Next Generation Sequencing Analysis of Cholangiocarcinoma Identifies Distinct *IDH1*-Mutated clusters

Margherita Rimini[1*], Eleonora Loi[2*], Carles Fabregat-Franco[3], Valentina Burgio[1], Sara Lonardi[4], Monica Niger[5], Mario Scartozzi[6], Ilario Giovanni Rapposelli[7], Giuseppe Aprile[8], Francesca Ratti[9], Federica Pedica[10], Helena Verdaguer[3], Mario Rizzato[11], Federico Nichetti[5], Eleonora Lai[6], Alessandro Cappetta[8], Teresa Macarulla[3], Matteo Fassan[12-13], Filippo De Braud[14], Andrea Pretta[6], Francesca Simionato[8], Francesco De Cobelli[15], Luca Aldrighetti[9], Lorenzo Fornaro[16], Stefano Cascinu[17], Zavattari Patrizia[2§], Andrea Casadei-Gardini[17§]

* Co-first authors

§Co-last authors

Affiliations:

1) IRCCS San Raffaele Scientific Institute Hospital, Department of Oncology, Vita-Salute San Raffaele University, Milan, Italy.
2) Department of Biomedical Sciences, Unit of Biology and Genetics, University of Cagliari, 09042, Cagliari, Italy.
3) Gastrointestinal Cancer Unit, Vall d'Hebron University Hospital & Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain.
4) Oncology Unit 3, Veneto Institute of Oncology – IRCCS, Padua, Italy.
5) Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano.
6) Medical Oncology, University and University Hospital, Cagliari, Italy.
7) Department of Medical Oncology, IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", 47014 Meldola, Italy.
8) Department of Oncology, San Bortolo General Hospital, Azienda ULSS8 Berica, Vicenza, Italy.
9) Hepatobiliary Surgery Division, Liver Center, Vita-Salute San Raffaele University, IRCCS San Raffaele Scientific Institute, Milan, 20132, Italy.

10) Department of Experimental Oncology, Pathology Unit, IRCCS San Raffaele Scientific Institute, Milan, 20132, Italy.
11) Oncology Unit 1, Veneto Institute of Oncology – IRCCS, padova, Italy.
12) Department of Medicine (DIMED), Surgical Pathology Unit, University of Padua, padova, Italy.
13) Veneto Institute of Oncology - IRCCS, padova, Italy.
14) Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy
15) School of Medicine, Vita-Salute San Raffaele University, Milan, 20132, Italy.
16) School of Medicine, Vita-Salute San Raffaele University, Milan, 20132, Italy.
17) Vita-Salute San Raffaele University, IRCCS San Raffaele Scientific Institute Hospital, Department of Oncology, Milan, Italy.

Corresponding author:

Margherita Rimini; Department of Medical Oncology, IRCCS San Raffaele Hospital, Via Olgettina n. 60, Milan, Italy; Email: margherita.rimini@gmail.com

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ABSTRACT

BACKGROUND: *IDH1* mutated intrahepatic cholangiocarcinomas (*IDH1*m iCCAs) could be treated with anti-IDH1 drugs although the high heterogeneity in this class of tumors could limit treatment's efficacy.

METHODS: We selected 125 *IDH1*m iCCAs treated as resectable, locally advanced or metastatic, 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. The most altered pathways in cluster 1 were Cell cycle and Apoptosis, RTK/RAS, PI3K and Chromatin Modification. Of note, *CDKN2A/2B* were mutated in 41/44 patients of this cluster. In cluster 2, the most affected pathways were: Chromatin Modification, DNA Damage Control, PI3K and RTK/RAS. In this cluster, the most frequently mutated genes were *ARID1A* and *PBRM1*. The most altered pathways in cluster 3 were: Cell cycle and Apoptosis, DNA Damage Control, TP53 and Chromatin modification. Importantly, *TP53* was mutated only in cluster 3 patients. In the cohort of patients treated with surgery, cluster 2 showed statistically significant better disease-free survival (DFS) and overall survival (OS) compared with patients in cluster 3 and cluster 1 (p=0.0014 and p=0.0003, respectively). In the advanced setting, cluster 2 experienced a statistically significant better PFS (p=0.0012), a tendency towards a better OS from first line treatment, and a better OS from first line progression compared with patients in cluster 1 and cluster 3 (p=0.0017). We proposed an easy-to-use algorithm able to stratify patients in the three clusters on the basis of the genomic profile.

CONCLUSION: We highlighted three different mutation-based clusters with prognostic significance in a cohort of *IDH1*m iCCAs.

1. INTRODUCTION

Intrahepatic Cholangiocarcinoma (iCCA) represents a heterogeneous group of malignancies characterized by dismal prognosis and challenging treatment (1-4). Surgery is the only curative treatment option for local stages. Unfortunately, the diagnosis occurs frequently at advanced stages, in which setting the survival decreases significantly (5). The platinum-based chemotherapy constitutes the backbone treatment for advanced and metastatic setting, but outcomes remain unsatisfactory, with a 5-year survival rate of about 2 % for stage IV (6). Recently, several new molecular targets with potential therapeutic implications in the advanced iCCA setting have been highlighted, including kinases such as FGFR family proteins, BRAF, PIK3CA, EGFR, ALK, EGFR, ERBB2, and AKT3, tumor suppressor genes involved in DNA damage repair pathway, such as BRCA 1/2, and oncogenes such as CCND3, MDM2 and, notably, Isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2)(1).

IDH1 has been investigated in selected onco-hematologic settings as a promising therapeutic target for patients harboring the mutated version of the IDH1 enzyme. In physiological condition, IDH enzymes play a fundamental role in the Krebs cycle and in cell metabolism (7-9). Gain of function mutations of the *IDH* genes lead to DNA and histone hypermethylation, genetic instability, aberrantly expressed hypoxia gene signature, oxidative stress and altered mitochondrial electron transport chain and mTOR pathway (10). In CCA setting, *IDH1* mutated forms have been highlighted to be exclusively present in iCCA, where represent the 25% of the cases, with several differences depending on geographical location (10). In the last years, the possible therapeutic implications of *IDH* genes' mutations have been explored. The promising results of the prospective randomized clinical trial ClarIDHy led to the FDA approval of the IDH1 inhibitor Ivosidenib as therapeutic choice for advanced and metastatic CCA patients harboring *IDH1* mutations (11), and several other IDH1 inhibitors are currently under investigation in the same setting (12). Nevertheless, if clinical trials are showing interesting results, it is possible that the high molecular heterogeneity existing in this group of neoplasia could limit the efficacy of Ivosidenib in advanced CCA patients carrying the *IDH1* mutations (13-15).

The aim of the present work is to deepen into the molecular landscape of *IDH1* mutated iCCAs (*IDH1*m iCCAs), thus performing a clustering analysis able to classify patients in different groups characterized by mutations in genes with clinical impact and prognostic significance in a cohort of patients surgically treated and in another one of patients receiving a first line systemic treatment.

2. MATERIAL AND METHODS

2.1. Patients' enrollment and sample collection

We selected 125 consecutive *IDH1* mutated iCCA patients treated for resectable, locally advanced or metastatic disease in six Italian institutions and one Spanish institute from January 2013 to March 2021. The sample included patients diagnosed at local stage who received radical surgical resection and relapsed after surgery, and patients diagnosed at locally advanced or metastatic stages with no indication for surgical treatment, thus exclusively receiving systemic treatment. All patients were reviewed to confirm the pathologic diagnosis of iCCA, the presence of *IDH1* mutation by next generation sequencing (NGS), and of disease stage as evaluated according to the 8th edition 2017 AJCC staging system. Formalin-fixed paraffin-embedded (FFPE) samples and hematoxylin-eosin staining slides of the 125 patients were collected from Pathology Department of each single institution. A genomic analysis of the primary tumors was performed by the FOUNDATION Cdx technology (FoundationOne essay).

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of each institution involved in the project (number of registry ethical committee: 113/INT/2021). Under the condition of retrospective archival tissue collection and patients' data anonymization, our study was exempted from the acquisition of informed consent from patients by the institutional review board.

2.2. Clinical Data

Clinical data including patients' age, gender, Eastern Cooperative Oncology Group (ECOG) Performance Status and kind of treatment received (surgical versus systemic, and type of systemic therapy), as evaluated at the baseline, were retrospectively collected. Pathological data, including

surgical records when available, primary tumor location, histological grading and TNM stage according to the 8th edition 2017 AJCC staging system were carefully collected as well at the baseline. Response to treatment (surgical and/or systemic ones) was assessed using RECIST 1.1 criteria. For both patients receiving a radical surgery and those treated with systemic therapy for advanced disease, the follow up and the oncologic assessment was planned as per standard of practice, according to guidelines and institutional protocols. Patients receiving systemic therapy were treated according to the physician choice.

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 essay (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 a total of 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 co-investigator and double-checked at the data manager center in order to perform a clustering analysis. For each patient, the mutational status of 324 genes screened in the FoundationOne assay was annotated.

Mutation-based clustering analysis has been performed with ccpw Model from Zhang et al (16).

This method permits to cluster tumor samples based on the somatic mutation spectra of the putative cancer driver genes. The list of cancer pathways including the putative cancer genes was manually curated from the core cancer pathway (ccpw) list (17) and the list of ten pathways curated by Sanchez-Vega et al. (18). The manually curated list named as merged cancer pathway (mcp) list included 511 putative cancer genes belonging to 16 pathways (Supplementary Table 1). The ccpw list includes the

following pathways: APC (13 genes), Cell cycle and apoptosis (21 genes), chromatin modification (31 genes), DNA damage control (31 genes), Hedgehog (HH) (6 genes), MAPK (8 genes), NOTCH (6 genes), PI3K (34 genes), RAS (32 genes), STAT (11 genes), TGF-β (10 genes) and Transcriptional regulation (13 genes). The list from Sanchez-Vega et al. (18) included Cell cycle (15 genes), HIPPO (38 genes), MYC (13 genes), NOTCH (71 genes), NRF2 (3 genes), PI3K (29 genes), RTK RAS (85 genes), TGF- β (7 genes), TP53 (6 genes) and WNT (68 genes). Since there were five pathways in common (cell cycle, NOTCH, PI3K, RAS and TGF-b), we merged the common pathways into a new one including all the genes from the two lists. We also merged APC list from ccpw list with WNT list from Sanchez-Vega work (18). Thus, we obtained the mcp list including the following pathways: Cell cycle 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- β (14 genes), TP53 (6 genes), transcriptional regulation (13 genes) and WNT (76 genes) (Supplementary Table 1). It should be noted that same genes could belong to multiple pathways.

By comparing our dataset of genomic alterations and the 511 cancer driver genes included in the mcp list, a total of 181 genes were included in our clustering analysis.

With this CCPW model, binary variables indicating the mutational status of cancer driver genes in tumors and the genes' involvement in the mcp list of 16 cancer pathways are treated as the features in Ward's hierarchical clustering.

2.5. Statistical Analysis

For clinical features, categorical variables were presented as totals and frequencies, then evaluated by Chi-squared test of Fisher exact test, as appropriate. Continuous variables were described as means with standard deviations or medians with ranges, and compared with T test. The genomic alterations present in ≥5% of the entire sample were considered for the analysis of distribution of genomic alterations in the sample of patients. A survival analysis according to the identified clusters were performed. For patients surgically treated, disease free survival (DFS) and overall survival (OS) from surgery were considered. DFS was measured from the date of surgery to the date of first recurrence or last follow-up/death for other reasons, whereas OS from surgery was measured from the date of surgery and the date of death or last follow-up. For patients diagnosed for a locally advanced or metastatic disease, who were stained not eligible for surgery, progression free survival (PFS) and OS

from the first line treatment were considered. PFS was measured from the date of the start of the first line therapy to the date of disease progression, death or last follow-up. OS was measured from the date of the first-line start and the date of death or last follow-up.
DFS and OS from surgery, as well as 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. DFS and OS from surgery as well as PFS and OS from the start of the first line treatment were estimated by the Kaplan-Meier method and curves were compared by the log-rank test. A p value <0.05 was considered statistically significant. .

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

3. RESULTS

3.1. Clinical Characteristics in Intrahepatic Cholangiocarcinoma patients

Overall, 125 *IDH1*m iCCA patients were retrospectively analyzed and considered for the survival and clustering analysis. The median age at diagnosis was 59 (range 28-85). At the baseline, 7% of patients were diagnosed of CCA at stage I, whereas 76% were diagnosed at more advanced stages (II, III and IV). At the start of treatment, 69/125 (55%) of patients presented an ECOG PS of 0. In our sample, the 46% of patients received surgical intervention with radical intention and 86% were treated with first line systemic therapy during their oncologic history; finally, 90/125 (72%) patients received the first line standard of care cisplatin plus gemcitabine, whereas 17/125 (13%) received other regimens (Table 1).

3.2. Genomic Alterations in *IDH1*-mutated patients

We firstly annotated alterations to specific genes. Overall, FoundatioOne assays allowed to identify a total of 359 genomic alterations in the entire sample which involved 75 genes, with a mean of 4.79 alterations per gene (range 1-36). Overall, 15/125 patients (12%) did not show any genomic alterations in addition to *IDH1* mutation; the rest of the sample presented at least one extra genomic alteration, with a median of genomic alterations for patients of 7.21 (range 1-13). The most common genomic alterations were found in *CDKN2A* (29%), *ARID1A* (22%), *CDKN2B* (22%),

PBRM1 (18%), *KRAS/NRAS* (13%), *BAP1* (13%), *PIK3CA* (11%), *TP53* (7%), *MTAP* (7%), *MUTYH* (5.5%), *MDM2* (5%), *MCL1* (5%), *DNMT3A* (5%) (Supplementary Table 2). It is important to note that *MTAP* and *MCL1* were not included in the clustering analysis since they were not classified as cancer genes in the list of pathways analyzed.

Mutations of all remaining genes were detected in less than 5% of the entire sample, and 24% of all the analyzed genes were mutated once in a single sample, including *CCND1, MAP3KI3, BRCA1, RNF43, IGF1R, IKBKE, FGF3, FGF4, RET* and *JAK3*.

3.3. Clustering analysis

From the clustering analysis, three main clusters resulted evident, since were characterized by mutations in genes belonging to different pathways (Figure 1A). Of note, no statistically significant differences were reported in terms of clinical characteristics between patients included in cluster 1, cluster 2 and cluster 3 (Table 2).

The cluster 1 included 44 patients showing mutations in 64 genes. The most altered pathways in cluster 1 were Cell cycle and Apoptosis (93.2% of patients), RTK/RAS (47.7% of patients), PI3K (43.2% of patients) and Chromatin Modification (40.9% of patients) (Figure 1B). The cluster 2 included 64 patients presenting 42 mutated genes. In this cluster, the most affected pathways were: Chromatin Modification (46.9% of patients), DNA Damage Control (28.1% of patients), PI3K (28.1% of patients) and RTK/RAS (26.6% of patients) (Figure 1C). Finally, the cluster 3 included a small number of patients (17) characterized by mutations in 27 genes. The most altered pathways in cluster 3 were: Cell cycle and Apoptosis (100% of patients), DNA Damage Control (100% of patients), TP53 (82.4% of patients) and Chromatin modification (52.9% of patients) (Figure 1D). It is important to mention that the alterations observed in these pathways are mostly due to mutations in the same genes.

Of note, for each pathway, the relative altered genes as well as their frequency in cluster 1, 2 and 3 were different.

The number of genes belonging to Cell cycle and Apoptosis pathway analyzed by Foundation was 26. This pathway resulted to be the most altered pathway in both cluster 1 and cluster 3, while it was not mutated in cluster 2. In cluster 1, *CDKN2A* (78%) and *CDKN2B* (58.5%) werethe most

frequently altered genes;in cluster 3, *TP53* was the most frequently altered (52.9%) (Supplementary Figure 1A). Importantly, *TP53* was mutated only in cluster 3 patients.

The number of genes belonging to DNA Damage Control pathway analyzed by Foundation was 21. This pathway resulted to be altered in all the three clusters, with predominance in cluster 2 and cluster 3. In cluster 1, *ERCC4* (33.3%) and *MUTYH* (33.3%) were the most frequently altered genes. In cluster 2, it was the second most mutated pathway, and *BAP1* (66.7%) was the most frequently altered gene. In cluster 3, *TP53* (52.9%) and *ATM* (29.4%) were the most frequently altered genes (Supplementary Figure 1B).

The number of genes belonging to Chromatin Modification pathway analyzed by Foundation was 26. This pathway resulted to be the most mutated pathway in cluster 2, but it showed to be significantly altered also in cluster 1 and in cluster 3. In cluster 1, *ARID1A* (38.9%), *PBRM1* (27.8%) and *DNMT3A* (16.7%) were the most frequently altered genes. In cluster 2, *ARID1A* (56.7%) and *PBRM1* (46.7%) were the most frequently altered genes. In cluster 3, *ARID1A* (44.4%) and *PBRM1* (44.4%) were the most frequently altered genes (Supplementary Figure 1C).

The number of genes belonging to *PI3K* pathway analyzed by Foundation was 37. This pathway resulted to be the third most altered pathway in cluster 1 and cluster 2, while it did not show alterations in cluster 3. In cluster 1, *PIK3CA* (21.1%), *EGFR* (15.8%), PDGFRA (10.5%), PTEN (10.5%) and *FGFR2* (10.5%) were the most frequently altered genes. In cluster 2, *PIK3CA* (55.6%) was the most frequently altered gene (Supplementary Figure 1D).

The number of genes belonging to RTK and RAS pathway analyzed by Foundation was 45, including *KRAS* and *NRAS*. This pathway resulted to be the second most altered pathway in cluster 1, whereas showed a lower mutation rate in cluster 2 and cluster 3. In cluster 1, *KRAS* (28.6%), *MAP3K1* (14.3%), *ERRFI1* (14.3%) and *EGFR* (14.3%) were the most frequently altered genes. In cluster 2, *KRAS* (47.1%) was the most frequently altered gene. In cluster 3, *KRAS* (66.7%), *IRS2* (66.7) and *FGFR4* (66.7%) were the most frequently altered genes (Supplementary Figure 1E).

The number of genes belonging to *TP53* pathway analyzed by Foundation was 5. This pathway resulted to be the second most altered pathway in cluster 3, whereas showed a lower mutation rate in cluster 1 and cluster 2. In cluster 1, the only mutated gene was *MDM4*. In cluster 2, the only mutated gene was *MDM2*. In cluster 3, *TP53* (64.3% of patients) and *ATM* (35.7% of patients) were the most frequently altered genes (Supplementary Figure 1F).

From the clinical point of view, no significant differences were reported in terms of baseline characteristics in patients included in the three clusters (Table 2).

3.4. An algorithm to stratify patients in clinical practice

We proposed an easy-to-use algorithm able to stratify patients in the three clusters on the basis of the genomic profile as revealed by FoundationOne analysis. According to the results of our clustering analysis, we chose the presence of alterations in *TP53*, *ATM*, *CDKN2A*/*2B*, *ARID1A* or *PBRM1* or *BAP1* or *PIK3CA* or *RAS* and *APC* as nodal points in our algorithm thus leading to stratify correctly patients in our sample (Figure 2).

3.5. Survival Analysis according the identified clusters

Firstly, we evaluated the correlation between patients' clusters based on mutations with clinical outcomes in patients treated with surgery (N=46). As reported in figure 3A, patients in cluster 2 showed a statistically significant better DFS compared with patients in cluster 3 and cluster 1 (Cluster 2 Reference HR 1, Cluster 3 HR 2.22, Cluster 1 HR 3.74, respectively; p=0.0014). In the same way, patients in cluster 2 showed a statistically significant better OS from surgery compared with patients in cluster 3 and cluster 1 (Cluster 2 Reference HR 1, Cluster 3 HR 2.50, Cluster 1 HR 6.76, respectively; p=0.0003) (Figure 3B).

Then, we evaluated the correlation between mutation-based clusters with clinical outcomes in patients receiving first line systemic treatment for advanced disease (N=86). In this setting, our analysis showed that patients in cluster 2 experienced a statistically significant better PFS compared with patients in cluster 1 and cluster 3 (Cluster 2 Reference HR 1, Cluster 1 HR 1.42, Cluster 3 HR 2.86, respectively; p=0.0012) (Figure 4A). Moreover, patients in cluster 2 showed a tendency towards a better OS from first line treatment, without reaching a statistical significance (Cluster 2 Reference HR 1, Cluster 1 HR 1.40, Cluster 3 HR 2.05, respectively; p=0.0828) (Figure 4B).

Finally, we evaluated the correlation between mutation-based clusters and OS from the time of progression to first line systemic treatment (N=86). In this setting patients in cluster 2 showed a statistically significant better OS compared with patients in cluster 1 and cluster 3 (Cluster 2 Reference HR 1, Cluster 1 HR 1.85, Cluster 3 HR 2.93, respectively; p=0.0017) (Figure 5).

4. DISCUSSION

At the best of our knowledge, the present study investigated for the first time the molecular landscape of a large sample of *IDH1*m iCCAs with the aim to uncover their mutational profile and to recognize clusters of patients characterized by mutations in genes belonging to different pathways with potential clinical impact. By performing a sophisticated clustering analysis on 125 *IDH1*m iCCA samples we individuated three clusters characterized by mutations in different genes, thus determining differences in terms of prognosis, also depending on the clinical setting. In particular, our results suggest a relation between cluster 2 and better survival outcomes, mainly in the surgical setting and in patients progressed to first line systemic treatment. The present results are of particular interest, if considering the data derived from the recently published ClarIDHy study (11). From the updated survival results, when adjusted for crossover, the 50% of patients treated with Ivosidenib showed a significant benefit in terms of OS when compared to placebo, but a genomic definition about which patients could be more likely to respond to IDH1 inhibitors are not yet available. From the clinical point of view, patients included in the three clusters did not show statistically significant differences. Nevertheless, an important difference which did not reach the statistical significance was highlighted between patients in the three clusters in terms of stage disease and first line therapy. In particular, the proportion of patients with a baseline stage disease IV was 54.5%, 56% and 29% in cluster 1, cluster 2 and cluster 3 respectively. Concerning the first line therapy, the 75%, 62.5% and 88% of patients received the standard treatment with cisplatin plus gemcitabine in cluster 1, cluster 2 and cluster 3, respectively. We could conclude that patients in cluster 2 showed the worse clinical characteristics at the baseline, thus making even more interesting the positive prognostic role we highlighted in our survival analysis. Previous works based on comprehensive genomic characterization have recently highlighted the complex molecular landscape of CCA and several clustering analyses have been performed on heterogeneous cohorts of patients (19-28). Nevertheless, no significant number of samples of *IDH1*m iCCAs have been already considered, thus a deeper understanding of this group of neoplasia is still lacking. Interestingly, Farshdifar and collaborators performed an integrated analysis by the Cancer Genome Atlas of a cohort of predominantly iCCA patients and highlighted an *IDH* mutant-enriched subtype (N=7) characterized by low expression of chromatin modifiers, an elevated expression of mitochondrial genes and an increased mitochondrial DNA copy number. In addition, the authors identified a prevalence of *ARID1A* hypermethylation and its decreased expression in *IDH1* mutated patients. More recently, Goeppert and collaborators performed an integrative analysis, which

identified four iCCAs subgroups with prognostic relevance, further designed as IDH, high, medium and low alteration groups. The IDH group (N=6) consisted of all samples with *IDH1* or *IDH2* mutations and showed, together with the high alterations group, a highly disrupted genome, characterized by frequent deletions of chromosome arms 3p and 6q compared with the other subtypes (27). Nevertheless, the small samples size considered by the two studies mentioned above did not permit to reach conclusive data about the molecular landscape of *IDH1*m iCCA patients. Differently, the present work has the merit to be the first integrative genomic analysis focused on a large sample of *IDH1*m iCCA patients. From the clinical point of view, a comparison between our finding and previous ones are quite difficult, due to the heterogeneity between the cohorts and to different inclusion and exclusion criteria. Nevertheless, an observation could be done: the clinical phenotype we highlighted in our cohort of IDH1m patients does not significantly differ from other ones reported in previous works, either if we consider cohorts of IDH1m or IDH1wt patients, or if we consider mixed cohorts (24-28). This point Is consistent with previous findings: in their analysis, Goyal and collaborators found that the clinical phenotype of IDH mutated and IDH wild type patients was similar (29). For sure, further investigations focused on the clinical characteristics of IDH1m and IDh1 WT CCA patients are needed in the next future in order to better define the clinical implications of the presence of IDH1 mutation. By referring to the mcp list of 16 cancer pathways involved in cancer genesis, we could optimize our analysis and clearly define three clusters based on the status of the most important driver genes. Consequently, we decided to use the most significative gene alterations as nodal points to design an easy-to-use algorithm, able to translate our clustering classification in clinical practice. The translation in clinical practice of our molecular clustering classification assumes a particular importance due to the correlation with clinical outcomes. In both cohorts of patients treated with surgery and patients progressed to the first line systemic treatment, cluster 2, showed better survival outcomes in terms of OS. Nowadays, Ivosidenib has been approved for patients harboring *IDH1* mutations and with a documented disease progression after at least one prior systemic treatment. Thus, the significative benefit in terms of OS revealed in patients progressed to first line therapy is of particular interest. In our cohort, no patient received Ivosidenib or another IDH1 inhibitor as subsequent therapy after progression to the first line. The next step would be aimed to verify which of these three clusters could benefit from treatment with Ivosidenib.

The transversal survival benefit highlighted for patients in cluster 2 could be related to the selection of negative molecular features in cluster 1 and cluster 3. Indeed, the presence of *TP53* mutations

exclusively defines the cluster 3, whereas the presence of *CDKN2A*/*2B* mutations mainly defines the cluster 1. A negative prognostic impact of *TP53* mutations as well as *CDKN2A*/*2B* mutations have been previously suggested in CCA (15,30). Simbolo and collaborators reported *TP53* mutations as exclusively present in poor prognosis patients, and the multivariate analysis confirmed its negative prognostic role (15). In a further integrative genomic analysis, the authors found that for all CCA patients considered, *TP53*, *KRAS* and *CDKN2A* alterations predicted worse OS across all stages; moreover, *CDKN2A* deletions tumors with associated high-risk clinical features were highlighted to not benefit of resection over chemotherapy (31). Importantly, even if consistent with our data, these previous analyses were conducted on heterogeneous cohorts of CCA patients without considering the only *IDH1* mutated subset. Our analysis suggested for the first time a prognostic stratification based on molecular and genomic features in a cohort of *IDH1* mutated patients.

Our research presents several limitations. Firstly, it was conducted as a retrospective investigation, thus several selection bias could be ascribed to the same nature of the study. Secondly, our genomic analysis focused on the presence of single genes' alterations, but excluded the analysis of the type of alterations. Moreover, for our clustering analysis we used data derived from an NGS-based panel, and not from a whole exome sequencing analysis, thus resulting in a kind of bias derived from the nature of our gene analysis. On the other hand, the panel we used to extract our genomic data is a large platform which evaluates the status of a significant number of genes highlighted to be relevant in cancer pathways. Thirdly, several clinical-pathological and familiar data have been excluded in our analysis, since our objective was to perform a pure genomic analysis with the definition of gene signatures by clustering analysis able to stratify our patients. Finally, the definition of three different mutation-based clusters of patients, if interesting and potentially useful from the clinical point of view, has to be validated on an external cohort of patients.

5. CONCLUSION

We performed a comprehensive genomic and clustering analysis on a large sample of *IDH1*m iCCA patients, thus highlighting the presence of three clusters characterized by different gene signatures with prognostic value and potential therapeutic implications. In the next future, it would be interesting to investigate the eventual predictive role of the three clusters on a cohort of *IDH1* mutated iCCA patients treated with Ivosidenib, with the aim to define and predict the subset of

patients which are more likely to respond to IDH1 inhibitors. Moreover, this first insight into the molecular heterogeneity of *IDH1* mutated iCCA patients could open new research's ways focused on the mechanisms of primary resistance to anti-IDH1, with the final aim to explore new combined and/or sequential therapeutic strategies and to improve the clinical management of *IDH1*m iCCA patients in an optic of precision medicine.

Figure Legends

Figure 1. 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.

Figure 2. Schematic view of the algorithm designed to stratify patients in the three clusters on the basis of the clustering analysis.

Figure 3. Kaplan-Meyer curves for DFS (A) and OS (B) according to the three genomic clusters in the cohort of patients resected.

Figure 4. Kaplan-Meyer curves for PFS (A) and OS (B) according to the three genomic clusters in the cohort of patients treated with first line systemic treatment.

Figure 5. Kaplan-Meyer curve for OS from the start of second line therapy according to the three genomic clusters in the cohort of patients treated with systemic treatments.

Supplementary Figure 1. Mutation frequency *per* genes in the altered pathways. Bar plots indicate the mutation frequency *per gene* in the three clusters. (A) Cell cycle and Apoptosis (B) DNA Damage Control (C) Chromatin modification (D) PI3K (E) RTK/RAS (F) TP53

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Table 1: Patients' Characteristics

Patients' Characteristics	IDH1 mutated (N=125) N (%)
Gender Male Female	 43 (34) 82 (66)
Age ≥70 <70	 25 (20) 100 (80)
Grading G1 G2 G3 NA	 2 (1.5) 16 (13) 26 (21.5) 80 (64)

Stage disease	
I	9 (7)
II	12 (10)
III	17 (14)
IV	65 (52)
NA	22 (18)
ECOG PS	
0	69 (55)
1	25 (20)
≥2	7 (6)
NA	24 (19)
Primary tumor resected	
Yes	57 (46)
No	68 (54)
Systemic Therapy for advanced disease	
Yes	
No	107 (86)
NA	14 (11)
	4 (3)
First line therapy	
Cisplatin/Gemcitabine	90 (72)
Others	17 (13)

Table 2: Patients' Characteristics according to the three genomic clusters

Patients' Characteristics	IDH1 mutated (N=125) N (%)	CLUSTER 1 (N=44)	CLUSTER 2 (N=64)	CLUSTER 3 (N=17)	P
Gender					
Male	43 (34)	18 (41)	16 (25)	9 (53)	0.0518
Female	82 (66)	26 (59)	48 (75)	8 (47)	
Age					
≥70	25 (20)	5 (11)	17 (26.5)	4 (23.5)	0.1537
<70	100 (80)	39 (89)	47 (73.5)	13 (76.5)	
Grading					
G1	2 (1.5)	0 (0)	2 (3)	0 (0)	0.2187
G2	16 (13)	9 (20.5)	5 (8)	2 (12)	
G3	26 (21.5)	5 (11)	17 (26.5)	4 (23.5)	
NA	80 (64)	30 (68.5)	40 (62.5)	11 (64.5)	
Stage disease					
I	9 (7)	3 (7)	6 (9)	0 (0)	0.0897
II	12 (10)	2 (4.5)	8 (12.5)	2 (12)	
III	17 (14)	6 (13.5)	8 (12.5)	3 (17.5)	
IV	65 (52)	24 (54.5)	36 (56)	5 (29)	

NA	22 (18)	9 (20.5)	6 (10)	7 (41.5)	
ECOG PS 0 1 ≥2 NA	 69 (55) 25 (20) 7 (6) 24 (19)	 26 (49) 9 (20.5) 0 (0) 9 (20.5)	 36 (56) 11 (17) 4 (6) 13 (21)	 7 (41.5) 5 (29) 3 (17.5) 2 (12)	0.1608
Primary tumor resected Yes No	 57 (46) 68 (54)	 17 (38.5) 27 (61.5)	 33 (52) 31 (48)	 7 (41.5) 10 (58.5)	0.3845
Systemic Therapy for advanced disease Yes No NA	 107 (86) 14 (11) 4 (3)	 40 (91) 3 (7) 1 (2)	 49 (76.5) 13 (20) 2 (3.5)	 16 (94) 0 (0) 1 (6)	0.1070
First line therapy Cisplatin/Gemcitabine Others	 90 (72) 17 (13)	 33 (75) 11 (25)	 40 (62.5) 24 (37.5)	 15 (88) 2 (12)	0.0838