumor's edge, which is surrounded by proliferating cells (Gomez-Zepeda et al., 2019; Patel et al., 2021).

Scientists at Pfizer have developed a novel algorithm called CNS multiparameter optimization (CNS MPO) to address some of the challenges in drug discovery for brain targets. This algorithm consists of physicochemical parameters: ClogP (lipophilicity, calculated partition coefficient), ClogD (calculated distribution coefficient at pH 7.4), MW (molecular weight), TPSA (topological polar surface area), pKa (most basic center), and HBD (the number of hydrogen bond donors) with a score of 0 for low probability and 1 for high probability. Thus, the summation of the scores is between 0 and 6. The study by Wager et al. (Wager et al., 2010) reported that a high score in the CNS MPO algorithm was associated with a higher probability of a compound being a successful CNS drug, as evidenced by the fact that 74% of marketed CNS drugs have a score of four or more. Shergalis et al. (Shergalis et al., 2018) identified 73 potential drug candidates for GBM and found that only 37% of the small molecule

candidates had a score of more than four in current clinical trials, indicating that the majority of these candidates may not have favorable physicochemical properties for effective CNS drug delivery. Therefore, this algorithm, accompanied by other available tools, can be used by medicinal chemists to expedite the identification of compounds with an enhanced probability of success at the design stage.

7.1.1 Limited brain penetration of TKIs

The drug candidate must have proper pharmacokinetics properties, like reaching therapeutic concentrations at the tumor site without diffusing into other tissue (Sun et al., 2022). Erlotinib and gefitinib efficacy is limited and efflux transporters such as P-gp and ABCG2 remove the drugs from the brain. Gefitinib is only effective in patients whose tumors have specific mutations in exons 19 and 21 of the EGFR domain (Agarwal et al., 2010; Lo, 2010; de Vries et al., 2012; Tournier et al., 2021). Osimertinib and afatinib, are substrates of P-gp, and hence, are effluxed back to the bloodstream (Wind et al., 2014; van Hoppe et al., 2019). Additionally, neratinib, a pan-EGFR inhibitor, is a substrate for P-gp and ABCG2 and has limited brain penetration. A pan-EGFR inhibitor is a type of drug that inhibits all members of the epidermal growth factor receptor family, which includes HER1, HER2, HER3, and HER4 (Feldinger and Kong, 2015). Furthermore, lapatinib, a dual HER1/HER2 inhibitor cannot efficiently cross the BBB (Higa and Abraham, 2007).

Perifosine is an inhibitor of AKT signaling, which is a key pathway involved in the growth and survival of cancer cells. However, preclinical studies have shown that perifosine has limited brain penetration, which could limit its effectiveness in treating brain tumors (Cole et al., 2015; Becher et al., 2017).

Foretinib and SGX523 are two inhibitors for c-MET but the data that show their penetration into the brain is inadequate. Significant side effects were observed for cabozantinib, which can inhibit c-MET and VEGFR2. However, the selective MET inhibitor, capmatinib (INC280), is under GBM clinical evaluation (NCT02386826) (Zhang et al., 2010).

Heffron et al. (Heffron, 2016) reported that many small molecule inhibitors designed to target VEGFR/PDGFR have limited brain penetration due to their substrate nature for efflux transporters such as P-gp and BCRP. Cediranib, pazopanib, sunitinib, sorafenib, regorafenib, tandutinib, axitinib, and vatalanib are examples of such inhibitors. However, cabozantinib and brivanib have been reported to exhibit minimal P-gp mediated efflux and could be potential targets for GBM treatment.

GDC-0084, pilaralisib, buparlisib, XL765, and PX-866 are PI3K/mTOR inhibitors that can cross the BBB leading to their advancement to clinical trials for the treatment of GBM (Zhao et al., 2017; Colardo et al., 2021). Unfortunately, buparlisib has been reported to induce mood changes (Wright et al., 2021). Everolimus and sirolimus are FDA-approved agents that inhibit mTORC1 but are substrates of P-gp. In addition, perifosine inhibits AKT signaling but has brain penetration limitation preclinically (Heffron, 2016).

Palbociclib and abemaciclib are CDK4 and CDK6 inhibitors that are substrates of both P-gp and BCRP (Groenland et al., 2020). *In vitro* studies have revealed that CDK1 and 2 inhibitors such as flavopiridol, seliciclib, dinaciclib, SNS-032, and AT7519 are still being evaluated through clinical trials and further research (Gojo et al., 2013; Sánchez-Martínez et al., 2015; Dichiara et al., 2017).

Additionally, imatinib, cediranib, pazopanib sunitinib, sorafenib, tivozanib, nintedanib, and dovitinib inhibit PDGF receptors but did not show a survival benefit due to poor BBB penetration (Wang et al., 2021). Crenolanib has been investigated in a phase II clinical trial (NCT02626364) involving GBM patients with PDGFRA gene amplification. This inhibitor selectively inhibits the signaling of wild-type and mutant isoforms of the PDGFR family. Crenolanib effectively inhibits phosphorylation of PDGFR-α and downstream AKT signaling in Ink4a/Arf$^{-/-}$. However, further research is needed to fully understand the potential of crenolanib and other PDGFR inhibitors in treating GBM (Paugh et al., 2013).

The modifications in the structure of gefitinib have been made to improve its physical properties and reduce transporter-mediated efflux. Similarly, AZD3759 (third-generation of TKIs), a pan-EGFR inhibitor, has been developed with reduced rotatable bonds and sufficient hydrogen bond donors, allowing it to cross the BBB more easily than gefitinib. Tucatinib, the inhibitor for phospho-HER2, was reported to be able to cross the BBB freely. Several clinical trials evaluating tucatinib have been completed or are currently ongoing (Borges et al., 2018; Kulukian et al., 2020). The third-generation EGFR inhibitor osimertinib (AZD9291) and GDC-0084 have demonstrated greater permeability in a Phase I dose-escalation study conducted in patients with high-grade GBM (Ballard et al., 2016; Wen et al., 2020).

7.2 pharmacokinetic and pharmacodynamic properties of TKIs

Although TKIs share similar mechanisms of action, they vary in their ability to target specific kinase profiles, pharmacokinetic properties, and potential side effects. Hartmann et al. (Hartmann et al., 2009) summarized the pharmacology, metabolism, and side effects of TKIs. TKIs are designed to bind to the ATP-binding site of the tyrosine kinase, thereby preventing ATP from binding and inhibiting the kinase activity. Most kinase inhibitors exhibit ATP-competitive binding, which is attributed to the presence of a large hydrophobic surface in the ATP binding pocket. This feature enables these inhibitors to bind with high affinity to the kinase, as they can effectively interact with the hydrophobic environment of the pocket (Knight and Shokat, 2005). While the exact structure of small TKIs can vary depending on the specific compound, there are some common features and structural motifs found in many TKIs, which are documented in databases such as PubChem and ChemSpider. The prevalent structure includes a core scaffold consisting of a central aromatic ring system or a heterocyclic ring, an ATP-mimetic moiety often including a substituted purine or pyrimidine ring, binding interactions that can involve hydrogen bonding, hydrophobic interactions, electrostatic interactions, and Van der Waals forces, as well as substituents that can influence their potency, selectivity, and pharmacokinetic properties. Additionally, TKIs exhibit variability, as different compounds are designed to target specific TKs or address specific disease indications (Roskoski, 2019). A new generation of allosteric kinase inhibitors has been discovered. These inhibitors target allosteric sites on kinases, providing a different approach compared to traditional ATP-competitive inhibitors. This allosteric targeting offers a promising strategy for developing highly selective and potent kinase inhibitors, which may lead to improved therapeutic outcomes.

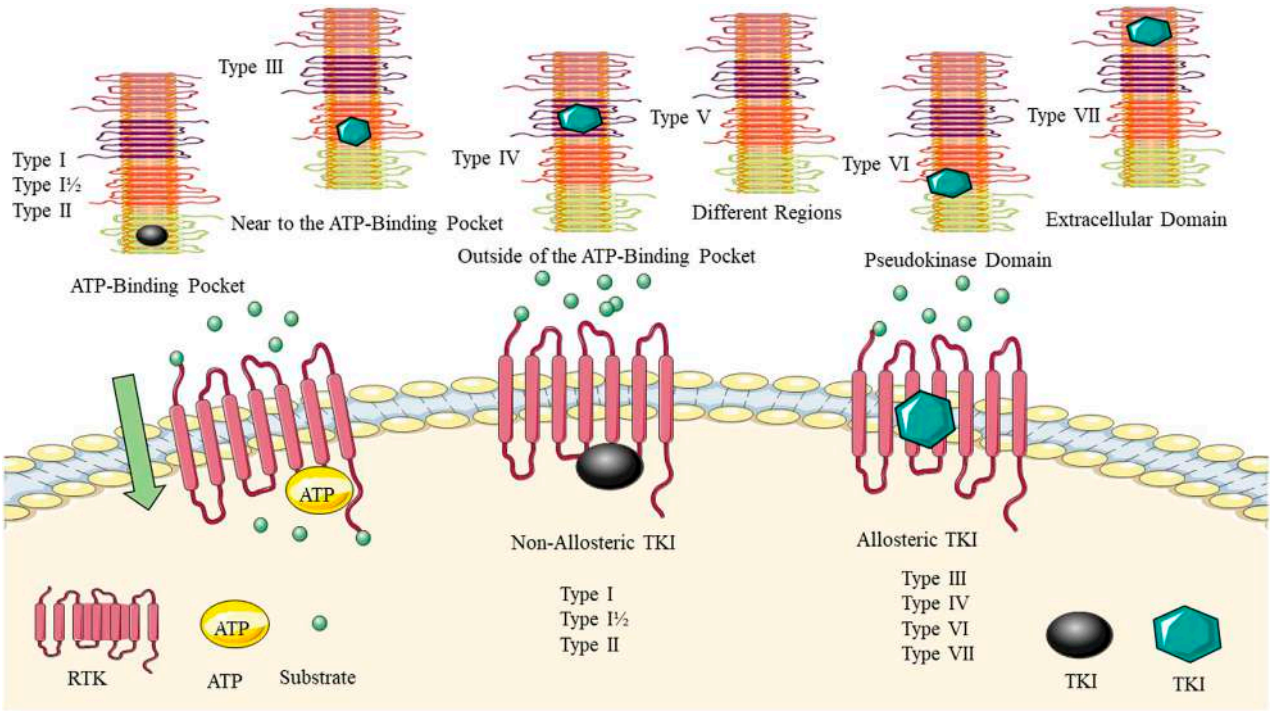

FIGURE 2
Allosteric and non-allosteric tyrosine kinase inhibitors. The binding site of the TKI for each group is highlighted. Type I–V inhibitors are reversible. Type VI kinase inhibitors can form covalent bonds with kinase sites, leading to the irreversible alteration of target activity.

Bhullar et al. (Bhullar et al., 2018) described the types of allosteric and non-allosteric inhibitors of TKs (Figure 2). Allosteric inhibitors bind to a site that is distinct from the ATP-binding pocket, called the allosteric site, and can induce conformational changes that inhibit kinase activity. Non-allosteric inhibitors, bind to the ATP-binding site and compete with ATP for binding to the kinase. Hence, Type I, such as cabozantinib and gefitinib, compete and bind to the ATP-binding pocket of the active conformation of proteins. In contrast, type II kinase inhibitors, including sorafenib, imatinib, and nilotinib, bind to the inactive conformation of protein kinases. While the binding sites of type III and IV are not located in the ATP pocket and function through allosteric mechanisms, only a few TKIs of these types, such as asciminib, have been approved. The type I–V inhibitors are reversible. Type VI kinase inhibitors can form covalent bonds with kinase sites, leading to the irreversible alteration of target activity. Osimertinib, afatinib, and ibrutinib possess better pharmacokinetic properties than reversible inhibitors (Hartmann et al., 2009; Lu et al., 2020).

Classifying ATP-competitive kinase inhibitors presents a challenge due to the variability in their molecular structures and the complexity of the conformational space occupied by kinase-inhibitor complexes. Inhibitors can bind to multiple conformational states of the kinase, making the classification process even more complicated (Arter et al., 2022). Robert Roskoski (Roskoski, 2023) described how small molecule protein kinase inhibitors can be classified into seven main groups based on their mechanism of action. The groups include reversible inhibitors (Groups I, I½, II, III, IV, and V) and targeted covalent irreversible inhibitors (VI). The type I½ and type II inhibitors are further divided into A and B subtypes, with subtype A inhibitors extending past the gatekeeper residue into the back cleft, while subtype B inhibitors do not. It is suggested that subtype A inhibitors may bind to their enzyme target with longer residence times compared to subtype B inhibitors. The example of sorafenib and sunitinib is given, with sorafenib being a type IIA VEGFR blocker with a residence time exceeding 64 min and sunitinib being a type IIB VEGFR inhibitor with a residence time of less than 2.9 min. Overall, the classification of small molecule protein kinase inhibitors into these groups and subtypes can aid in understanding their mechanisms of action and potential therapeutic benefits (Roskoski, 2023).

TKI resistance is a major challenge that significantly reduces patients' survival and quality of life. The abnormal activation of protein kinase-related signaling pathways due to gene mutations is the main reason for TKI resistance, and the tumor microenvironment also plays a crucial role. Cell death resistance, immune reprogramming, tumor metabolism, and epigenetic modifications are other mechanisms involved in TKI resistance (Yang et al., 2022). Therefore, due to the heterogeneity of TKI resistance mechanisms, a single therapeutic strategy may not be effective in all patients, and a deeper understanding of the mechanisms is essential.

8 Rational drug design of TKIs by computer-aided

The binding pockets found in kinase proteins are highly similar in structure, making it challenging to develop inhibitors that specifically target one particular kinase and can contribute to

adverse effects (Ravikumar et al., 2019). Various methods have been developed over the years to improve kinase selectivity. The first generation of TKIs was developed as ATP-competitive inhibitors. Second-generation TKIs were developed as allosteric inhibitors. Third-generation TKIs have been developed to address resistance mutations that occur during treatment with first- and second-generation TKIs. These mutations can occur in the kinase domain and lead to structural changes that hinder the binding of earlier TKIs. By selectively binding to the mutant kinases, these inhibitors aim to restore the efficacy of kinase inhibition and improve treatment outcomes (Huang et al., 2020; Kim and Ko, 2020; Hirschbühl et al., 2021).

Bioinformatics plays a pivotal role across various stages of the drug design process, including lead compound screening, target protein discovery, understanding the mechanism of drug action, and clinical statistical analysis (Li K. et al., 2020). Bioinformatics facilitates the identification of molecules with specific chemical structures for desired pharmacological effects in lead compound screening and, for target protein discovery, involves analyzing known effective target genes by quantifying their characteristics and comparing homologies with potential new target genes (Behl et al., 2021). In addition, bioinformatics plays a crucial role in drug development by assessing target druggability to reduce project failure risks, examining the similarity between different drugs to enhance understanding of drug mechanisms, utilizing clinical statistical analysis to evaluate the clinical effectiveness of compounds, and employing computational techniques to explore drug-target interactions and the role of proteins in drug mechanisms (Woolle et al., 2017; Wang et al., 2023).

Rational drug design, also known as computer-aided drug design (CADD), is a powerful tool used in the development of TKIs. CADD allows researchers to use computer simulations and modeling to predict how drug molecules interact with their targets and optimize the drug's properties such as selectivity, affinity, and pharmacokinetics (Yu and MacKerell, 2017). One approach to rational drug design is to use the crystal structures of protein kinases to design inhibitors that fit into the active site of the kinase. By using computational modeling and molecular dynamics simulations, researchers can predict which compounds are likely to bind with high affinity to the kinase and selectively inhibit its activity and named as structure-based drug design (SBDD) (Prieto-Martínez et al., 2019). Another approach is to use virtual screening methods to identify potential kinase inhibitors from large compound libraries, similarity searching, quantitative structure-activity relationship (QSAR) modeling, and pharmacophore generation which is named ligand-based drug design (LBDD) (Giord et al., 2022). Gagic et al. (Gagic et al., 2020) reviewed the CADD methods for the design of TKIs as anticancer drugs. The authors also provided examples of how to design new inhibitors for specific targets such as EGFR, VEGFR, PI3K, and MAPK (Gagic et al., 2020). Furthermore, several databases provide information on TKIs like ChEMBL (Gaulton et al., 2017), Kinase Knowledgebase (KKB), (Sharma et al., 2016), Protein Kinase Inhibitor Database (PKIDB) (Carles et al., 2018), and BindingDB (Gilson et al., 2016) that can be helpful for researchers to search for potential protein kinase inhibitors and their properties, as well as to analyze the structure-activity relationships of known inhibitors.

9 Future direction

The emergence of multi-omics data facilitates computational predictions for anticancer drugs by revealing potential repositioning opportunities. To address the complexity of patient responses in cancer treatment, bioinformatics methods leverage patient-specific genetic, epigenetic, metabolomic, and transcriptomic profiles for precise drug selection, ultimately improving clinical outcomes. Omics technologies play a crucial role in unraveling the mechanisms of cancer progression and identifying biomarkers and treatment targets (Baysoy et al., 2023). Large-scale initiatives, such as the Pan-Cancer Analysis of Whole Genomes (PCAWG) Consortium, have generated extensive omics data, enabling advanced studies on gene mutations and expression profiles across diverse cancers. Notable datasets, including the NCI-60 Human Tumor Cell Lines Screen, Genomics of Drug Sensitivity in Cancer (GDSC), The Cancer Genome Atlas (TCGA), Cancer Therapeutic Response Portal (CTRP), L1000 profiles from The Library of Integrated Network-Based Cellular Signatures (LINCS) Program, Cancer Cell Line Encyclopedia (CCLE), and the Catalogue of Somatic Mutations In Cancer (COSMIC), have proven valuable in understanding drug-resistant cancer cells. These datasets provide novel insights, and the increasing volume is expected to drive the development of computational models that systematize approaches to studying drug-resistant cancer cells more effectively (Nicora et al., 2020; Cai et al., 2022). Particularly, the integration of multi-omics analyses with advanced tools like genome engineering like CRISPR-Cas9 will remain pivotal for the comprehensive characterization of drug-resistant cancer cells. The growing abundance of omics data is expected to contribute to the development of diverse computational models. Consequently, the outcomes predicted by these models will enable a more systematic design of experiments focused on drug-resistant cancer cells (Jung et al., 2021). In recent years, machine learning (ML) and artificial intelligence (AI) have also been applied to the rational drug design of TKIs. These methods can rapidly process large amounts of data and generate predictive models that can guide the design of novel inhibitors with improved properties (Urbina et al., 2021; Moriwaki et al., 2022; Bao et al., 2023).

10 Conclusion

GBM is characterized by high molecular and transcriptional heterogeneity, which contributes to therapy resistance. Despite recent advancements in targeted therapies, particularly TKIs against GBM, their success has been limited. This is primarily due to their poor penetration of the BBB and inadequate achievement of pharmacokinetic concentrations. Additionally, resistance to TKIs poses a significant challenge in cancer treatment, especially with long-term use. Resistance can arise from genetic alterations, alternative signaling pathways, or changes in the tumor microenvironment. Understanding the mechanisms of resistance and developing new strategies to overcome it is crucial for enhancing the efficacy of TKIs in cancer treatment.

To address this, several reliable methodologies have been developed to profile kinome activity by monitoring substrate or kinase phosphorylation in a high-throughput manner. These

techniques have greatly contributed to our understanding of biological and pathological processes, enabling the identification of key kinases involved in disease progression. Such approaches play a vital role in discovering druggable targets and provide valuable insights into potential therapeutic interventions.

Moreover, the integration of bioinformatics in TKI development has expedited the drug discovery and optimization process, leading to the creation of more effective and selective TKIs for cancer treatment. Although some TKIs in clinical trials have demonstrated limited specificity and efficacy, the future of TK-targeted therapeutics in GBM holds promise.

Author contributions

MR: Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. SJ: Investigation, Writing–original draft, Writing–review and editing. HB: Investigation, Writing–original draft, Writing–review and editing. LS: Funding acquisition, Supervision, Validation, Writing–review and editing. VS: Conceptualization, Supervision, Validation, Writing–review and editing.

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Rapid response to fifth-line brigatinib plus entrectinib in an *ALK*-rearranged lung adenocarcinoma with an acquired *ETV6-NTRK3* fusion: a case report

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The management of non-small cell lung cancer (NSCLC), specifically targeting the anaplastic lymphoma kinase (*ALK*) with tyrosine kinase inhibitors (TKIs), is challenged by the emergence of therapeutic resistance. Resistance mechanisms to *ALK* TKIs can be broadly classified into ALK-dependent and ALK-independent pathways. Here, we present a case with lung adenocarcinoma (LUAD) harboring an *ALK* rearrangement. The patient had developed resistance to sequential ALK TKI therapies, with an acquired *ETV6-NTRK3* (E4:N14) fusion as a potential mechanism of *ALK*-independent resistance to lorlatinib. Subsequently, the patient was treated with the combination of brigatinib plus entrectinib and demonstrated a positive response, achieving an 8-month progression-free survival. Our case provides a potential treatment option for LUAD patients with *ALK* rearrangements and highlights the utility of next-generation sequencing (NGS) in uncovering genetic alterations that can guide the selection of effective treatment strategies.

KEYWORDS

ALK* rearrangement, brigatinib, entrectinib, resistance mutations, *ETV6-NTRK3

Introduction

Tyrosine kinase inhibitors (TKIs) targeting driver alterations have been an established modality in treating non-small cell lung cancer (NSCLC) (1). However, the duration of response to TKIs was often limited by acquired drug resistance (2). Resistance to treatment in anaplastic lymphoma kinase (*ALK*)-rearranged NSCLC involve both ALK-dependent

and ALK-independent mechanisms (3). ALK-dependent resistance typically arises from secondary mutations within the ALK tyrosine kinase domain. On the other hand, ALK-independent resistance is often due to the activation of bypass signaling pathways, highlighting the complexity and challenges in the management of resistance. Notably, oncogenic alterations in bypass signaling pathways, such as mutations in the epidermal growth factor receptor signaling pathway have been reported (3, 4). In addition, a rare genetic abnormality involving the neurotrophic tyrosine receptor kinase 3 (*NTRK3*) gene fusion has recently been identified in an *ALK*-rearranged NSCLC patient following lorlatinib treatment (5). It is noteworthy that genetic testing was not performed prior to the initiation of ALK inhibitor treatment in the case, so it is unclear whether the *NTRK3* fusion was primary or therapy-induced. In this case report, we delve into the clinical consequences of the *ETV6-NTRK3* (E4:N14) fusion as a resistance mechanism and evaluate the therapeutic potential of a combination treatment with brigatinib and entrectinib in a heavily pre-treated *ALK*-rearranged NSCLC patient.

Case presentation

A 56-year-old male former smoker (25 pack years) was diagnosed with lung adenocarcinoma (cT2N2M1c) with right pleural metastasis, effusion and left iliac bone metastasis in June 2019. His medical history was otherwise unremarkable, though it is noteworthy that his sister had been diagnosed with colorectal cancer. Genetic testing revealed the presence of an *EML4-ALK* (E20:A20) gene fusion. The patient was treated with frontline alectinib, achieving a best response of partial response (PR) (Figure 1A). In February 2021, computed tomography (CT) scans demonstrated the enlargement of pre-existing lesions, emergence of new lesions, and aggravated pleural effusion. According to the RECIST v1.1 criteria, these findings collectively indicate progressive disease (PD), with the patient achieving a progression-free survival (PFS) of 19 months (Figure 1A). After the initial progression, the patient underwent thoracentesis and began second-line treatment with ceritinib. Unfortunately, he experienced PD just two months later. Treatment was then switched to lorlatinib, which was associated with adverse effects, including persistent fever, aggravated pleural effusion, tumor growth, mediastinal lymph node enlargement, and pleural thickening. To manage these complications, the patients underwent thoracentesis for pleural empyema, pleural decortication, and cautery division of pleural adhesions. Then the patient was started on a combination therapy of lorlatinib with pemetrexed and carboplatin, which led to a best response of stable disease. However, the disease progressed again, with the detection of liver metastases in a CT scan in April 2022. Subsequently, the patient received six cycles of pemetrexed plus carboplatin and experienced PD five months later, as evidenced by new and enlarged liver metastases and new bone lesions on CT and bone scans. A pathological analysis of a liver biopsy verified the presence of metastatic adenocarcinoma (Figures 1B, C). The liver

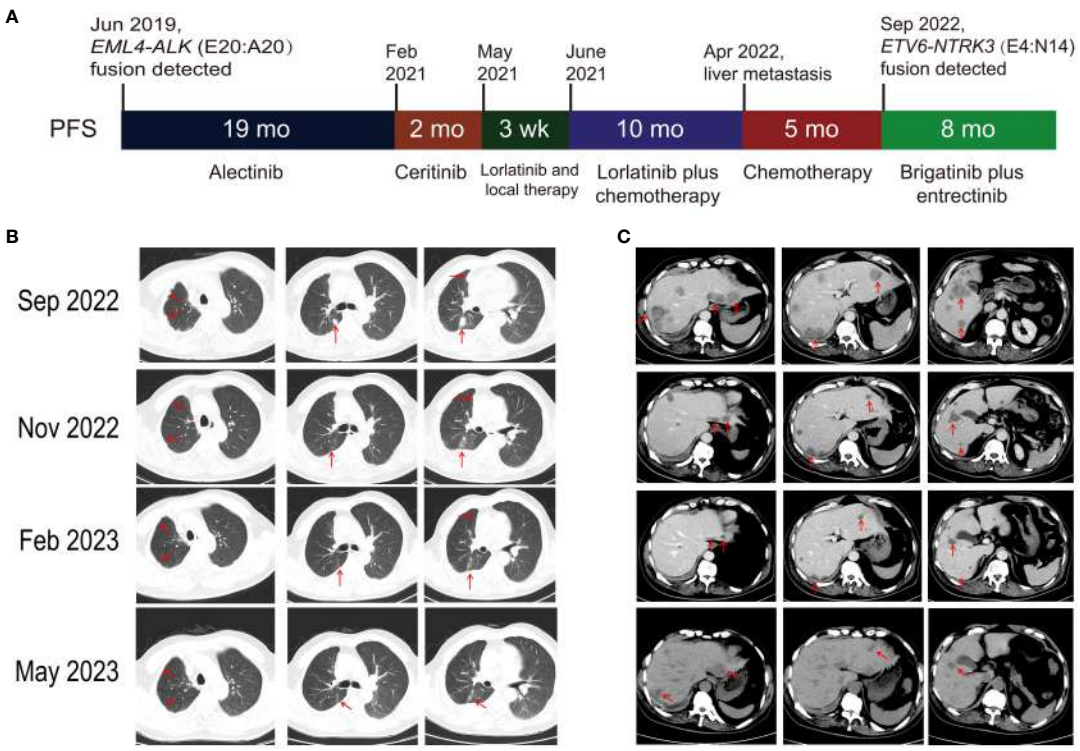

FIGURE 1
Patient's clinical progression and radiologic response to brigatinib plus entrectinib therapy. **(A)** Schematic overview of clinical progression. **(B)** Pulmonary and **(C)** liver computed tomography findings: pre-treatment (Sep 2022) and 2 months post-treatment (Nov 2022), 5 months post-treatment (Feb 2023), and 8 months post-treatment (May 2023) with brigatinib plus entrectinib. PFS, progression-free survival.

biopsy sample contains 50% normal liver tissue and 50% metastasized lung cancer tissue (Supplementary Figure S1). In the tumor tissue area, there is heavily filled with cancer cells, with only a small amount of connective tissue, and occasional lymphocytes and neutrophils are present. A smaller section of the sample displays an increase in fibrous tissue along with a higher count of lymphocytes, neutrophils, and a few eosinophils. The genetic profiles of the surgical samples and plasma were assessed using capture-based targeted deep sequencing, employing the GeneseeqPrime® panel with a sequencing depth of 3000X. This comprehensive panel analyzes the full exons, fusion-related introns, variable splicing regions, and specific microsatellite (MS) sites of 437 genes associated with cancer, spanning approximately 1.53 megabase pairs across the human genome (provided by Nanjing Geneseeq Technology Inc., China). This enhanced depth of sequencing facilitates a more accurate detection and characterization of genetic alterations, contributing to a deeper understanding of the cancer profile in each sample. A comparative analysis using the test results procured during diagnosis not only disclosed the persistence of the *EML4-ALK* (E20:A20) fusion that occurred with a mutation abundance of 35.38%, but also unveiled the emergence of the *ETV6-NTRK3* (E4:N14) fusion that appeared with a mutation abundance of 24.05%. In the following sections, we will provide a detailed discussion on the patient's response to various treatments, as well as any adverse events that might occur for each treatment. There were no mutations in *EGFR*, *KRAS*, *ROS1* and *MET*. The tumor mutation burden (TMB) was 4.1 mutations/Mb and microsatellite status were stable (MSS). The variant frequencies of these fusions were 23.4% in the tumor tissue and 24.1% in the plasma, respectively, as shown in Figure 2. This newly identified *ETV6-NTRK3* (E4:N14), alongside the persistent *EML4-ALK* (E20:A20), might play a significant role in the observed resistance mechanism. Based on these findings, the patient initiated a fifth-line treatment regimen with brigatinib (90 mg d1-7 qd po followed by 180 mg qd po). Two weeks into brigatinib therapy, entrectinib was added to the regimen (400 mg d1-7 qd po followed by 300 mg qd po). A follow-up CT evaluation conducted two months after initiating this combination therapy revealed significant improvement in both pulmonary and liver lesions, indicative of a PR (Figures 1B, C). As of the most recent follow-up in May 2023, the patient's disease has remained control, achieving a PFS of 8 months. Notably, genomic profiling on the plasma sample collected during the latest visit identified an *NTRK3* p.G623R mutation with a variant frequency 0.64%. This mutation may potentially be associated with resistance to entrectinib (6, 7), underscoring the

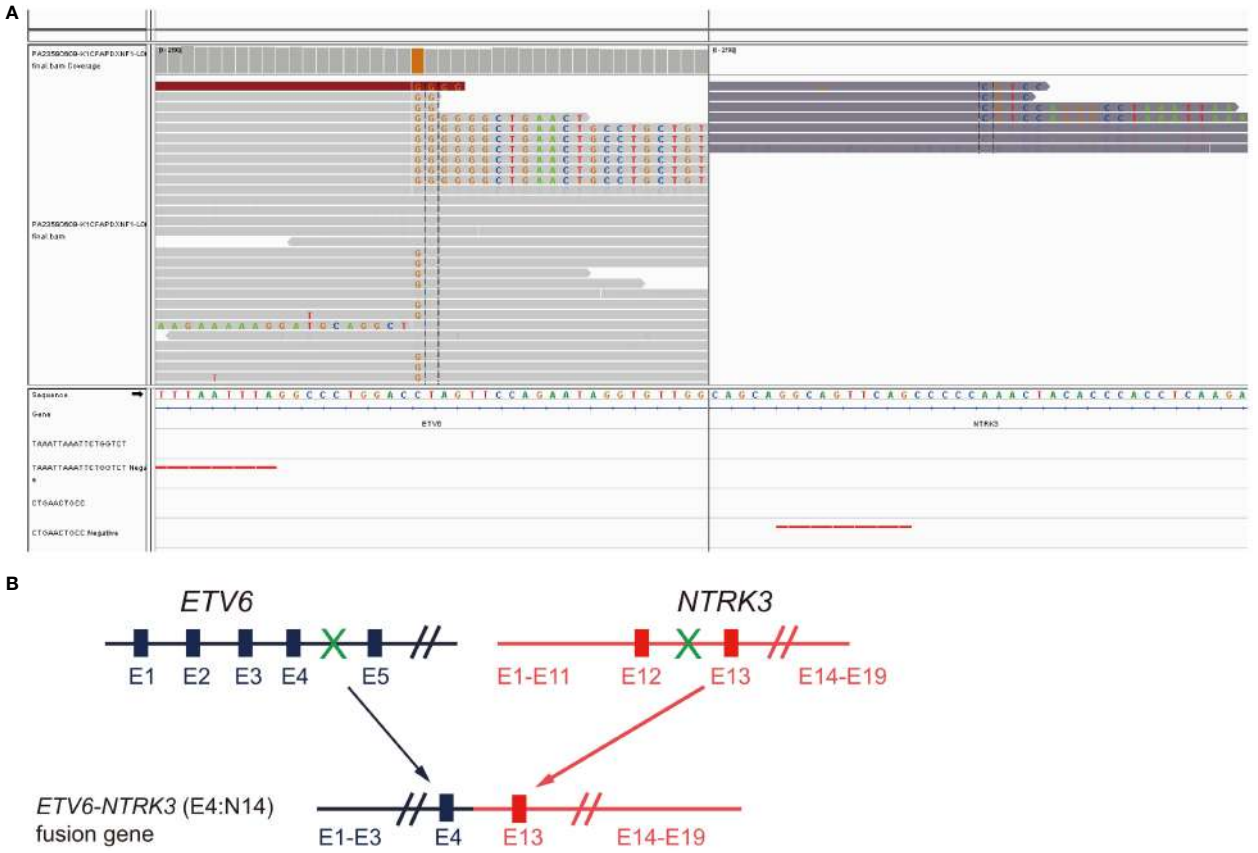

FIGURE 2
Detection of *ETV6-NTRK3* fusion following progression on fourth line brigatinib plus chemotherapy. **(A)** Integrative Genomics Viewer (IGV) snapshot of the fusion. **(B)** Diagram illustration of the chimeric protein generated.

importance of continuous genomic monitoring. The patient's disease progressed, but he refused chemotherapy. Unfortunately, he passed away from liver failure in August 2023.

Discussion

Long-term management of *ALK*-altered NSCLC poses significant challenges due to acquired resistance, necessitating the development of multiple generations of ALK TKIs to address ALK-dependent resistance mechanisms (8). However, overcoming ALK-independent resistance remains an ongoing challenge that requires further research and novel approaches. *NTRK3* is a member of the *NTRK* family of kinases, which are rare oncogenic driver genes in cancer, occurring at frequencies of 0.31% in adult tumors and 0.34% in pediatric tumors (9). In NSCLC, the most common partner of *NTRK3* fusion is *ETV6* (10). *NTRK* fusions are generally considered as mutually exclusive with *ALK* fusions, yet they have been identified as a resistance mechanism to EGFR TKI therapies (11). In the case report published by Garrido, *EML4-NTRK3* (E4:N14) fusion was detected after progression on lorlatinib. However, it remained inconclusive whether this fusion was acquired, as genetic testing had not been conducted prior to the initiation of ALK inhibitor treatment in that patient (5). Fortunately, in our case, genetic testing was performed both at baseline and upon progression on fourth-line treatment comprising lorlatinib, pemetrexed and carboplatin, which confirmed the *ETV6-NTRK* (E4:N14) fusion as a secondary event. However, the precise timing of the fusion's emergence and the potential for earlier intervention remains unknown. Both of these cases highlight the significance of genomic profiling in re-biopsies for uncovering novel resistance mechanisms, and thereby facilitating timely and appropriate adjustments to the management strategy of the disease.

The differential response to entrectinib observed in our case and those reported in previous studies presents an intriguing aspect. In the previous reported case, entrectinib monotherapy was administered after progression on lorlatinib, yet it failed to elicit a clinical response. On the contrary, in our case, after observing the ineffectiveness of alectinib, ceritinib, and lorlatinib, a combination therapy incorporating both brigatinib and entrectinib was selected, which resulted in a notable clinical improvement. Several factors could account for the disparate outcomes between these two instances, including the potential superior efficacy of brigatinib over entrectinib in inhibiting EML4-ALK, the presence of entrectinib-resistant *ALK* mutations not detected in genetic testing in the reported case, or yet unknown resistance mechanisms. More research is warranted to reveal the underlying mechanism that led to the different outcomes in these two cases.

A final point to note in our study is the detection of *NTRK3* p.G623R mutation in the plasma eight months after the initiation of brigatinib plus entrectinib. This mutation was initially reported in patients with *ETV6-NTRK3* (E4:N14) fusion-positive patients manifesting secondary resistance to NTRK inhibitors (6). *NTRK* p.G623R is a solvent-front mutation homologous to *ALK* p.G1202R and *ROS1* p.G2032R mutations, all of which confer resistance to entrectinib (12). Hanf et al. reported response to cabozantinib following acquired entrectinib resistance in an *ETV6-NTRK3* (E4:N14) fusion-positive patient harboring *NTRK3* p.G623R (6). The significance of *NTRK* p.G623R on our patient's clinical course and further treatment options awaits further follow-up.

Conclusion

In summary, we report a case of *ALK*-rearranged NSCLC in which acquired *ETV6-NTRK3* (E4:N14) fusion was detected, and the patient derived positive clinical outcome to a combination treatment approach incorporating brigatinib plus entrectinib. Our findings provide clinical evidence supporting the role of *NTRK3* fusions in mediating acquired resistance to ALK inhibitor therapy and highlight the efficacy of combination therapy with ALK and NTRK inhibitors as a promising treatment option.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

This research was approved by the Ethics Committee of The Second Hospital of Dalian Medical University. Written informed consent to publish the clinical details and images were obtained from the patient.

Author contributions

DL: Writing – review & editing, Data curation. YZ: Writing – review & editing, Methodology. JS: Writing – original draft, Resources. DY: Writing – original draft, Supervision. SC: Writing – review & editing, Investigation. XL: Writing – original draft, Visualization. LW: Writing – original draft, Project administration. JZ: Writing – original draft. EP: Writing – review & editing. ZD: Conceptualization, Writing – review & editing.

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Conflict of interest

Authors JZ and EP were employed by the company Nanjing Geneseeq Technology Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1339511/full#supplementary-material

SUPPLEMENTARY FIGURE 1
H&E staining of the liver biopsy sample (**A** with magnification x100, **B** with magnification x200). The liver biopsy sample contains 50% normal liver tissue and 50% metastasized lung cancer tissue.

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Case report: successful response to bevacizumab combined with erlotinib for a novel *FH* gene mutation hereditary leiomyoma and renal cell carcinoma

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FH-deficient Renal Cell Carcinoma (*FH*-deficient RCC) are inherited tumors caused by mutations in the fumarate hydratase (*FH*) gene, which plays a role in the tricarboxylic acid cycle. These mutations often result in aggressive forms of renal cell carcinoma (RCC) and other tumors. Here, we present a case of *FH*-deficient RCC in a 43-year-old woman with a history of uterine fibroids. She exhibited a new heterozygous mutation in exon six of the *FH* gene (c.799_803del, c.781_796del). The patient had multiple bone metastases and small subcutaneous nodules in various areas such as the shoulders, back, and buttocks. Biopsy of a subcutaneous nodule on the right side revealed positive expression of 2-succinate-cysteine (2SC), and *FH* staining indicated *FH* expression deletion. The patient underwent treatment with a combination of erlotinib and bevacizumab, which resulted in significant efficacy with moderate side effects. This treatment combination may be recommended as a standard regimen. This case underscores the importance of genetic testing in patients with advanced renal cancer to enhance diagnostic accuracy. Furthermore, it provides insights into potential treatment approaches for *FH*-deficient RCC.

KEYWORDS

hereditary leiomyoma and renal cell carcinoma, *FH* mutation, peripheral blood genetic testing, targeted therapy, bevacizumab combined with erlotinib

Introduction

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is a kind of hereditary disease caused by germline mutation of fumarate hydratase (*FH*) gene, which is manifested as renal malignant tumor of skin and uterine smooth muscle myoma. *FH*-deficient renal cell carcinoma (RCC) is associated with HLRCC syndrome, which is characterized by *FH* germline mutation or bi-allelic cell *FH* deletion without germline mutation. *FH* system mutation may also lead to renal cell carcinoma. And it has very similar biological functions to HLRCC caused by *FH* germline mutation (Lau et al., 2020).

FH-deficient RCC is aggressive, and patients may develop metastatic diseases. Therefore, when diagnosed with *FH*-deficient renal cell carcinoma, timely surgical treatment should be performed to prevent the occurrence of metastatic cancer (Ohe et al., 2018).

FH is an enzyme involved in the tricarboxylic acid cycle, facilitating the conversion of fumarate to L-malate. Heterozygous mutations in the *FH* gene can lead to *FH*-deficient RCC, predisposing individuals to aggressive forms of renal cell carcinoma and other tumors

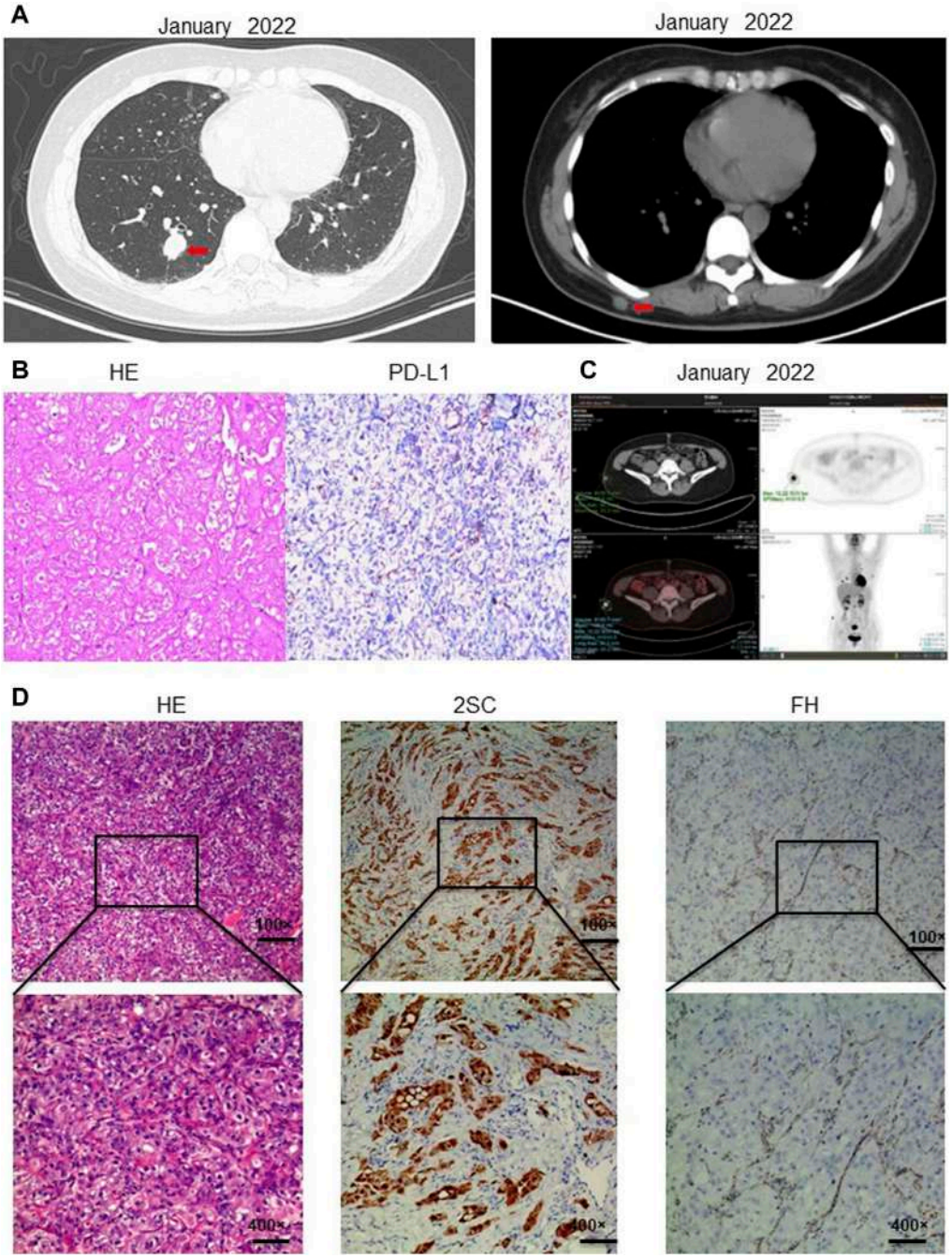

FIGURE 1
Diagnosis of hereditary smooth muscle tumor and renal cell carcinoma. **(A)** CT in January 2022 showed that the patient had subcutaneous nodules in both lungs and a subcutaneous nodule on the right back. **(B)** Immunohistochemistry showed PD-L1 expression TPS positive, TPS = 5%; PD-L1 expression CPS positive, CPS = 6. **(C)** PET-CT showed cystic lesions in the right kidney; unequal-sized nodules in both lungs; localized bone destruction and uneven density in the left scapula, part of the concha and adnexa, sacrum, and right ilium; bone metastasis was considered. **(D)** Immunohistochemistry showed that the metastatic tumor cells lacked the expression of 2SC; Hematoxylin-eosin staining showed that the metastatic tumor cells had large nuclei with obvious phagocytic nuclei and obvious halos around the nuclei; immunohistochemistry showed that the metastatic tumor cells lacked the expression of fumarate hydratase FH, which supported the diagnosis of renal cell carcinoma with FH deficiency. Information of antibodies for PD-L1, FH, 2SC is shown in Supplementary Table S2.

(Zyla and Hodgson, 2021). *FH*-deficient RCC typically carries a poor prognosis, with metastatic *FH*-deficient RCC often showing resistance to conventional therapies, necessitating exploration of novel treatment modalities.

The morphological diagnosis of *FH*-deficient RCC is difficult. Immunohistochemistry (IHC) is used to detect the deletion of *FH* expression in tumor cells to diagnose *FH*-deficient renal cell carcinoma, which has been proved to be closely related to the inactivation mutation

of the *FH* gene (Smith et al., 2016). In addition, the positive rate of 2SC in *FH*-deficient renal cell carcinoma was 100%, and the positive manifestations were strong positive in diffuse nucleus and cytoplasm, which could be used for auxiliary diagnosis of *FH*-deficient renal cell carcinoma (Muller et al., 2018).

In addition, in order to determine whether patients have metastatic carcinoma, the immunohistochemistry of PAX8, CD10 and Vimentin plays an important role in the diagnosis of metastatic renal cell carcinoma. Among them, CD10 (renal tubular epithelial enzyme) is a common marker of renal cell carcinoma, which can help determine the presence of renal cell carcinoma in immunohistochemical staining (Sangoi et al., 2010). Vimentin is an intermediate filament protein that is associated with metastasis of renal cell carcinoma (Yao et al., 2020).

Here, we present a case of renal cell carcinoma deficient in *FH* in a 43-year-old woman, who harbored a novel heterozygous variant in the sixth exon of the *FH* gene (c.799_803del, c.781_796del). Treatment with a combination of erlotinib and bevacizumab resulted in remarkable efficacy. The successful outcome of this case offers promising insights into HLRCC treatment strategies. Given the limited effective systemic treatments available for *FH*-associated RCC, further investigation into the combination of bevacizumab and erlotinib in a larger patient cohort is warranted.

Case report

The patient, a 43-year-old middle-aged woman with a history of uterine fibroids, noticed a subcutaneous nodule on her back in February 2022, measuring approximately 2 cm × 2 cm. The nodule felt hard and was non-tender upon palpation. Computed tomography (CT) scans revealed small subcutaneous nodules on her right back and right buttock, along with variable-sized nodules in both lungs, suggestive of metastasis (Figure 1A). An excisional biopsy of the subcutaneous nodule on her right back was conducted, and immunohistochemistry results indicated characteristics consistent with metastatic cancer: Ckpan (+), Villin (−), CK20(−), CK7(−), P40 (−), Vim (−), Ki67(45%+), S100 (−), SOX10 (−), HMB45(−), TFE3 (−), DES (−), PLAP (−), SALL4 (−), CD10 (focal+), PAX8 (2+), AR (−). Subsequently, another excisional biopsy was performed on a subcutaneous nodule on her right posterior dorsal region. Immunohistochemistry revealed high expression of programmed cell death ligand 1 (PD-L1), with a TPS of 5% and a CPS of 6 (Figure 1B). Further evaluation with positron emission tomography (PET)-CT showed a cystic lesion in the right kidney with a thick capsule wall and increased fluorodeoxyglucose (FDG) uptake, consistent with renal carcinoma, with a possibility of cystic adenocarcinoma. Additionally, nodules of unequal sizes in both lungs displayed increased FDG uptake, indicative of metastasis. Localized bone destruction, uneven density, and increased FDG uptake were observed in the left scapula, part of the concha and adnexa, sacrum, and right ilium, suggesting bone metastasis (Figure 1C). Based on the collective imaging and pathological findings, the patient received a final diagnosis of high-grade renal cell carcinoma.

In February 2023, a resection biopsy of the patient's right dorsal subcutaneous nodule was conducted due to the persistence of severe subcutaneous nodules 1 year post-treatment. HE staining revealed enlarged nuclei with prominent eosinophilic nucleoli and a clear halo around the nucleolus (Figure 1D). Immunohistochemistry for 2SC demonstrated positive staining (Figure 1D), while staining for *FH* showed loss of *FH* expression (Figure 1D). Following the patient's informed consent, whole exon sequencing was performed on the patient's tissue, revealing suspicious pathogenic mutations that could account for the patient's phenotype. Sequencing results identified heterozygous mutations in the *FH* gene (NM_000143: c.799_803del, p. P267fs; NM_000143: c.781_796del, p. R261fs) (Supplementary Table S1). Integrating the genetic testing and pathological findings, the patient was diagnosed with *FH* genotype-deficient renal cell carcinoma. Given the hereditary nature of *FH* gene-deficient renal cell carcinoma, whole exon gene sequencing was conducted on peripheral blood samples from the patient's mother and two daughters. Results indicated that the patient's mother and one daughter harbored the same mutations at the identical sites within the *FH* gene (Figure 2A). Wild-type Sanger sequencing is depicted in Figure 2B. The patient's family pedigree is illustrated in Figure 2C.

In February 2022, following a diagnosis of high-grade renal cell carcinoma, the patient commenced immediate treatment with two cycles of pembrolizumab combined with sunitinib (Pembrolizumab 200mg, every 3 weeks; sunitinib 50 mg, once daily for 2 weeks, with a 1-week break). Subsequent CT scans in April 2022 revealed significant reductions in the size of subcutaneous nodules and pulmonary metastases on the right back compared to previous scans (Figure 3A). However, due to intolerance to sunitinib, the treatment was modified to pembrolizumab combined with axitinib for 2 weeks (Pembrolizumab 200 mg, every 3 weeks; Axitinib 5mg, twice daily). In May 2022, CT scans indicated an increase in multiple small nodules in various subcutaneous areas and an increase in metastatic tumors in both lungs compared to April 2022 (Figure 3B). Subsequently, the patient underwent treatment with anlotinib in combination with pembrolizumab for eight cycles (Pembrolizumab 200 mg, every 3 weeks; Anlotinib 12 mg, once daily for 2 weeks, with a 1-week break). PET-CT results in October 2022 demonstrated significant progression of bone metastases throughout the body compared to May 2022 (Supplementary Figure S1A). In December 2022, the patient received treatment with pembrolizumab alongside oral ST1898 targeted therapy. However, a CT scan in January 2023 revealed significant enlargement of bilateral lung metastases compared to October 2022 (Figure 3C). Subsequently, in February 2023, following the diagnosis of *FH*-deficient renal cell carcinoma, the patient's treatment regimen was adjusted. Treatment with pembrolizumab, erlotinib, and bevacizumab was initiated, although immunization was temporarily suspended due to significantly increased pituitary prolactin levels. In March 2023, the patient underwent eight cycles of treatment with bevacizumab and erlotinib. A CT reexamination in June 2023 showed a significant reduction in metastatic lesions, with the patient's condition stabilized (Figure 3D). The timeline of the case is illustrated in Figure 4, with the top axis depicting the diagnostic process and the bottom axis showing the treatment process. Consent for publication of this case report was obtained from the patient.

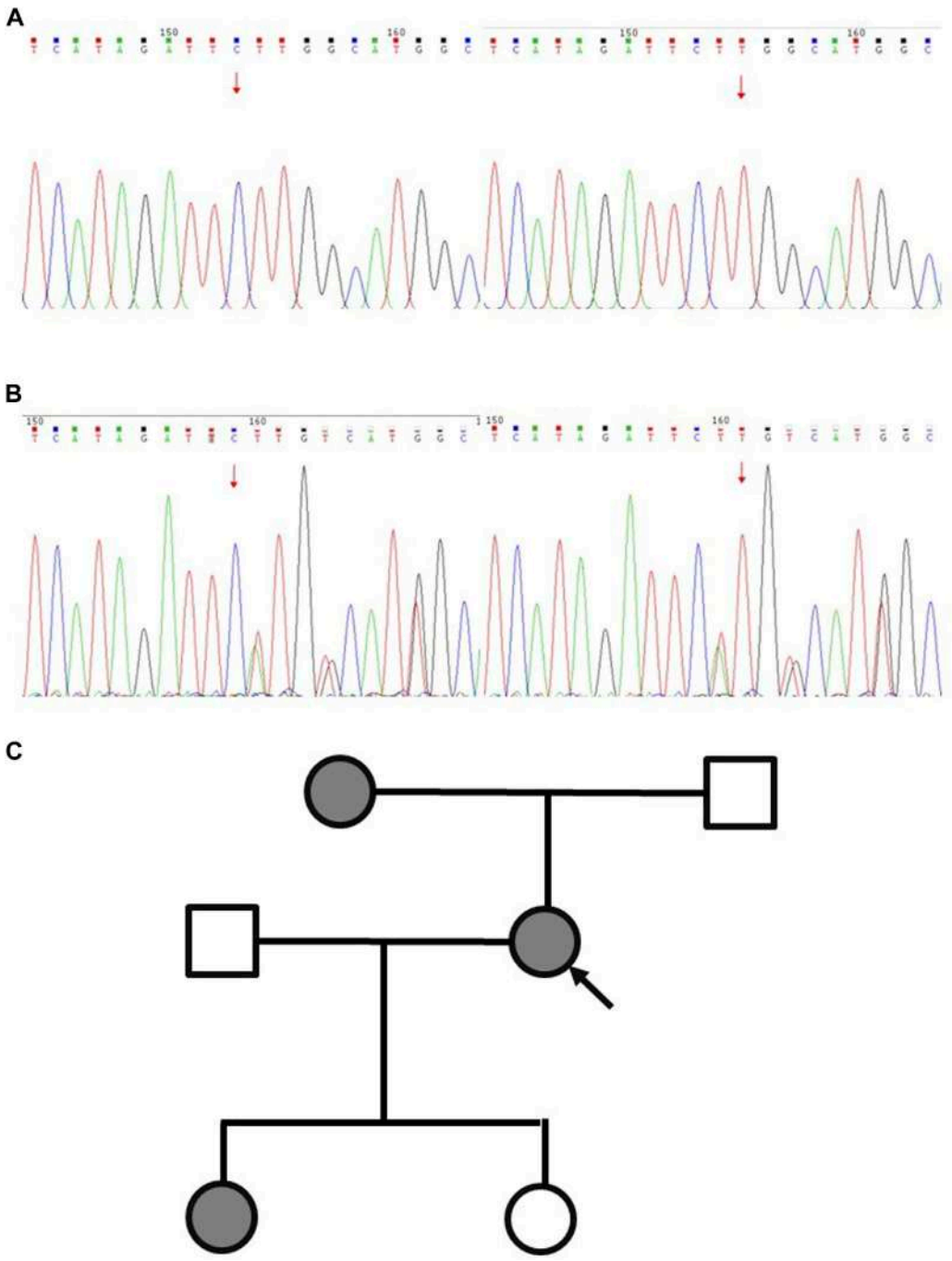

FIGURE 2
Schematic diagram of Sanger sequencing validation results for the proband and wild-type FH variants. **(A)** Wild type. **(B)** The proband. **(C)** Pedigree of the family with three patients. The black symbols represent the affected members with renal carcinoma, and the arrow indicates the proband.

Discussion

HLRCC is an autosomal dominant genetic disorder linked to inactivating mutations in the *FH* gene. Typically, individuals with HLRCC exhibit a genetic predisposition to skin and uterine leiomyomas, as well as kidney tumors (Linehan and Ricketts, 2019). The *FH* gene mutation leads to dysfunction or structural abnormalities in the *FH* protein, which plays a crucial role in catalyzing the conversion of fumarate to malate within the tricarboxylic acid (TCA) cycle—a fundamental process in cellular energy metabolism. Disruption of this enzymatic activity due to the *FH* mutation results in fumarate accumulation and decreased malate levels within cells. This perturbation in the TCA cycle adversely affects cellular energy metabolism and ATP production. Moreover, the *FH* mutation may induce excessive free radical generation, thereby promoting cellular oxidative stress, apoptosis, and potentially contributing to tumor development (Valcarcel-Jimenez and Frezza, 2023). *FH* serves as a pivotal metabolic

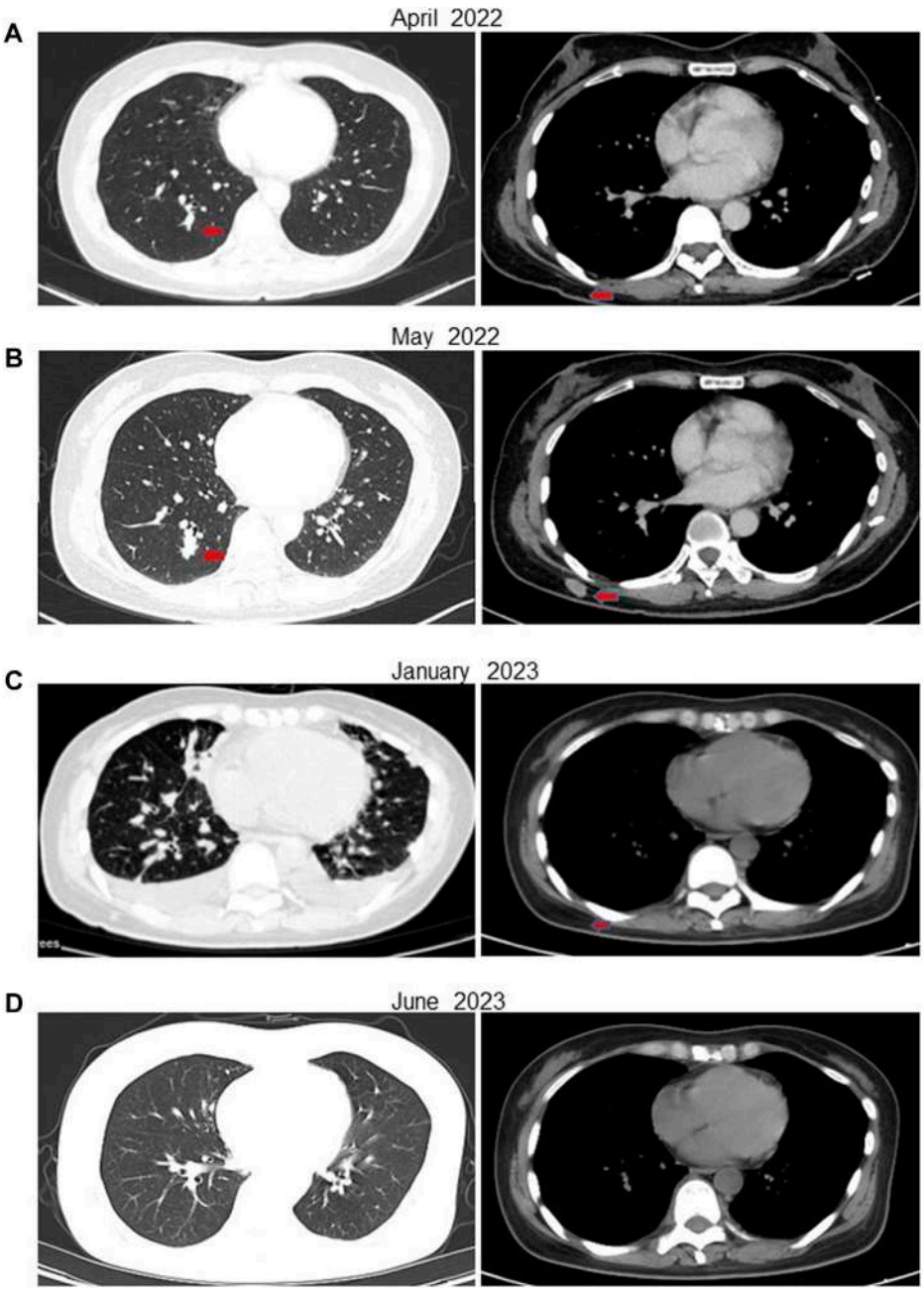

FIGURE 3
CT of the patient at various stages after receiving treatment. **(A)** In April 2022, after 6 weeks of treatment with pembrolizumab in combination with sunitinib, the patient's metastases were significantly reduced. **(B)** In May 2022, after 5 weeks of treatment with pembrolizumab in combination with axitinib due to the patient's intolerance of sunitinib, the subcutaneous nodule on the right side of the back was enlarged compared with the previous one. **(C)** After eight cycles of the original regimen, a CT in January 2023 showed multiple metastases in both lungs that were significantly more advanced than before. **(D)** ACT in July 2023 showed a significant reduction in the patient's metastatic lesions.

enzyme in the TCA cycle, and its deficiency leads to intracellular fumarate accumulation. Fumarate buildup within mitochondria and subsequent leakage into the cytoplasm, termed "tumor metabolites," is associated with the development of skin leiomyomas, uterine fibroids, and kidney cancer (Lindner et al., 2022). HLRCC represents a subtype of RCC characterized by notable invasiveness, predominantly affecting young individuals and often accompanied by early metastasis (Yu et al., 2021). Concurrently, intracellular fumarate accumulation can induce a stable chemical modification of intracellular proteins known as abnormal succinylation. The presence of modified proteins can be detected using 2SC antibodies. While immunohistochemical detection of *FH* protein remains crucial for diagnosing HLRCC, some HLRCC tumor cells may still express *FH* protein. Therefore, combined detection of *FH* and 2SC can enhance the diagnostic accuracy of HLRCC (Zheng et al., 2023).

A recent study documented a case of HLRCC in which a patient remained free of tumor recurrence or metastasis for 24 months

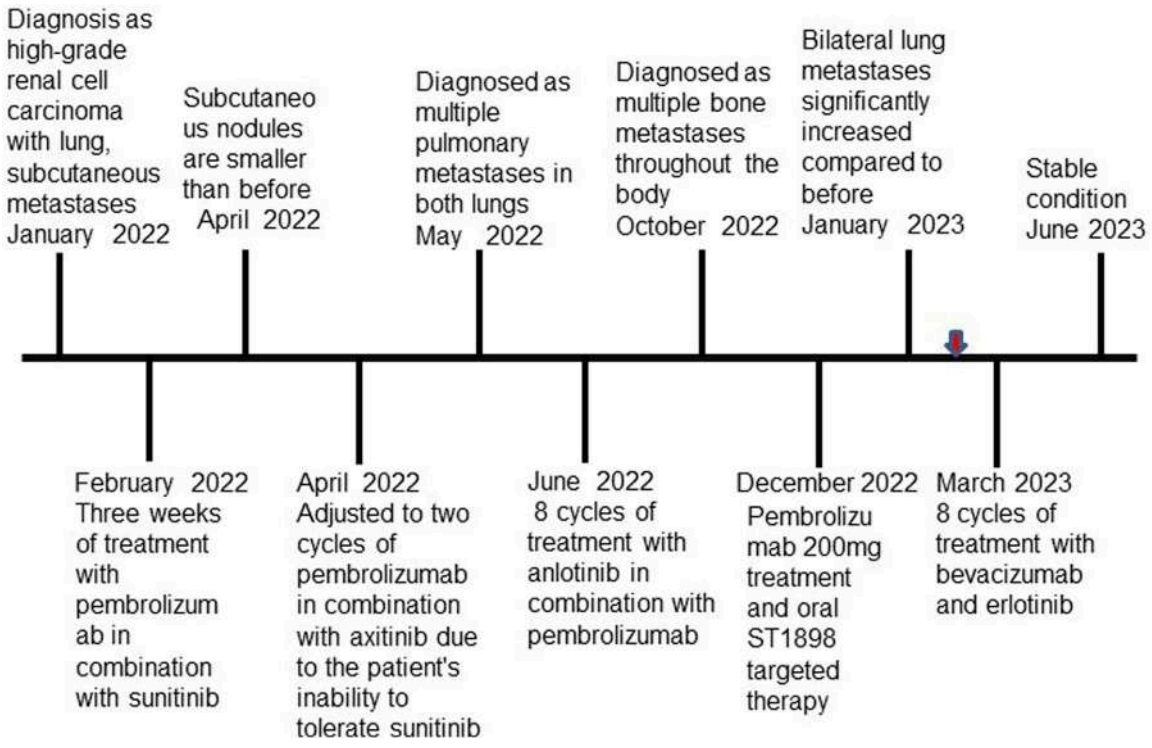

FIGURE 4
Timeline of the case. Red arrow indicates that the patient was diagnosed with FH-deficient renal cell carcinoma.

following treatment with a PD-1 inhibitor, Pembrolizumab (Wang et al., 2021). PD-1 inhibitors have emerged as the preferred therapeutic option for many cases of RCC (McDermott et al., 2018; Aggen et al., 2020; Brown et al., 2020). PD-1 is expressed on B cells, T cells, and regulatory T cells, and its expression is indicative of T-cell exhaustion. PD-L1, found to be upregulated in both hemangiomas and solid tumors, acts as a checkpoint molecule that inhibits the host's anti-tumor immunity (Jiang et al., 2019). Consequently, inhibitors targeting PD-1 and PD-L1 have been employed in tumor treatment (Shi et al., 2011). Research findings suggest that PD-L1 expression is prevalent in the majority of HLRCC cases, rendering immunotherapy a promising therapeutic avenue for HLRCC (Sun et al., 2021). Moreover, elevated expression of PD-L1 has been observed in the subcutaneous metastases of patients discussed in our reported case. Therefore, it is imperative to assess the immune microenvironment, including PD-L1 expression and $CD8^+$ T cells, in HLRCC. Such evaluations can provide valuable insights to guide the development of more precise clinical treatment strategies.

In addition, we evaluated the pathogenicity of *FH* gene defects in patients, including the following aspects: Gene mutation analysis; through sequencing and analysis of the *FH* gene, deletion mutations with the *FH* gene (c.799 _ 803del, c.781 _ 796del) can be detected. Determination of enzyme activity; the expression of *FH* gene was determined by immunohistochemistry. The patient's immune results showed that the expression of *FH* gene was missing. Based on the above evaluation results, the pathogenicity of *FH* gene defects can be determined, and corresponding diagnosis and treatment suggestions can be provided for patients.

FH-RCC is relatively rare, posing challenges in standardized diagnosis due to the lack of data from multicenter clinical trials with large sample sizes. Real-world treatment outcomes exhibit considerable heterogeneity, and there is a lack of uniform standardized treatment protocols. In this context, we present a case of an HLRCC-RCC patient with a history of uterine fibroids, wherein gene testing revealed a heterozygous mutation in the *FH* gene. The patient underwent treatment with a combination of bevacizumab and erlotinib, resulting in symptom relief. The combination of bevacizumab and erlotinib is a strategy to enhance the anti-tumor effect of drugs based on two different mechanisms. Bevacizumab is an anti-vascular endothelial growth factor (VEGF) monoclonal antibody, which can selectively bind to human vascular growth factor (VEGF) and block its biological activity. It can inhibit the binding of VEGF to its receptors VEGFR-1 and VEGFR-2 located on endothelial cells, so that VEGF loses its biological activity and reduces tumor angiogenesis, thus inhibiting tumor growth (Garcia et al., 2020). Erlotinib is a targeted therapy drug, which belongs to the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) class, by inhibiting the activity of EGFR, thereby preventing the growth and spread of tumor cells. EGFR is a protein expressed on the surface of tumor cells, which can promote the growth and survival of tumor cells. Erlotinib can bind to EGFR and block its activity, thereby inhibiting the growth and spread of tumor cells (Grépin et al., 2020). The combination of bevacizumab and erlotinib, abbreviated as the E-B regimen, has shown efficacy in treating *FH*-deficient RCC (Carril-Ajuria et al., 2021). The main purpose of the combination of these two drugs is to enhance the anti-tumor effect through two

different mechanisms. This combined effect can theoretically improve the therapeutic effect and is expected to reduce the development of drug resistance. The results of first-line treatment showed that the objective remission rate of *FH*-deficient RCC patients treated with E-B regimen was 50%, the median progression-free survival was 13.3 months, and the disease control rate was 90% (Zhou et al., 2021). The successful outcome of this case may offer novel insights into the treatment of *FH*-deficient RCC, suggesting the potential utility of the E-B regimen in managing this condition.

Conclusion

In this case report, the patient's diagnosis of *FH*-deficient RCC was delayed due to the lack of prompt genetic testing. *FH*-deficient RCC involves a mutation in the *FH* gene, and genetic testing holds significant importance for its treatment. The patient exhibited a novel heterozygous mutation (c.799_803del, c.781_796del) in the sixth exon of the *FH* gene. Following treatment with a combination of bevacizumab and erlotinib, metastases decreased or disappeared, leading to disease stabilization. This underscores the necessity of genetic testing for patients and their relatives with advanced RCC, aiding in the early detection of *FH*-deficient RCC and facilitating appropriate treatment. The treatment approach employed in this case offers insights for managing *FH*-deficient RCC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

MH: Writing–review and editing, Project administration, Methodology, Investigation. YC: Writing–review and editing, Resources, Methodology, Funding acquisition, Formal Analysis, Conceptualization. XB: Writing–original draft, Visualization, Investigation, Data curation. DX: Writing–original draft, Visualization, Project administration, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1373020/full#supplementary-material

SUPPLEMENTARY TABLE S1
Gene mutation information of the patient.

SUPPLEMENTARY TABLE S2
Information of antibodies for PD-L1, FH, 2SC.

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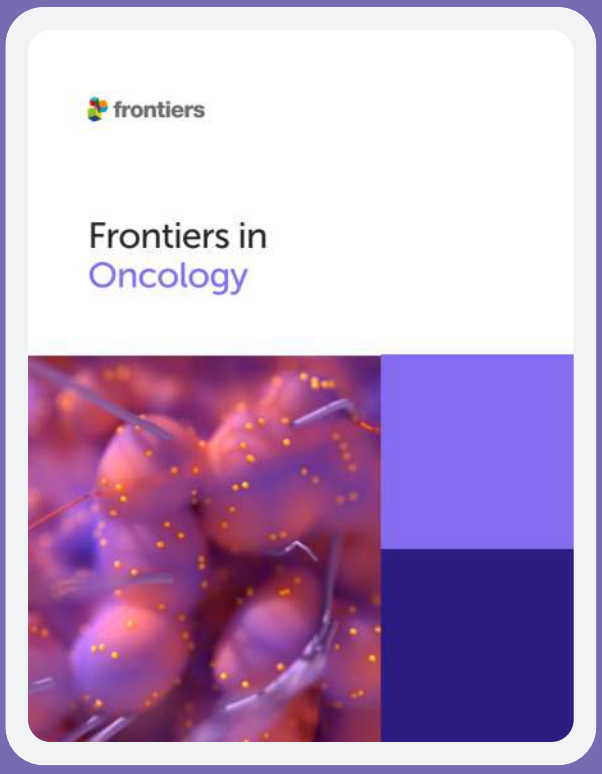

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