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Original article

Custom Next-Generation Sequencing panel for cholangiocarcinoma diagnostic profiling

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Summary

Objective. Cholangiocarcinoma (CCA) is an aggressive malignancy with limited therapeutic options and poor prognosis. Advances in next-generation sequencing (NGS) revealed distinct molecular profiles between intrahepatic (iCCA) and extrahepatic (eCCA) subtypes, identifying recurrent mutations with diagnostic and therapeutic significance. We evaluated the performance of a laboratory-developed NGS panel designed for the integrated diagnostic and predictive profiling of CCA.

Methods and Results. In this multicentric retrospective study, 50 biopsies were analyzed, achieving 92% success rate. Pathogenic variants were identified in 58.7% of samples, with *TP53* (26.1%) and *KRAS* (21.7%) as the most frequently mutated genes. Multiple mutations were observed in 14 cases, the most frequent co-mutation being *KRAS* plus *TP53* (6 cases). Non-druggable but diagnostically relevant genes (*e.g.*, *ARID1A*, *BAP1*) were added to enhance classification accuracy.

Conclusions. Despite limitations such as the inability to detect gene fusions (*e.g.*, FGFR2), the panel demonstrated strong analytical feasibility and clinical utility. Integrating DNA/RNA sequencing, optimized pre-analytical workflows, and multidisciplinary interpretation will refine precision oncology approaches for CCA management in the future.

Keywords: cholangiocarcinoma, custom panel, IDH; KRAS, next-generation sequencing

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Introduction

Cholangiocarcinoma (CCA) is a malignancy with a high mortality rate and few therapeutic options at the moment of diagnosis, so that, although still considered a low-incidence cancer, it has become a significant health problem [1]. CCAs include intrahepatic (iCCA) and extrahepatic (eCCA) subtypes, with the distinction becoming increasingly clear thanks to next-generation sequencing (NGS) studies, which have revealed distinct genetic, transcriptomic, and proteomic profiles between iCCA and eCCA [2,3]. The advent of high-throughput sequencing technologies has enabled the identification of recurrent, potentially actionable mutations in a significant proportion of small-duct iCCA, which accounts for most cases amenable to targeted therapy. Among the most relevant alterations, *IDH1* mutations account for 1-18% (10-20% among iCCA) [4,5], *FGFR2* gene fusions are observed in approximately 5-7% (10-15% iCCA), *HER2* amplifications have been detected in 5-10% of cases, and

BRAF V600E variant in 1-7% [6,7]. To date, these alterations have become a primary therapeutic target and are classified as ESCAT I variants [6,8]. In fact, selective *FGFR* inhibitors such as Pemigatinib and Futibatinib have demonstrated clinical efficacy [7,9]. Similarly, *IDH1* mutations are now targetable with IDH1 inhibithors (*e.g.*, Ivosidenib), which significantly improved progression-free survival in previously treated patients [10]. Moreover, further single-cell NGS studies allowed the identification of molecular subtypes, paving the way for targeted therapies and immunotherapy [2,3].

On the other hand, eCCA and iCCA of the large duct subtype more commonly harbour mutations in *KRAS*, *TP53*, and *SMAD4* [7]. While the molecular distinction between iCCA and eCCA, together with the definition of small- and large-duct tumours is now well established, with implications for diagnosis, prognosis, and therapy, NGS studies are allowing the identification of actionable targets for precision therapy not possible since a few decades ago [2], albeit some feasibility issues remain, particularly for eCCA where available tissue is often scarce [2,11,12]. Other alterations with clinical relevance include *BRAF* V600E mutation, *HER2* amplification or mutations (predominantly in extrahepatic CCA), and *NTRK* fusions, although the latter are very rare (< 1%) [13]. Tumors harboring *BRAF* mutations, *HER2* amplifications, or *NTRKs* alterations may benefit from targeted therapies approved in other tumor types, such as BRAF/MEK inhibitors, anti-HER2 agents, and TRK inhibitors, respectively. Furthermore, mismatch repair deficiency (dMMR) and high microsatellite instability (MSI-H), although rare (< 5%), are predictive of response to immune checkpoint inhibitors, such as pembrolizumab [14].

However, other alterations, even if they are not "druggable", may be helpful to help in the diagnosis of CCA, mainly in those cases with a limited amount of material, as preoperative biopsies. For example, the presence of *ARID1A*, *BAP1*, and *PBRM1* mutations has been associated with iCCA, particularly of the small duct subtype, and may aid in distinguishing it from other biliary or hepatic neoplasms [15]. The co-occurrence of *KRAS*, *TP53*, and *SMAD4* mutations may support a diagnosis of large-duct iCCA or eCCA and may help differentiate these from small-duct iCCA, which tend to harbour *IDH1*/*IDH2* mutations and *FGFR2* fusions [16]. Moreover, the presence of one of these alterations may help pathologists in distinguishing a lesional sample from a non-neoplastic one [17]. The identification of these molecular subtypes has prompted the integration of molecular profiling into clinical practice for CCA, underscoring the importance of next-generation sequencing (NGS) as a standard diagnostic tool to guide therapeutic decision-making.

Nonetheless, these non-druggable alterations are usually not included in "classic" commercial NGS panels, which are more focused on the predictive markers. For this reason, we have tested a lab-developed NGS panel for routine diagnostic purposes.

Materials and methods

Patient selection, ethics, and histopathological analysis

This is a retrospective, multicentric, explorative study. Fifty biopsies from CCA patients were retrospectively enrolled in 2 years, from IRCCS S.Orsola Hospital of Bologna (N = 20 cases) and Santa Maria delle Croci Hospital of Ravenna (N = 30 cases). The present study received the approval of the Ethical Committee of the Area Vasta Emilia Centrale (AVEC), protocol number 128/2023/Sper/AOUBo. Patients signed informed consent for the study, and they are kept anonymous. This study follows the guidelines of the 1975 Declaration of Helsinki (and following amendments).

From formalin-fixed and paraffin-embedded (FFPE) tissue blocks, 4-5 slides of 8-10 µm were cut for NGS analysis. Archival slides were revised by a dedicated hepatobiliary pathologist in order to confirm diagnosis, to assess the presence of enough tissue for NGS, and to collect the main histopathological tumor features, such as grade and CCA subtype (small duct, large duct, eventual special histotypes).

NGS

Two to four unstained 10-µm-thick sections were used for DNA extraction and DNA was quantified using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, approximately 30 ng of input DNA was used to prepare NGS libraries using the AmpliSeq Plus Library Kit 2.0 (Thermo Fisher Scientific). Sequencing was performed using a laboratory-developed NGS panel designed in the Molecular Pathology Laboratory of Solid Tumors - IRCCS Policlinico di S.Orsola, allowing the analysis of the entire coding sequence (CDS) or hot-spots regions of the following genes (567 amplicons, 43.03 kb, human reference sequence hg19/GRCh37): *ARID1A* (CDS), *BAP1* (CDS), *BRAF* (exons 11, 15), *CDKN2A* (CDS), *CTNNB1* (exons 3, 7, 8), *FGFR1* (CDS), *FGFR2* (CDS), *FGFR3* (CDS), *FGFR4* (CDS), *GNAS* (CDS), *HRAS* (exons 2, 3, 4), *IDH1* (exon 4), *IDH2* (exon 4), *KRAS* (exons 2, 3, 4), *NRAS* (exons 2, 3, 4), *POLE* (CDS), *SMAD4* (CDS), *STK11* (CDS), *TERT* (promoter region, g.1295171-g.1295375), and *TP53* (CDS). Templates were then sequenced by using an Ion 530 chip

on a S5 Prime sequencer (Thermo Fisher Scientific). The results were analyzed with the IonReporter tools (version 5.20, Thermo Fisher Scientific). Thanks to the small size of the panel, the samples were run alongside routine samples analyzed by different NGS panels, simulating a routine pipeline. To achieve a minimum coverage of 1000x per amplicon, a total of 32-35 samples can be run on a single Ion 530 chip.
According to previous validation, only mutations present in at least 5% of the total number of reads analyzed and observed in both strands were considered for mutational calls [18]. The Varsome (https://varsome.com/) and Franklin by Genoox (https://franklin.genoox.com/clinical-db/home) tools were used to evaluate the ACMG classification of each reported variant (last accessed 30 th July 2025). Only Pathogenic/Likely Pathogenic and VUS (Variant of Uncertain Significance) variants were reported.

FISH analysis

Samples were evaluated for *FGFR2* rearrangement using FISH. The ZytoLight SPEC FGFR2 Dual Color Break Apart Probe (Z-2169-50, Zytovision) was used to detect *FGFR2* gene translocation. The hybridization signals of the probe appear green (proximal to the FGFR2 breakpoint region) and orange (distal to the FGFR2 breakpoint region). In interphases of normal cells or cells without a translocation involving the FGFR2 gene region, two green/orange fusion signals appear. On the contrary, the *FGFR2* gene affected by a translocation is indicated by one separate green signal and one separate orange signal.

Statistical analysis

Statistical analyses were performed using Jamovi software for Windows, version 2.3 (*The jamovi project (2022). [Computer Software]. Retrieved from https://www.jamovi.org*). Continuous variables are expressed as means±standard deviation and range, and discrete variables as frequencies and percentages. The χ^2 test was performed to correlate variables, assuming a p-value less than or equal to 0.05 is significant for rejecting the null hypothesis.

Results

Patients and histopathological variables

The 50 cases were 28 (56%) males and 22 (44%) females, with a mean age at diagnosis of 66.5±12.9 years (range 22-91). The analysed tissue came from peripheral liver biopsies in 23 (46%) cases, peri-hilar liver biopsies in 11 (22%), and extrahepatic neoplasia (during endoscopic retrograde cholangiopancreatography, ERCP) in 16 (32%).
At histopathological diagnosis, depending on the tumor site, 29 (58%) cases showed a large duct morphology, according to WHO[1], and 18 (36%) a small duct morphology; in 3 (6%) cases, the morphological subtype was not assessable due to the scarce bioptic material and the poor differentiation. Tumor grade was G3 in 26 (52%) cases, G2 in 17 (34%), G1 in 1 (2%) case, and not assessable in 6 (12%) cases.

NGS results

At NGS, 2 of 50 cases (8%) were not sequenced due to low cellularity (< 10%). Moreover, two cases were considered not evaluable after NGS sequencing because of low coverage (less than 100x per amplicon), due to low quality of DNA (Fig. 1).
Among the 46 evaluable cases, 19 (41.3%) showed no mutations (wild-type – WT, Fig. 1). In the other 27 (58.7%) cases, at least one variant was detected: *TP53* was the most frequently altered gene (12 cases, 26.1%), *KRAS* was mutated in 10 cases (21.7%), and both *SMAD4* and *ARID1A* were mutated in 4 cases (8.7%) (Tab. I). The vast majority of detected mutations were pathogenic/likely pathogenic, except for variants in ARID1A (4 VUS of 4), POLE (1 VUS of 1), and CDKN2A (1 VUS of 3) (Tab. I, Fig. 2). FISH analysis was performed on 30 samples, because no remnant material was available for the other specimens. Of these samples, 1 was positive for *FGFR2* rearrangement.

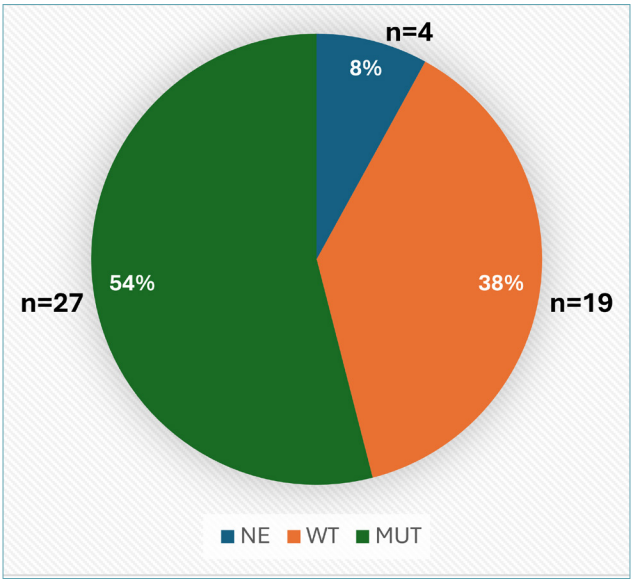


Figure 1. Distribution of NGS results in the overall population.
NE: not evaluable; WT: wild type; MUT: mutated.

Table I. Genes altered in the 27 mutated cases.

Gene	Number of mutated cases	Freq (%) N=346	ACMG significance
TP53	12	26.1	P/LP
KRAS	10	21.7	P
SMAD4	4	8.7	P/LP
ARID1A	4	8.7	VUS (n=4)
CDKN2A	3	6.5	P/LP (1 VUS)
BRAF	2	4.3	P
FGFR1	2	4.3	P/LP
IDH1	2	4.3	P
IDH2	1	2.2	P
NRAS	1	2.2	P
BAP1	1	2.2	P
TERT	1	2.2	P
POLE	1	2.2	VUS
CTNNB1	1	2.2	P

P/LP: Pathogenic/Likely Pathogenic; VUS: Variant of Uncertain Significance

Consistent with the literature, the cases mutated in *IDH1*, *IDH2*, and *BAP1* were all small-duct iCCA ($p = 0.016$, χ^2 test), while most of the *KRAS*-mutated cases were large-duct CCA or of not assessable subtype ($p = 0.010$).

In 14 cases, concomitant mutations were found. The more common combination of variants was between *KRAS* and *TP53* (observed in 6 of 14 samples). Four out of these 6 cases were eCCA, albeit no statistical correlations were found with histotype and grade (*data not shown*). As expected, the oncosuppressors *CDKN2A, SMAD4,* and *ARID1A* were consistently detected as altered in combination with other genes, except for one sample that harbored only an *ARID1A* mutation and one sample that harbored only an *SMAD4* mutation.

Discussion

In this study, we evaluated an NGS panel developed in the laboratory for the diagnostic-predictive profiling of cholangiocarcinoma (CCA) on FFPE biopsy material, obtaining 46 evaluable cases out of 50 (92% of analytical success after exclusion due to poor cellularity or degraded DNA). In our cohort, the overall frequency of cases with at least one variant was 58.7%. *TP53* (26.1%) and *KRAS* (21.7%) were the most frequently altered genes, while mutations in *IDH1* and *IDH2* were relatively rare (4.3% and 2.2%, respectively), as well as mutations in tumor suppressor genes such as *SMAD4* and *ARID1A*, both found in 8.7% of cases.

The mutation profiles observed in our sample reflect the known molecular differences between anatomical/histological subtypes of CCA. The high frequency of alterations in *TP53* and *KRAS* is consistent with the predominance, in our series, of tumors with large duct

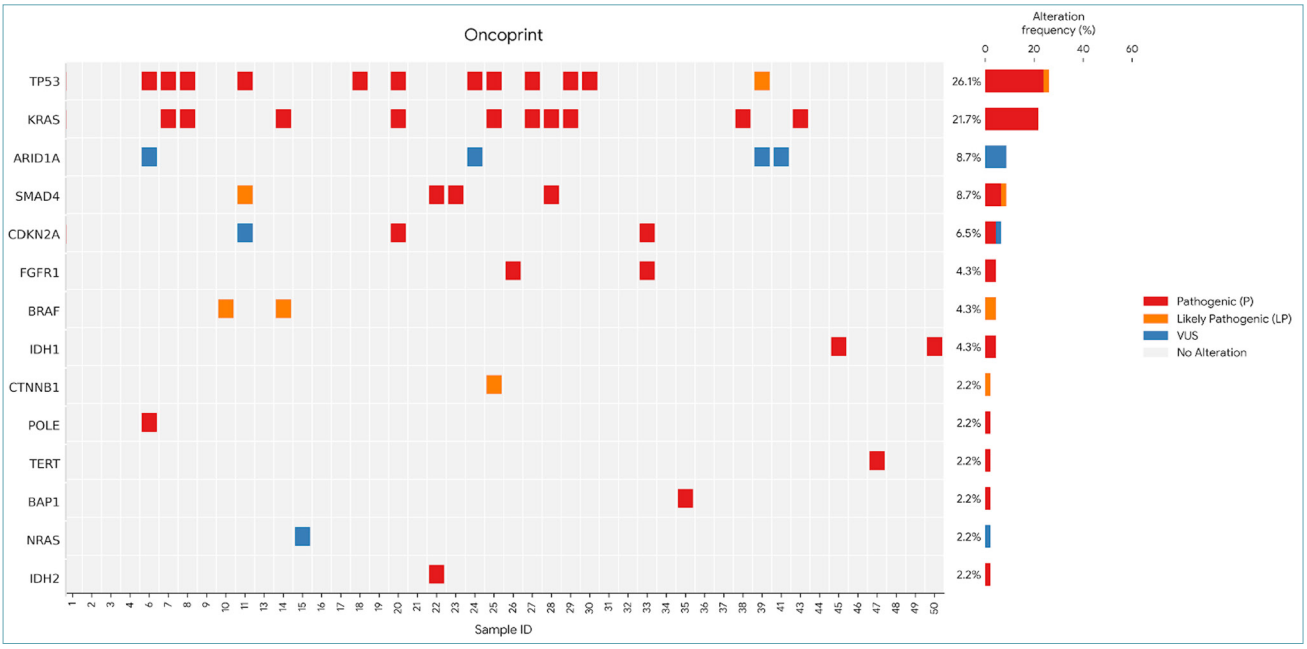


Figure 2. Oncoprint illustrating the main mutations found and their pathologic significance.

morphology and eCCA cases, entities that are commonly reported as associated with recurrent alterations in *KRAS* and *TP53* [11].

Our results support the practical value of including in the panel, in addition to "druggable" predictive markers, genes whose mutations have diagnostic value (e.g., *ARID1A*, *BAP1*), as they can aid in the diagnosis and classification of those cases in which the limited tissue quantity or equivocal morphology limit the histopathological analysis [18]. This approach is recommended by the literature, which emphasizes the integration of molecular and histopathological data with predictive targets [19].

In our case series, we observed co-occurrences of mutations, with "*KRAS*+*TP53*" association being the most common combination (6 of 14 cases with concomitant alterations). The co-occurrence of these alterations has been associated in previous studies with more aggressive phenotypes and possible unfavorable prognostic implications; therefore, the simultaneous detection of multiple drivers/tumor suppressors can provide useful information not only for classification but also for prognostic stratification [11,20].

Our study has some limitations: 8% of the analyzed cohort were found to be not evaluable due to poor cellularity or low-quality DNA, underscoring the critical nature of the pre-analytical phase in optimizing NGS analyses on endoscopic/needle core biopsies and the need for rigorous standardized pre-analytical procedures (SOPs) for managing FFPE material [21,22]. Moreover, the design of our panel (targeted amplicon-based panel) is focused on the diagnosis of CCA, and it allows for effective DNA-based alteration detection (SNVs, small indels) but does not cover the identification of gene fusion events (*e.g.*, FGFR2-fusion). The panel could also be used for predictive purposes, but in that case, it should be paired with an orthogonal analysis that enables the detection of *FGFR2* rearrangements, such as FISH. In this study, we combined the custom panel with the *in situ* method to cover this type of marker as well, although the panel was designed specifically for diagnostic purposes. Another possibility to cover possible rearrangements as well, not only that of FGFR2, it should be combined with an RNA-based panel. Therefore, the combined use of DNA/RNA or NGS/*in situ* approaches should be considered if the clinical request is for predictive biomarkers [23].

The main strength of this work is the applied evaluation of an in-house panel designed to integrate diagnostic and predictive markers, intended for use on clinical biopsy materials. The analytical success rate (92%) and the ability to identify relevant drivers and co-mutational patterns confirm the practical feasibility of the molecular diagnostic pathway in the institutional context. Furthermore, the deliberate inclusion of genes with diagnostic significance (*e.g.*, *ARID1A*, *BAP1*) provides added value for histological classification in cases of limited morphology.

According to our data, the following operational recommendations are suggested in the management of CCA specimens for molecular characterization: (i) to establish SOPs to optimize the quality and representativeness of biopsy material; (ii) to integrate, if requested, gene fusion-specific approaches to improve the detection of *FGFR2* fusions; (iii) to consider the complementary use of ctDNA analysis in cases where tissue is insufficient; (iv) to keep the classification databases (ACMG classification) updated, to ensure clinical-diagnostic classification of variants, and set up multidisciplinary pathways (i.e. molecular tumor boards) for the clinical translation of results. These measures are also consistent with the latest international guidelines recommending multi-gene NGS testing for patients with advanced BTC and emphasizing the importance of integrated diagnostic strategies [24].

Conclusions

The NGS lab-developed panel here described has proven to be a valid and feasible tool for the molecular diagnostic evaluation of CCA on FFPE biopsy material, allowing the identification of alterations of diagnostic and, in part, predictive relevance. However, maximizing the clinical value of molecular profiling in CCA requires an integrated approach that includes rigorous pre-analytical procedures, methods capable of detecting fusions and CNVs, and the complementary use of non-invasive techniques (ctDNA) when necessary. Prospective studies on larger case series and with multimodal diagnostic workflows will help define the real-world sensitivity/specificity of these pathways and in optimizing the transfer to clinical practice of precision oncology in cholangiocarcinoma.

Conflicts of interest statement

The are no conflicts of interest to declare.

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Author contributions

DdB: conceptualization, study design, paper drafting; TM, methodology, data analysis; GCi and GCa, methodology; LP, data analysis; DM, data retrieval; MC, data retrieval; LS, supervision, critical revision; GT, critical revision; FV, conceptualization, paper drafting.

Ethical consideration

This study was approved by the Institutional Ethics Committee of the Area Vasta Emilia Centrale (AVEC), protocol number 128/2023/Sper/AOUBo.
The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.
Written informed consent was obtained from each participant/patient for study participation and data publication.

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