Different genomic clusters impact on responses in advanced biliary tract cancer treated with cisplatin plus gemcitabine plus durvalumab.
An exploratory multicenter analysis

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ABSTRACT

BACKGROUND:

The results reported in the TOPAZ-1 phase 3 trial led to the approval of the combination of cisplatin and gemcitabine with durvalumab as the new first-line standard of care.

OBJECTIVE:

We performed a clustering analysis to classify patients into different groups based on their mutation profile, correlating the results of the analysis with clinical outcomes.

PATIENTS AND METHODS:

We selected 51 patients with cholangiocarcinoma who were treated with the combination of chemotherapy and durvalumab and were screened by the NGS-based FoundationOne gene panel. We conducted a mutation-based clustering of tumors and survival analysis.

RESULTS:

Three main clusters were identified. Cluster 1 is mostly characterized by mutations in genes belonging to the chromatin modification pathway, altered in 100% of patients. Cluster 2 is characterized by the alteration of several pathways, among which DNA damage control, chromatin modification, RTK/RAS, cell cycle apoptosis, TP53, and PI3K resulted the most affected. Finally, most altered pathways in cluster 3 were RTK/RAS and cell cycle apoptosis. Overall response rate (ORR)

was 4/13 (31%), 12/24 (50%) and 0/10 (0%) in cluster 1, cluster 2 and cluster 3, respectively and the difference between the three clusters was statistically significant (p=0.0188).

CONCLUSION:

By grouping patients into three clusters with distinct molecular and genomic alterations, our analysis showed that patients included in cluster 2 had higher ORR, whereas patients included in cluster 3 had no objective response. Further investigations on larger and external cohorts are needed in order to validate our results.

KEY POINTS:

- Three main clusters were identified from a clustering analysis conducted on a cohort of advanced CCA who received cisplatin/gemcitabine plus durvalumab.
- Patients included in cluster 2 had higher ORR, whereas patients included in cluster 3 had no objective response.
- Further investigations on larger and external cohorts are needed in order to validate our results.

1. INTRODUCTION

Biliary tract cancers (BTCs) are a heterogeneous group of malignancies with dismal prognosis and poor therapeutic options (1-4). For early stage, surgery followed by chemotherapy is the only curative option, but the proportion of recurrences after radical treatment remains high (5). For locally advanced and metastatic stages, platinum-based chemotherapy constituted the standard of care since 2010, when the randomized ABC-02 phase 3 trial reported a survival benefit with the addition of cisplatin to gemcitabine compared to gemcitabine alone. Nevertheless, survival rates reported for the chemotherapy combination were about 2% at 5 years for patients with metastatic disease (6,7). Recently, the development of new high throughput molecular analysis techniques led to the highlight of the genomic heterogeneity of BTC, and several potential molecular targets with therapeutic implications have been investigated (1, 8-12). Moreover, recently there has been the advent of immunotherapy for these patients: the phase 3 TOPAZ-1 trial reported a survival benefit

in terms of both overall survival (OS) and progression free survival (PFS) for patients with locally advanced or metastatic BTC who received the anti-programmed cell death ligand 1 (anti-PD-L1) durvalumab in addition to the standard chemotherapy combination (13). More precisely, the combination of cisplatin/gemcitabine and durvalumab achieved a median OS of 12.8 months compared to 11.5 months for chemotherapy alone, with a significant reduction in the risk of death of 20% in favor of experimental arm (13). These results led to the approval of the combination with durvalumab as the new first-line standard of care by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA). Another chemo-immunotherapy combination has been recently proposed for patients with advanced BTC: the phase 3 KEYNOTE-966 trial demonstrated improved survival outcomes with the addition of the anti-programmed cell death 1 (anti-PD1) pembrolizumab to the standard chemotherapy backbone cisplatin/gemcitabine (15), thus confirming the benefit of chemotherapy combined with immunotherapy in this setting. Despite the recent progress in BTC field, no biomarkers able to identify which patients could benefit most from immunotherapy have been identified, and only scarce data about the clinical impact of genomic features in this setting are available. Of note, the TOPAZ-1 trial reported no significant interaction between PD-L1 status and treatment efficacy, since the clinical benefit of the addition of durvalumab to chemotherapy was not significantly different in PD-L1 positive and negative patients. . The impact of molecular features on efficacy outcomes in patients enrolled in the same trial has been recently presented: efficacy expressed in terms of OS, overall response rate (ORR) and PFS with the addition of durvalumab to standard chemotherapy has been reported consistently in all patients regardless of the mutational profile, including patients with actionable alterations (14). A deeper knowledge on the molecular profile of these patients ad on the therapeutic implications could lead to the identification of those patients who are more likely to respond to a treatment rather that another one. Moreover, the identification of those patients who could be more responsive to immunotherapy could open the way to further investigations focused on other clinical settings, including the neoadjuvant and adjuvant setting. In order to gain insight into the molecular heterogeneity of BTC and the therapeutic impact of genomic profiling, we performed a clustering analysis to classify patients into different groups based on their mutation profile. In details, we analyzed a cohort of patients with locally advanced or metastatic BTC who received durvalumab plus cisplatin/gemcitabine as first-line treatment in a real-world setting, correlating the results of the analysis with clinical outcomes.

2. MATERIAL AND METHODS

2.1. Patients' enrollment and sample collection

The overall population included patients with unresectable, locally advanced or metastatic BTC, including intra- and extra-hepatic cholangiocarcinoma (CCA) and gallbladder carcinoma. Data were prospectively collected from 11 Italian institutions. Formalin-fixed paraffin-embedded (FFPE) samples and hematoxylin-eosin staining slides of the patients included were collected from the Pathology Department of each single institution. A genomic analysis of primary tumors was performed by the FOUNDATION Cdx technology (FoundationOne assay).

Patients included in the cohort received gemcitabine (1000mg/m2) plus cisplatin (25mg/m2) intravenously on days 1 and 8 of 21-day cycle for up to eight cycles. Durvalumab (1500 mg) was administered on day 1 of each cycle, in combination with chemotherapy. After completion of up to eight cycles, durvalumab monotherapy was administered once every 4 weeks until clinical or imaging disease progression or unacceptable toxicity. Since durvalumab is not yet reimbursed by the Italian Medicines Agency (AIFA), it was provided free of charge at the request of physicians for each individual patients by AstraZeneca Italy as early access program. AstraZeneca Italy had no role in planning this study, collecting, or analyzing patient data.

The present study was approved by local Ethics Committee at each center, complied with the provisions of the Good Clinical Practice guidelines and the Declaration of Helsinki and local laws, and fulfilled the Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the processing of personal data.

2.2. Clinical Data

Clinical data including patients' age, gender, and Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) were retrospectively collected at baseline. Pathological data, including primary tumor location, histological grading and TNM stage according to the 8th edition 2017 AJCC staging system were collected at baseline. Response to treatment was assessed using RECIST 1.1 criteria. For each patient, follow up and oncology assessment were planned as per standard of practice, according to guidelines and institutional protocols.

2.3. Identification of Genomic Alterations

FFPE tumor tissues containing at least 20% of tumor cells were collected at each center and sent for genomic analysis by the NGS-based FoundationOne assay (FoundationOne®, Foundation Medicine Inc., MA, USA) gene panel. Identified alterations included insertions/deletions (indel, 1-40 bp), base substitutions, copy number alterations-amplifications (ploidy<4, amplification with copy number ≥8), copy number alterations-deletions (ploidy<4, homozygous deletions) and fusion/rearrangements in 324 genes. The variants of uncertain significance (VUS) were included in the analysis. In addition, microsatellite status (determined by assessing indel characteristics at 114 homopolymer repeat loci in or near the targeted gene regions of the FoundationOne test) was assessed.

A descriptive analysis of the molecular landscape in the entire sample was performed.

2.4. Clustering Analysis

Genomic data were collected into electronic data files by each participating center and centrally reviewed at the coordinating center (IRCCS San Raffaele Hospital) in order to perform a clustering analysis. For each patient, the mutational status of 324 genes screened in the FoundationOne assay was annotated.

Genes showing mutations in more than 5% of patients were selected for the clustering analysis resulting in 37 genes.

Mutation-based clustering analysis was performed with ccpw Model from Zhang et al (16), using a manually curated cancer pathway list (mcp list), including 511 putative cancer genes belonging to 16 pathways, as described in our previous work (9). In brief, binary values indicate the mutational status of cancer driver genes and are used as features in the in the Ward's hierarchical clustering process. The mcp list includes the following pathways: Cell cycle and apoptosis (32 genes), chromatin modification (31 genes), DNA damage control (31 genes), HH (6 genes), HIPPO (38 genes), MAPK (8 genes), MYC (13 genes), NOTCH (73 genes), NRF2 (3 genes), PI3K (57 genes), RTK RAS (99 genes), STAT (11 genes), TGF-b (14 genes), TP53 (6 genes), transcriptional regulation (13 genes), and WNT (76 genes). It should be noted that the same genes could belong to multiple pathways.

2.5. Statistical Analysis

Categorical variables were presented as totals and frequencies and evaluated by Chi-squared test or Fisher exact test, as appropriate. Genomic alterations observed in ≥5% of the entire cohort of patients were considered for the analysis of distribution of genomic alterations. A survival analysis according to the identified clusters was performed. In the survival analysis, PFS and OS were considered. PFS was measured from the date of the start of first-line therapy to the date of disease progression, death, or last follow-up. OS was measured from the date of the first-line therapy start and the date of death or last follow-up. The last follow-up was not counted as an event, but patients were censored at that point.

PFS and OS from first-line therapy were calculated by Kaplan-Meier method and assessed by log-rank test for univariate analysis. The results were recorded as hazard ratios (HR) and 95% confidence intervals (CIs). A two-tailed P value less than 0.05 was considered statistically significant. For the response rate analysis, both ORR and disease control rate (DCR) were considered. ORR was defined as the proportion of patients who achieved a complete response (CR) or partial response (PR). DCR was defined as the proportion of patients who achieved a CR or PR or a stable disease (SD).

A MedCalc package (MedCalc® version 16.8.4) was used for statistical analysis.

3. RESULTS

3.1. Clinical Characteristics

Overall, the cohort of patients included 138 patients with advanced BTC who received treatment from January 2017 to November 2022 ; 51 of them were studied with a 324 gene NGS panel (FoundationOne), thus being including in the survival and clustering analysis. Median age at diagnosis was 62 years old (range: 36-80). Overall, 74.5% of the sample was affected by an intrahepatic CCA, whereas less than 20% presented an extrahepatic CCA and 6% a gallbladder carcinoma. 35% of the patients received previous surgery, and 21.5% of patients were treated with adjuvant chemotherapy. Most of the cohort (96%) had metastatic disease at baseline, with only 2 patients with locally advanced disease. 57% of the entire population presented an ECOG PS 0-1, and 55% was classified as normal-weight. A significant proportion of patients (65%) had high blood levels of CA 19-9 at baseline, whereas approximately 50% presented neutrophil-lymphocyte ratio >3. Baseline characteristics are reported in table 1.

3.2. Selection of Genomic Alterations

First, we performed a descriptive molecular analysis and selected the genomic alterations observed in at least 5% of patients. Overall, the 324-gene NGS panel allowed to identify 183 genomic alterations observed in at least 5% of the entire cohort. These genomic alterations involved 37 genes, with a median of 5.0 genomic alterations per gene (range 3-15) and a median of genomic alterations for patients of 3.6. The most common genomic alterations were found in *ARID1A*, (29%) *CDKN2A/2B* (21.5%), *MLL2* (17.5%), *BRCA2* (15.5%), *PBRM1* (15.5%), *NRAS/KRAS* (15.5%), *BAP1* (15.5%), *TP53* (14%), *IDH1* (12%), *MTAP* (12%), *MDM2* (10%), *ATM* (10%), *MSH3* (10%), *SMAD4* (10%) and *PIK3C2B* (10%) (Figure 1). The entire list of genomic alterations observed in ≥5% of patients is reported in table 2.

3.3. Clustering analysis

Overall, 30 out of the 37 genes with a mutation frequency ≥5% were present in the mcp list and therefore included in the clustering analysis (table 2). Figure 2A shows two main clusters: one including 27 patients (cluster 2) and the second including 24 patients, further subdivided into one cluster of 13 patients (cluster 1) and one of 11 patients (cluster 3). The three main clusters are characterized by alterations in genes belonging to different pathways (Figure 2B, C, D). Patients included in the three different clusters had similar baseline characteristics, except for baseline blood levels of CA 19-9, since patients in cluster 3 showed less patients with elevated CA 10-9 levels compared to those included in cluster 1 and 2 (18% Vs 59% Vs 62%, respectively, p=0.0395) (Table 1).

By comparing the number of mutated genes in the three clusters, cluster 2 had the highest number of mutated genes (36/37 analyzed genes) while patients belonging to cluster 1 and cluster 3 showed genomic alterations in a similar number of genes (15/37 and 13/37 analyzed genes, respectively).

Cluster 1 is mostly characterized by mutations in genes belonging to the chromatin modification pathway, altered in 100% of patients (Figure 2B). Cluster 2 is characterized by the alteration of several pathways, among which DNA damage control (81%), chromatin modification (78%), RTK/RAS (63%), cell cycle apoptosis (59%), TP53 (48%), and PI3K (30%) resulted the most affected (Figure 2C). Finally, most altered pathways in cluster 3 were RTK/RAS (36%) and cell cycle apoptosis (27%) (Figure 2D).

By considering the single pathways, several differences were found in terms of altered genes and frequencies in cluster 1, cluster 2, and cluster 3.

Eight genes belonging to the chromatin modification pathway were found to be altered in our sample (*ARID1A*, *PBRM1*, *MLL2*, *IDH1*, *IDH2*, *SETD2*, *TET2* and *ASXL1*). This pathway was altered in both cluster 1 and cluster 2 (Fig. S1A). Overall, 7/8 genes belonging to this pathway were mutated in cluster 1 and cluster 2, in particular *IDH2* was mutated only in cluster 1, while *SET2D* was mutated only in cluster 2. Mutations in *ARID1A* (6/13, 46% of patients), *IDH1* (4/13, 31% of patients) and *IDH2* (3/13, 23% of patients) defined almost the entire cluster 1 (12/13 patients, 92%) and were mutually exclusive except for one patient that presented mutations in both *ARID1A* and *IDH2*. On the other hand, mutations in *ARID1A* (9/27, 33% of patients), *PBRM1* (7/27, 26% of patients) and *MLL2* (7/27, 26% of patients) defined 67% of cluster 2. No genes included in the chromatin modification pathway were altered in cluster 3.

Cluster 2 and cluster 3 shared alterations in the same pathways not altered in cluster 1, i.e., DNA damage control, RTK/RAS, cell cycle and apoptosis and TP53, but with striking differences.

Four genes included in the DNA damage control pathway were found to be altered in our sample (*BRCA2*, *BAP1*, *TP53* and *ATM*) (Fig. S1B). This pathway was altered almost only in cluster 2, showing mutations in 4/4 genes included in this pathway: *BRCA2* (8/27, 30% of patients), *TP53* (7/27, 26% of patients), *BAP1* (6/27, 22% of patients) and *ATM* (5/27, 19% of patients). Only two patients included in cluster 3 were reported to have mutations in *BAP1* (2/11, 18% of patients).

Six genes included in the RAS/RTK pathway were found to be altered in our patient sample (*KRAS*, *NFK1*, *ERBB3*, *FGFR3*, *IGF1R* and *MAP3K1*) (Fig. S1C). Mutations in 6/6 genes of RAS/RTK pathway were observed in cluster 2: *RAS* (5/27, 19% of patients), *NF1* (4/27, 15% of patients), *FGFR3* (3/27, 11% of patients), *IGF1R* (3/27, 11% of patients), *MAP3K1* (3/27, 11% of patients) and *ERBB3* (2/27, 7% of patients). The only altered genes included in this pathway in cluster 3 were: *RAS* (3/11, 27% of patients) and *ERBB3* (1/11, 9% of patients).

TP53, *CDKN2A/2B* and *CCND1* genes included in the cell cycle apoptosis pathway were found to be altered in our patient sample (Fig. S1D). While *TP53* was mutated in cluster 2 (7/27, 26% of patients), *CDKN2A/B* and *CCND1* were mutated in both clusters with similar frequencies (*CDKN2A/B*: 8/27, 30% of patients in cluster 2 and 3/11, 27% of patients in cluster 3; *CCND1*: 2/27, 7% of patients in cluster 2 and 1/11, 9% of patients in cluster 3).

Finally, three genes included in the TP53 pathway were found to be altered in our patient sample (*TP53, MDM2* and *ATM*). A high percentage of the alterations in this pathway were observed in cluster 2 with mutations in all three genes: *TP53* (7/27, 27% of patients), *ATM* (5/27, 19% of patients), and *MDM2* (3/27, 11% of patients). In contrast, the only mutated gene in cluster 3 was *MDM2* (2/11, 18% of patients). Of note, mutations in in CDKN2A/B and RAS defined 86% (6/7) of mutated patients cluster 3.

3.4 An algorithm to stratify patients in clinical practice according to the clustering analysis

We proposed an easy-to-use algorithm able to stratify patients in clinical practice according to our clustering analysis. We chose the following genes as nodal points of our algorithm based on the presence of genomic alterations: *TP53*, *BRCA2*, *IDH1/2* or *ARID1A*, *CDKN2A/B* or *RAS* and *SMAD4*, *BAP1* or *PBRM1* (Figure 3).

3.5 Outcome analysis according to the identified clusters

A survival analysis based on the clustering analysis was performed. In terms of PFS, a trend toward better outcome was reported for cluster 2, without reaching statistical significance with a median PFS of 8.88 months versus 6.45 and 6.25 months for cluster 1 and cluster 3, respectively (cluster 2 reference HR 1, cluster 1 HR 1.79, cluster 3 HR 1.44, p=0.5605) (Figure 4A). In terms of OS, no statistically significant differences were found between cluster 1, cluster 2 and cluster 3 (cluster 3 reference HR 1, cluster 2 HR 2.01, cluster 3 HR 2.09, p=0.7834) (Figure 4B).
Overall, 13, 24 and 10 patients were available for response rate analysis in cluster 1, cluster 2 and cluster 3, respectively. ORR was 4/13 (31%), 12/24 (50%) and 0/10 (0%) in cluster 1, cluster 2 and cluster 3, respectively and the difference was statistically significant (p=0.0188). In terms of DCR, 8/13 (61.5%), 12/24 (50%) and 9/10 (90%) patients had disease control in cluster 1, cluster 2 and cluster 3, respectively (p=0.0900).
In terms of adverse events, no statistically significant differences were reported in the three clusters of patients (Table 3).

4. DISCUSSION

To the best of our knowledge, the present work reported the first clustering analysis performed on a sample of patients with advanced BTC treated with durvalumab plus cisplatin/gemcitabine as first-line treatment in a real-world setting. Our analysis highlighted three clusters characterized by different molecular and genomic features. Patients included in the three clusters showed significant differences in terms of response rate, with patients in cluster 2 showing the highest ORR (50%) and no patients included in cluster 3 achieving an objective response. Of note, the negative impact in terms of response rate was observed despite more favorable levels of CA 19-9. Finally, we developed an easy-to-use algorithm in order to transfer our results into clinical practice, thus providing a useful tool to stratify patients who could benefit from treatment with cisplatin/gemcitabine and durvalumab in terms of ORR.

Several considerations arise from our results. Cluster 1 has been shown to be mainly characterized by genomic alterations in genes involved in the chromatin modification pathway, thus including *IDH1* and *ARID1A*. In contrast, cluster 2 has been highlighted as the most mutated, probably due to frequent mutations in genes involved in DNA damage repair (primarily, *BRCA2* and *ATM*), thus conferring a sort of genomic instability on this group of patients. Finally, cluster 3 was shown to be characterized by genomic alterations in genes involved in RAS/RTK, cell cycle and apoptosis. In the survival analysis, no statistically significant differences in OS or PFS were found between the three clusters, possibly due to the small sample size and low rate of events. However, despite the small sample size, this result is of particular interest in the selection of patients who could benefit from neoadjuvant and/or conversion treatment. To date, no standard neoadjuvant treatment has been approved for BTC, data are scarce, and the selection of patients who could benefit from neoadjuvant or conversion treatment is still challenging. Indeed, in other cancer types, systemic treatment has been shown to reduce the risk of recurrence after surgery and make patients who were not resectable at baseline resectable. From the TOPAZ-1 trial, patients with locally advanced disease were found to have a larger survival benefit with cisplatin/gemcitabine plus durvalumab compared to metastatic patients. Furthermore, the chemotherapy triplet cisplatin/gemcitabine plus nab-paclitaxel was shown to confer a survival benefit in locally advanced patients and not in patients with metastatic disease (19). The distinct response rates by molecular cluster we found in the advanced setting, may lead to some speculations on the impact of cisplatin/gemcitabine plus durvalumab also in a setting of conversion/neoadjuvant treatment, where shrinkage is even more needed to reach better outcomes. Our results might suggest that patients included in cluster 3 might not benefit from conversion/neoadjuvant treatment before surgery. In contrast, patients

with locally advanced disease included in cluster 2 could be the best candidates to conversion treatment with cisplatin/gemcitabine plus durvalumab. More investigations are needed in order to verify our hypothesis. The improved response rate reported in cluster 2 could be ascribed to the molecular profile. Indeed, cluster 2 is enriched with mutations in genes like *BRCA2* and *ATM*, which are known to confer the "BRCAness" phenotype. As already demonstrated in several oncological settings, including breast, ovarian and pancreatic cancer, the "BRCAness" phenotype confers a special susceptibility to platinum compounds and PARP inhibitors (20-24, 11). In a previous retrospective analysis, our group demonstrated a significant benefit in terms of PFS and a trend toward better OS in a cohort of advanced BTC patients with "BRCAness" phenotype treated with cisplatin plus gemcitabine as first-line therapy compared to "BRCAness wild type" patients (25). In addition, a growing body of evidence suggests that changes in the DNA damage repair system could alter genomic stability (26), as they lead to the accumulation of DNA damages thus promoting local antigen release and enhancing immunogenicity in tumors (27-29). The immunogenicity promoted by alterations in DNA damage repair systems may underlie the improved response to immune checkpoint inhibitors, which has been reported in various oncology settings (30). Thus, "BRCAness" patients seem to be biologically prone to respond well not only to cisplatin and PARP inhibitors, but also to immunotherapy, which is consistent with our findings. Interestingly, molecular analysis performed on patients included in the TOPAZ-1 study showed a benefit on ORR regardless of any genomic alteration.

From a molecular point of view, our work has shown that the most commonly altered genes are *ARID1A*, (29%) *CDKN2A/2B* (21.5%), *MLL2* (17.5%), *BRCA2* (15.5%), *PBRM1* (15.5%), *NRAS/KRAS* (15.5%), *BAP1* (15.5%), *TP53* (14%), *IDH1* (12%), *MTAP* (12%), *MDM2* (10%), *ATM* (10%), *MSH3* (10%), *SMAD4* (10%) and *PIK3C2B* (10%). Recently, Valle and colleagues presented the results of the genomic analysis from the TOPAZ-1 trial: this analysis showed that *TP53* (48.8%), *CDKN2A/B* (25.2%), *KRAS* (24.0%), *ARID1A* (20.9%), *SMAD4* (14.5%), *IDH1* (8.8%) and *PIK3CA* (8.2%) were the most frequently altered genes, with differences depending on primary tumor site and geographic location. Moreover, they presented the impact of clinically actionable genomic alterations such as in *KRAS, IDH1, ERBB2, BRCA1/2, BRAF* and *FGFR2* on objective response rate. Interestingly, in the cisplatin/gemcitabine plus durvalumab arm, patients with *BRCA1/2* mutations reported a higher ORR compared to wild-type patients, which is consistent with our results.

Our research has several limitations. First, this is a retrospective analysis, thus selection biases cannot be excluded due to the nature of the investigation. Second, the small sample size and short

follow-up period do not allow to draw definitive conclusions, thus validation on a larger cohort of patients is needed. Nevertheless, durvalumab has been recently introduced in the therapeutic armamentarium for BTC, and our cohort is the largest cohort of patients with advanced BTC who received durvalumab in a real-world setting and who were screened with a comprehensive NGS panel at baseline, thus making our results, although preliminary, of interest. Another limitation is related to the NGS panel used: no whole exome sequencing analysis was performed in our cohort, but all patients were tested with the FoundationOne assay, to ensure consistent data quality and, consequently, more reliable results. In addition, the choice to include the VUS has to be considered and justified. Since scarce data are already available concerning the role of molecular alteration and clinical outcomes to cisplatin/gemcitabine plus durvalumab in this setting of patients, as well as the role of many genomic alterations in advanced BTC has not conclusively already defined, we decided to include all the genomic alterations highlighted, thus including the VUS. If it could be considered a methodological choice, we need to consider that in the interpretation of the present results. Moreover, no complete data about the variant allele frequency were available, so this important information has not been considered in the present analysis, thus configuring an important limitation. Finally, our data resulting from a sophisticated clustering analysis, need to be confirmed and validated on larger cohorts of patients.

5. CONCLUSION

The present analysis is one of the first comprehensive genomic analyses performed on a cohort of patients with advanced BTC who received cisplatin/gemcitabine plus durvalumab. The clustering analysis highlighted the presence of three clusters characterized by different genomic profile with interesting clinical impact, mainly in terms of response rate. No prognostic value has been shown, and further investigations are needed. The present analysis aimed to identify molecular and genomic biomarkers to select which patients are more likely to respond to the combination of immunotherapy plus chemotherapy. By grouping patients into three clusters with distinct molecular and genomic alterations, despite the limitation of the small sample size, our analysis showed that patients included in cluster 2 had a higher ORR, whereas patients included in cluster 3 had no objective response. Further investigations on larger and external cohorts are needed in order to validate our results. Nevertheless, this preliminary insight into the molecular heterogeneity of BTC patients treated with cisplatin/gemcitabine plus durvalumab as first-line therapy could open

new avenues of research focusing on mechanisms of primary resistance to treatments, with the ultimate goal of improving stratification of patients and clinical outcomes.

Figure Legends

Figure 1. Diagram of the most frequently altered genes according to the NGS test.

Figure 2. Classification of patients according to their mutation profile. (A) Mutation-based clustering. (B) Main altered pathways in the three clusters. Bar plots indicate the mutation frequency for the most altered pathways in the three clusters. Cluster 1 is mostly characterized by mutations in genes belonging to the chromatin modification pathway, altered in 100% of patients (Figure 2B). Cluster 2 is characterized by the alteration of several pathways, among which DNA damage control (81%), chromatin modification (78%), RTK/RAS (63%), cell cycle apoptosis (59%), TP53 (48%), and PI3K (30%) resulted the most affected (Figure 2C). Finally, most altered pathways in cluster 3 were RTK/RAS (36%) and cell cycle apoptosis (27%) (Figure 2D).

Figure 3. Schematic view of the algorithm designed to stratify patients in the three clusters based on clustering analysis.

Figure 4. Kaplan-Meyer curves for PFS (A) and OS (B) in months according to the three genomic clusters.

Supplementary Figure 1. Mutation frequency *per* genes in the altered pathways. Bar plots indicate the mutation frequency *per gene* in the three clusters. (A) Chromatin modification (B) DNA Damage Control (C) RTK/RAS (D) Cell cycle and apoptosis (E) TP53

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