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Original article

Harmonization trial of *FGFR1-3* testing strategies in cholangiocarcinoma patients: an Italian multicenter experience

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Summary

Aims. Molecular analysis of *FGFR2* aberrant transcripts became crucial for clinical stratification of intrahepatic cholangiocarcinoma (iCCA) patients. Several strategies, including fluorescent in situ hybridization (FISH) and next generation sequencing (NGS), are commonly used to investigate *FGFR* aberrations. Here, we evaluated the technical performance of clinically implemented diagnostic strategies in 8 referral Italian institutions on artificial reference formalin-fixed paraffin-embedded (FFPE) samples.

Methods. Each participating institution was requested to apply its own diagnostic testing strategy on 8 sections obtained from artificial reference specimens built to harbor *FGFR3(17)-TACC3(11)* rearrangement and unbalanced *FGFR2.* A second-round slide set hosting *FGFR2(17)-BICC1(3)* aberrant transcript was shared to detect clinically relevant *FGFR2* fusion. Artificial reference sample was previously validated by the University of Naples Federico II before arranging the shipment. Technical procedures (e.g. extraction methods, testing platforms and assays) were recorded.

Results. Overall, cell resuspension yielded higher amounts of DNA and RNA (SNU16 61.5 ng/µl, 38100.0 pg/µl; RT112 118.0/µl, 2140.0 pg/µl, respectively) in comparison with SNU16+ RT112 mixing cell block (0.7 ng/µl DNA and 412.0 pg/µl RNA). Moreover, FFPE samples showed a higher fragmentation index (DIN 1.2 and RIN not calculated) compared with cell line resuspension (DIN 2.2 and 9.5 for SNU16 and RT112; RIN 3.9 and 6.8 for SNU16 and RT112). All participating institutions identified *FGFR2(17)-BICC1(3)* and *FGFR3(17)-TACC3(11)* aberrant transcripts. Moreover, ID#2, ID#4, ID#7 institutions also detected *FGFR2(3)-CD44(1)* rearrangement on RNA, whereas institutions ID#1, ID#2, ID#3, ID#5, ID#6, ID#8 identified *FGFR2* CNVs on DNA.

Conclusions. NGS represents the most suitable approach in molecular profiling of *FGFR* aberrant transcripts. Rings trial based on artificial reference samples play a pivotal role in optimizing routine diagnostic procedures filling the gap in clinical stratification of iCCA patients.

Key words: cholangiocarcinoma, molecular biomarker, *FGFR*

Key bullet points

- Cholangiocarcinoma (CCA) covers a heterogeneous group of intrahepatic or extrahepatic biliary tract tumors.
- Fibroblast growth factor receptor type 2 (*FGFR2*) aberrant transcripts may be found in 15.0% of intrahepatic CCA patients (iCCA) predicting iCCA sensitivity to target drugs.
- A plethora of testing procedures are commercially available to detect clinically relevant aberrant transcripts selecting iCCA patients for personalized therapy. Here, an artificial standard reference sample was built to arrange a "mimetic diagnostic specimen" for evaluating inter-laboratory reproducibility among referral Italian institutions.
- Among 8 participating institutions, NGS was the unique testing strategy to evaluate *FGFR1*-3 aberrant transcripts. Amplicon-based and hybridization-based NGS platforms were adopted in diagnostic routine practice. Remarkably, *FGFR3*(17)-*TACC3*(11) aberrant transcript was successfully detected by all institutions inspecting RNA molecular data. In addition, two institutions (ID#4, ID#7A) were also able to identify *FGFR2*(3)-*CD44*(1) on the same RNA sample. The remaining institutions identified DNA-based *FGFR2* CNVs in the same standard reference sample. Second round slide set, previously validated by the coordinating institution, was successfully tested in all instances: *FGFR2(17)-BICC1(3)* aberrant transcript was detected by each system.
- Preanalytical procedures play a crucial role successfully evaluating *FGFR2* aberrant transcripts in iCCA patients. Fragmentation rate of diagnostically available nucleic acids dramatically impacts on the detectability of aberrant transcripts in diagnostic routine samples.
- Harmonized ring trials built to optimize diagnostic testing strategies to analyze *FGFR1*-3 aberrant transcripts may play a pivotal role into definition of diagnostic algorithm for iCCA patients.

Introduction

Cholangiocarcinoma (CCA) consists of a heterogeneous group of tumors classified as intrahepatic or extrahepatic on the basis of biliary tract localization [1]. In terms of incidence, CCA represents the most common liver cancer and CCA mortality is rapidly increasing, worldwide [2]. Although CCA patients may undergo surgery (35.0% of cases), gemcitabine plus cisplatin for first line treatment is warranted as standard of care for relapsing patients within 2 years [3]. Molecular profiling identifies potentially druggable alterations in up to 40.0% of CCA patients [4]. On this basis, intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC) have distinct molecular patterns where *TP53*, *ARID1A*, *IDH1/2*, *PBRM1*, *BAP1* and *PIK3CA* and *KRAS* are frequently mutated in ICC and ECC patients, respectively [5,6]. As a consequence, prognostic implications and therapeutic options are widely influenced by histological-molecular classifications. Patients with ECC frequently undergo surgery, but the 5- year survival rate is 18.0-23.0% . Conversely, ICC patients were surgically treated in a lower percentage of cases, but 5-year survival rates were 30-40% [7]. Of note, fibroblast growth factor receptor *(FGFR)* encodes for a family of four extracellular membrane tyrosine kinase (TK) receptors activating pro-angiogenetic and proliferative molecular pathways [8]. Noteworthy, *FGFR2* aberrant transcripts and rearrangements play a pivotal role for malignant transformation occurring in 15.0% of iCCA patients. Recently, *FGFR2* has been identified as a clinically useful biomarker for personalized therapy [9-12]. The open-label phase II FIGHT-202 study aimed to assess the efficacy and safety of a novel TKIs against aberrant *FGFR2* (pemigatinib) in a series of 146 iCCA patients [13], showing a disease control rate (DCR) of 82.0% of iCCA patients with *FGFR2* aberrant transcript [14]. Moreover, post-hoc analysis revealed a PFS of 7.0 months in *FGFR2* rearranged iCCA patients compared with other *FGFR2* mutations and *FGFR2* wild-type groups [14]. Additionally, two independent real-world cohorts confirmed a similar clinical efficacy in terms of DCR and median PFS (84.7% and 8.7 months, respectively) [15]. To date, fluorescent in situ hybridization (FISH) using break-apart probes or dual fusion probes is the most common tool to detect *FGFR2* rearrangements [16]. Unfortunately, FISH is affected by high false negative rate, unknown fusion partners (break apart probes) and a low inter-laboratories reproducibility rate. In the recent era of precision oncology, the widespread diffusion of next generation sequencing (NGS) platforms successfully detects clinically informative molecular alterations from diagnostic routine samples [1]. Several NGS assays (both DNA and RNA based panels) covering *FGFR2* rearrangements have been developed to optimize diagnostic workflow for iCCA patients. Target specific and unbalanced *FGFR2* assays may be adopted to detect clinically relevant aberrant fusions [17]. The successful implementation of testing strategy depends on several factors such as turnaround time (TAT), heterogenous sampling approaches (small biopsy, surgical resection, cytological samples) technical costs, and skilled personnel [10]. Remarkably, the lack

of harmonized procedures drastically impacts on the accuracy of molecular analysis in diagnostic routine samples [14]. We aimed to evaluate the technical performance of *diagnostic testing strategies for FGFR* among 8 representative Italian institutions. Each participating institution was asked to analyze artificial reference samples built on engineered cell lines carrying *FGFR2-3* aberrant transcripts. Concordance rates were assessed by comparing molecular records among participating institutions.

Methods

Study design

An artificial reference specimen including *FGFR3(17)-TACC3(11)* and unbalanced *FGFR2* aberrant transcripts were built to comprehensively cover *FGFR* rearrangements. Each participating institution received a slide set of standard reference samples built to evaluate aberrant *FGFR* rearrangements adopting own internal diagnostic workflow. Artificial control was prepared by mixing two engineered cell lines harboring *FGFR2-3* rearrangements in CCA patients (Supplementary Table I). Following our previous experience [17], a standard reference sample, internally validated by the coordinating institution (University of Naples Federico II) prior to shipment, was arranged as formalin-fixed and paraffin-embedded (FFPE) specimens to standardize pre-analytical managing procedures. After the training set, a second standard reference sample certified for *FGFR2* aberrant transcript *FGFR2(17)-BICC1(3)* was shared.

Briefly, nucleic acid fragmentation was inspected by a microfluidic system; molecular analysis was carried out on DNA and RNA using a fully automated NGS system with an optimized bioinformatic pipeline combining automatic and visual data inspection.

Each participating institution investigated reference slides from FFPE samples to evaluate *FGFR* rearrangements. Molecular records were shared with the coordinator center inspecting technical performance of real-world diagnostic workflow. Biological material was managed under the authorization of the Department of Public Health at the University of Naples Federico II, Naples. Statistical analysis was performed using binomial test (R software, v.4.5.0) to calculate the probability of "success" vs "failure" (dichotomic variables) among independent measurements.

In addition, a real-world retrospective series of iCCA patients previously tested leveraging comprehensive genomic profiling (CGP) assay was retrieved to validate *FGFR2* testing algorithm in diagnostic setting.

Standard Sample generation and validation

Human bladder carcinoma cell line RT112, which shows an *FGFR3-TACC3* rearrangement, and the human gastric cancer cell line SNU16, harboring CNV in *FGFR2*, were adopted. The adherent RT112 cell line was grown in RPMI1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Euroclone, Milan, Italy), 1% L-glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich). The SNU16 cell line was cultured in the same medium. Both cell lines were amplified incubating at 37 °C with an atmosphere containing 5% CO2. Each cell line underwent standard testing for the presence of mycoplasma using the EZ-PCR Mycoplasma Detection Kit (Biological Industries Israel Beit Haemek Ltd., Kibbutz Beit Haemek, Israel). Additional quality checks were performed on each cell line to ensure its purity and integrity. Beyond mycoplasma testing, viability assays using trypan blue confirmed a high percentage of live cells in the final suspension. Morphological checks were regularly carried out by microscopically observing cells to rule out any bacterial or cross-contamination. For the RT112 cells, a trypsin (Sigma-Aldrich) solution was used to split cells at specific ratio after sub-confluency. The adherent RT112 cells were enzymatically detached from dishes with trypsin. In contrast, the suspension SNU16 cells did not require trypsinization for passaging or sample preparation. Both cell lines were washed twice in PBS (phosphate buffered saline) and were counted in a Bürker cell counting chamber. A standard formula was used to calculate the total cell concentration and the average number of viable cells.

Finally, aliquots of these two cell lines were mixed in an equimolar ratio (RT112_50% / SNU16_50%) for the internal validation step [18]. To generate the second standard reference sample, BaF3 [FGFR2(17)-BICC1(3)] (Creative Biogene Inc., Shirley, NY, USA) (BaF3) the same growth medium and environmental conditions of RT112 and SNU16 cell lines were maintained (BaF3_100%). Like SNU16 cells, BaF3 cell line did not need trypsin for routine subculturing and expansion.

DNA and RNA extraction

DNA and RNA were simultaneously purified from mixed SNU16 (*FGFR2* CNV), and RT112 (*FGFR3(17)-TACC3(11)* cell pellet and FFPE sample adopting AllPrep DNA/RNA Kits (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. Finally, DNA and RNA were eluted in 30 µl of nuclease-free water and stored at -80 Cº until molecular analysis. Nucleic acids were also manually recovered from BaF3 [*FGFR2(17)-BICC1(3)*] cell line

and FFPE samples following the same technical approach.
DNA and RNA were run on automatized microfluidic system (TapeStation 4200, Agilent Technologies, Santa Clara, California, USA) to evaluate nucleic acid concentration (ng/µl and pg/µl for DNA and RNA, respectively) and fragmentation index (calculating DNA and RNA integrity number) following manufacturer procedures.

Molecular analysis

Overall, cell pellet and FFPE samples from training (SNU16 and RT112) and validation (BaF3) standard reference specimen were validated by the coordinator center adopting a fully automated NGS platform (Ion Torrent Genexus™ Integrated Sequencer, Thermo Fisher Scientific) that enables a single-step NGS workflow. This system was integrated with Oncomine Precision Assay (OPA, Thermo Fisher Scientifics) that covers 50 actionable genes (including *FGFR1-3* aberrant transcripts) across different solid tumors [19]. In particular, 10 ng of nucleic acid was dispensed in a 96-well plate on the Genexus™ system following manufacturer procedures. Samples were sequenced into GX5TM chips able to simultaneously analyze 8 samples in a single lane. Data analysis was carried out as follows: *FGFR1-3* aberrant rearrangements were automatically identified using Oncomine Knowledgebase Reporter Software (Oncomine Reporter 5.0). Samples showing a coverage of 500X and a median uniformity rate > 90.0% were accepted. In addition, aberrant transcripts both with common fusion partner and unbalancing *FGFR2-3* (CNVs > 3) were reported.

Results

Standard Sample generation and validation

Nucleic acid extraction

Overall, cell resuspension yielded the following data: SNU16 (61.5 ng/µl, 38100.0 pg/ µl, respectively) and RT112 (118.0 ng/µl, 2140.0 pg/µl, respectively). Moreover, the SNU16+ RT112 cell block showed 0.7 ng/µl DNA and 412.0 pg/µl RNA demonstrating that the FFPE sampling strategy had an impact on the amount of nucleic acids recovered from cell block. Additionally, the DNA fragmentation index (DIN) was 2.2 and 9.5 for SNU16 and RT112 cell resuspension, respectively, whereas the RNA fragmentation index (RIN) was 3.9 and 6.8 values for SNU16 and RT112 cell resuspension, respectively. Cell block analysis also showed a RIN of 1.2 whereas DIN was not successfully calculated due to high DNA fragmentation. Similarly, DNA and RNA from BaF3 cell pellet highlighted 19.9 ng/µl and 25800.0 pg/µl, respectively in association with a DIN and RIN of 9.2 and 5.3, respectively.

Molecular analysis

NGS analysis was successfully carried out in all instances: (number of total reads 1954437.0 and 1756776.0, mean read length 91.0 and 102.0, number of mapped reads 1940034.0 and 1744086.0, percent reads on target 78.3% and 93.3%, mean depth 4606.0 and 6463.0, uniformity of amplicon coverage 59.2% and 93.9%) for DNA-based analysis of SNU16 and ST112 cell line suspension, respectively. Moreover, RNA based analysis of SNU16, and ST112 cell line suspension also highlighted valuable technical performance (number of total reads 2570851.0 and 1677449.0, mean read length of 90.0 and 97.0,

Table I. Validation step. Technical parameters from NGS analysis performed on Genexus platform (Thermo Fisher Scientifics) at University of Naples Federico II.

	Sample	Nucleic Acid Type	Total Reads	Mean Read Length	Mapped Reads	On Target Reads	Mean Depth	Uniformity of amplicon coverage
Training Set	DNA_RT112	DNA	1756776	102	1744086	93.3%	6463	93.9%
	DNA_SNU16	DNA	1954437	91	1940034	78.3%	4606	59.2%
	RNA_RT112	RNA	1677449	97	NA	NA	NA	NA
	RNA_SNU16	RNA	2570851	90	NA	NA	NA	NA
	DNA_MIXED	DNA	754202	80	680192	75.7%	1534	83.2%
	RNA_MIXED	RNA	1384997	95	NA	NA	NA	NA
Validation Set	RNA_BaF3	RNA	1897258	97	NA	NA	NA	NA

Abbreviations: DNA (*Deoxyribonucleic Acid*); NA (*Not Available*); RNA (*Ribonucleic Acid*).

Table II. List of DNA and RNA extraction kits used by participating centers and nucleic acid quantification data.

	Training set				Validation set	
Center ID	DNA Extraction Kit	DNA ng/µl	RNA Extraction Kit	RNA ng//µl	RNA Extraction Kit	RNA ng//µl
1	MagCore® Genomic DNA FFPE One-Step Kit	4.9	MagCore® Genomic RNA FFPE One-Step Kit	4.6	MagCore® Genomic RNA FFPE One-Step Kit	0.1
2	MagMAX™ FFPE DNA/RNA Ultra Kit	4.9	MagMAX™ FFPE DNA/ RNA Ultra Kit	73.0	MagMAX™ FFPE DNA/RNA Ultra Kit	8.0
3	Maxwell CSC DNA/RNA FFPE Kit	2.8	Maxwell CSC DNA/RNA FFPE Kit	12.2	Automatic Genexus Purification System (GPS) ThermoFisher	6.7
4	-	-	Maxwell® CSC RNA FFPE Kit	31.6	Maxwell® CSC RNA FFPE Kit	15.6
5	Maxwell CSC DNA/RNA FFPE Kit	2.0	Maxwell CSC DNA/RNA FFPE Kit	12.3	Maxwell CSC DNA/RNA FFPE Kit	8.6
6	Automatic Genexus Purification System (GPS) ThermoFisher (RNA/DNA)	15.5	Automatic Genexus Purification System (GPS) ThermoFisher (RNA/DNA)	16.7	Automatic Genexus Purification System (GPS) ThermoFisher (RNA/DNA)	16.2
7	Maxwell CSC DNA/RNA FFPE Kit	45.9	Maxwell CSC DNA/RNA FFPE Kit	45.9	Maxwell CSC DNA/RNA FFPE Kit	10.8
8	MagCore Automated Nucleic Acid Extractor Super	9.7	MagCore Automated Nucleic Acid Extractor Super	17.1	DSP Qiamp DNA FFPE Kit/Qiamp RNA FFPE Kit - Qiagen	105.1
9	QIAAMP DNA FFPE TISSUE KIT - QIAGEN	4.9	RNeasy DSP FFPE Kit - QIAGEN	60.0	RNeasy DSP FFPE Kit- QIAGEN	0.4

Abbreviations: DNA (*Deoxyribonucleic Acid*); RNA (*Ribonucleic Acid*).

mapped reads respectively). Molecular analyses on the cell blocks from mixed engineered cell lines also met all technical quality checks in terms of number of total reads (754202.0 and 1384997.0), mean length (80.0 and 95.0), mapped reads (680192.0), percent reads on target (75.7%), mean depth (1534.0), uniformity of coverage (83.2%) on DNA and RNA based molecular analysis (Tab. I). Considering cell pellet and FFPE samples from BaF3 cell line, NGS analysis yielded consistent technical parameters on both DNA and RNA samples (Tab. I).

Standard Sample analysis

Overall, a series of 4 slide sets (5 µn), from previously validated FFPE samples, and 2 back-up tubes containing SNU16 and RT112 cell resuspension were shipped to 8 participating institutions. After the training set, a series of 4 slide sets (5 µn) from BaF3 were shared by coordinator group to other members. In two cases cell resuspension was also shipped integrating (ID#5) or substituting (ID#8) slide set. Each laboratory was asked to share molecular results with coordinator institution within 30 working days, as suggested by experimental design of the study. Participating institutions were geographically distributed as follows: 3 of 8 (37.5%) were in Northern Italy, 3 (37.5%) in Central Italy, and 2 (25.0%) in Southern Italy. Of note, all centers were able to successfully carry out molecular analyses and to share molecular records with the coordinating institution in templated format within the deadline of the project.

During the training set, DNA and RNA purification was simultaneously approached in 7 of 8 (87.5%) institutions adopting an automated technical approach. In a single case, only RNA was purified for molecular analysis of referenced genes (ID#4). Of note, a median DNA concentration of 12.3 ng/µl (ranging from 2.0 to 45.9 ng/ µl) and a median RNA concentration of 27.1 ng/µl (ranged from 4.6 to 73.0 ng/µl) were identified (Tabs. II, III, Fig. 1). RNA was successfully purified from BaF3 slides by each participating laboratory showing a median RNA concentration of 19.1 ng/µl (range 0.1 to 105.1 ng/µl) (Tab. II).

Testing strategies and molecular analysis

Each institution approached *FGFR1-3* molecular evaluation following its own internal diagnostic routine workflow. Interestingly, an NGS-based strategy was selected by all participating centers to detect *FGFR1-3* aberrant fusion transcripts on RNA and DNA samples.

Table III. List of assays and platforms used by participating institutions to test standard reference sample.

Center	Analysis	Analysis Technologies	Analysis Kit
1	**DNA**	Illumina NextSeq 550	Myriapod NGS Cancer Probe plus
	RNA	Illumina NextSeq 550	Myriapod NGS Cancer Probe plus
2	**DNA**	Thermo Fisher Genexus	Oncomine Precision Assay GX
	RNA	Thermo Fisher Genexus	Oncomine Precision Assay GX
2b	**DNA**	NA	NA
	RNA	Thermo Fisher Genexus	Oncomine Comprehensive Assay Plus
3	**DNA**	Thermo Fisher S5 GS Prime	Oncomine Focus Assay
	RNA	Thermo Fisher S5 GS Prime	Oncomine Focus Assay
4	**DNA**	NA	NA
	RNA	Thermo Fisher S5 GS	Oncomine Comprehensive Assay Plus RNA
5	**DNA**	Thermo Fisher S5 GS Prime	Oncomine Focus Assay
	RNA	Thermo Fisher S5 GS Prime	Oncomine Focus Assay
6	**DNA**	Illumina MiSeq	Myriapod NGS Cancer Probe plus
	RNA	Thermo Fisher S5 GS	Archer Solid Tumor panel
7	**DNA**	Illumina MiSeq	Archer FUSIONPlex Core Solid Tumor panel
	RNA	Illumina MiSeq	Archer FUSIONPlex Core Solid Tumor panel
7b	**DNA**	Illumina NextSeq 550	Myriapod NGS Cancer Probe plus
	RNA	Illumina NextSeq 550	Myriapod NGS Cancer Probe plus
8	**DNA**	Illumina MiSeq	Myriapod NGS Cancer panel DNA
	RNA	Illumina MiSeq	Myriapod NGS Cancer panel RNA (NG33-NG101)
9	**DNA**	Illumina Novaseq6000	TruSight Oncology 500 High-Throughput
	RNA	Illumina Novaseq6000	TruSight Oncology 500 High-Throughput
Technology variations for validation set			
3	**RNA**	Thermo Fisher Genexus	Oncomine Precision Assay GX
6	**RNA**	Thermo Fisher S5 GS	Oncomine Precision Assay GX
6b	**RNA**	Thermo Fisher S5 GS	Oncomine Comprehensive Assay Plus
8	**RNA**	Illumina MiSeq	Myriapod NGS Cancer Probe plus

Abbreviations: DNA (*Deoxyribonucleic Acid*); RNA (*Ribonucleic Acid*).

In a single case a real-time PCR (RT-PCR) system was adopted to confirm the *FGFR* molecular result (ID#1). Of note, two different hybridization-based NGS panels (Archer Solid Tumor panel and Myriapod NGS cancer probe PLUS) were used on MiSeq and NextSeq550 (Illumina) systems, respectively, to test both DNA and RNA samples in a single institution (ID#7). In addition, two distinct technical strategies (amplicon-based Archer Solid Tumor panel on S5 GS system and hybridization based Myriapod NGS cancer probe PLUS on Miseq platform) were implemented to assess *FGFR* molecular analysis on RNA and DNA, respectively, by participating institution ID#6. Overall, 3 of 5 (60.0%) and 2 of 5 (40.0%) remaining institutions adopted amplicon based and hybridization-based testing strategy for DNA based application whereas RNA based molecular analysis was carried out adopting amplicon based and hybridization-based testing approach in 4 (66.7%) and 2 out of 6 (33.3%) respectively (Tab. III). The same diagnostic algorithm was used by 6 of 9 participating members to detect *FGFR2(17)-BICC1(3)* aberrant transcript in the second round slide set. In two cases (ID#3, #8) an automatized system (Genexus Purification System, Thermofisher Scientifics) and a manual assay (QIAamp DNA FFPE Tissue Kit for DNA/RNA Extraction, Qiagen) were adopted switch-

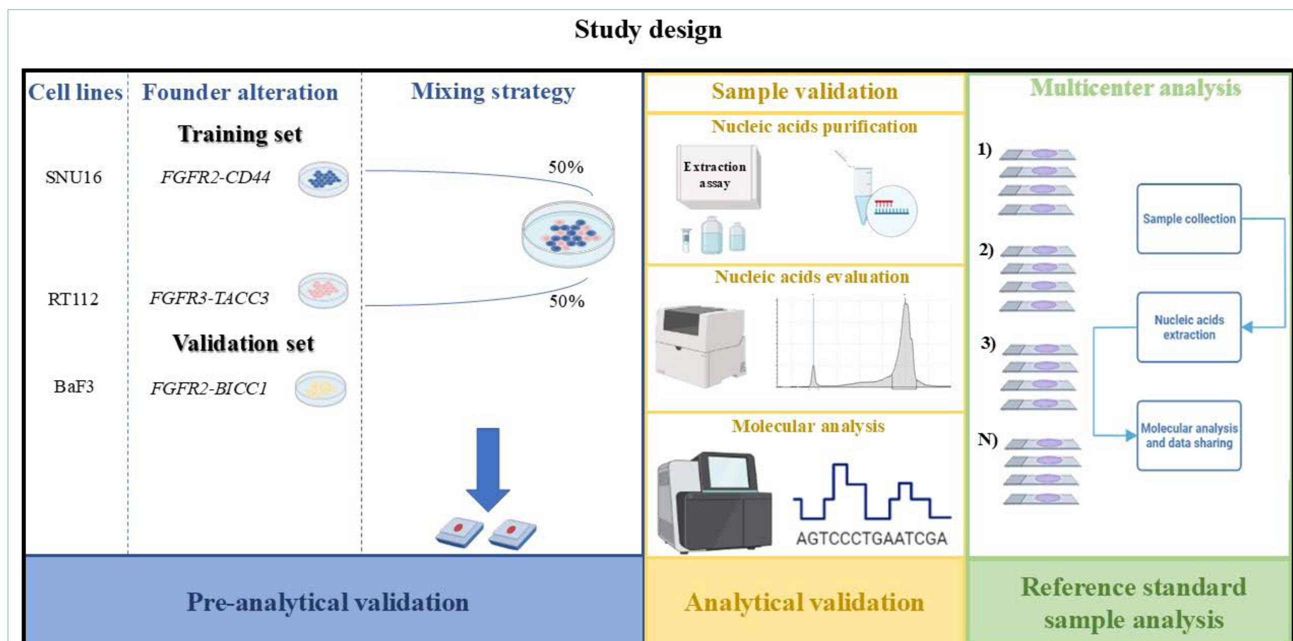

Figure 1. Schematic representation of the study design. Briefly, after internal validation of standard reference sample at University of Naples Federico II, a set of 8 slides were distributed at each center, where internal diagnostic workflow (from nucleic acid extraction to molecular data interpretation) were carried out. Data were shared with coordinator center. Abbreviations: BEC (Beclin 1); CEP110 (Centriolin); FGFR1-2-3 (Fibroblast Growth Factor Receptor 1-2-3); PPHLN1 (Periphilin 1); TACC1-3 (Transforming Acidic Coiled-Coil Containing Protein 1-3).

ing from previously reported methods (Tab. II). Different NGS panels were used in the remaining cases compared with first round slide set. Institutions #3, 6, 8 upgraded NGS assays, as shown in Table III. A simultaneous NGS analysis using Oncomine precision assay (OPA) and Oncomine comprehensive assay (OCA) on Genexus ™ system (Thermofisher Scientifics) was used in two institutions (ID#2, #6). In addition, ID#5 successfully tested both slides and RNA extracted shared by coordinator institution in two dedicated shipments slides vs cell pellet, 1859 vs 5974 reads) (Tab. III) *FGFR3(17)-TACC3(11)* aberrant tran-

Table IVA. Molecular results of standard reference samples for each participating institution in training set. The list of *FGFR* aberrant transcripts were reported.

Center ID	RNA aberrations	Reads count	DNA CNV	CNV
1	**FGFR3** FGFR3-TACC3 F17:T11	48545	**FGFR2**	5
2	**FGFR3** FGFR3-TACC3 F17:T11	5641	**FGFR2**	79.0
2b	**FGFR3** FGFR3-TACC3 F17:T11 **FGFR2** CD44-FGFR2 C1:F3	172611 1750	**-**	**-**
3	**FGFR3** FGFR3-TACC3 F17:T11	72216	**FGFR2**	196.0
4	**FGFR3** FGFR3-TACC3 F17:T11 **FGFR2** CD44-FGFR2 C1:F3	122102 485	**-**	-
5	**FGFR3** FGFR3-TACC3 F17:T11	126608	**FGFR2**	ND
6	**FGFR3** FGFR3-TACC3 F17:T11	1622	**FGFR2**	5.1
7	**FGFR3** FGFR3-TACC3 F17:T11 **FGFR2** CD44-FGFR2 C3:F3	4795 6	**-**	-
7b	**FGFR3** FGFR3-TACC3 F17:T11	28270	**FGFR2**	5.3
8	**FGFR3** FGFR3-TACC3 F17:T11	71296	**FGFR2**	4.8

Abbreviations: DNA (*Deoxyribonucleic Acid*); FGFR (*Fibroblast Growth Factor Receptor*); ND (*Not defined*); RNA (*Ribonucleic Acid*).

Table IVB. Molecular results of standard reference samples for each participating institution in validation set. The list of *FGFR* aberrant transcripts is reported.

Center ID	RNA aberrations	Reads count	Sample type
1	**FGFR2** FGFR2-BICC1 F17:B3	1244	CB Slide
2	**FGFR2** FGFR2-BICC1 F17:B3	186	CB Slide
2b	**FGFR2** FGFR2-BICC1 F17:B3	1471710	CB Slide
3	**FGFR2** FGFR2-BICC1 F17:B3	340	CB Slide
4	**FGFR2** FGFR2-BICC1 F17:B3	162230	CB Slide
5	**FGFR2** FGFR2-BICC1 F17:B3	5974	Pellet
5b	**FGFR2** FGFR2-BICC1 F17:B3	1859	CB Slide
6	**FGFR2** FGFR2-BICC1 F17:B3	226	CB Slide
6b	**FGFR2** FGFR2-BICC1 F17:B3	568664	CB Slide
7	**FGFR2** FGFR2-BICC1 F17:B3	5072	CB Slide
8	**FGFR2** FGFR2-BICC1 F17:B3	5680	Pellet
9	**FGFR2** FGFR2-BICC1 F17:B3	3583	CB Slide

Abbreviations: CB (*Cell Block*); DNA (*Deoxyribonucleic Acid*); FGFR (*Fibroblast Growth Factor Receptor*); ND (*Not defined*); RNA (*Ribonucleic Acid*).

script was successfully identified by all institutions inspecting RNA derived molecular records. In this regard, a median range of 53455.0 (ranging from 1622.0 to 126608.0) mapped reads were detected across institutions. Moreover, *FGFR3(17)-TACC3(11)* was also confirmed by ID#1 institution adopting RT-PCR platform (Cq 26.7) (Tab. IVA). Noteworthy, ID#8 successfully detected *FGFR3(17)-TACC3(11)* aberrant transcript with an amplicon based, and a hybridization based NGS panel. In addition, three institutions (ID#2, ID#4, ID#7A) detectedFGFR2(3)-CD44(1) on the same RNA sample leveraging CGP assay. Considering DNA-based molecular analysis, n = 7 institutions (ID#1, ID#2, ID#3, ID#5, ID#6, ID#7, ID#8) identified *FGFR2* CNVs (median ratio of 49.2, range from 4.8 to 195.9). All institutions implementing DNA-based NGS analysis were able to detect a series of molecular alterations listed in Supplementary Table II. Particularly, *PIK3CA* exon 9 p.E545K hotspot mutation was successfully detected in all cases. Noteworthy, *FGFR2(17)-BICC1(3)* was successfully identified in all instances (Tab. IVB). A median read count of 185564.0 (ranging from 186.0 to 1471710.0) was assessed on *FGFR2(17)-BICC1(3)* analysis. As shown in Table IV A-B, significant variation was observed among participating institutions in terms of read count on referenced alterations.

CGP strategy targeting FGFR2 aberrant transcripts in iCCA patients

To validate CGP based diagnostic algorithm targeting *FGFR2* aberrant rearrangements on real world iCCA samples, a retrospective series of FFPE samples from iCCA samples were retrieved from internal archive of Fondazione Policlinico Universitario (FPG) "Agostino Gemelli" IRCCS (Rome).

In 2022, FPG500 promoted a CGP program (Ethical committee approval number: ID#3837) recruiting 11 different cancer types to be tested leveraging in-house CGP strategy [19]. In brief, FFPE specimens showing a tumor cell (TC) content ≥ 20.0%, combined with adequate DNA (≥40 ng/μL) and RNA (≥90 ng/μL) amounts required by CGP assay, were tested using TruSight Oncology 500 High Throughput (TSO500HT, Illumina Inc., San Diego, CA) [20,21]. DNA/RNA were simultaneously extracted from 2×5-μm FFPE scrolls using AllPrep® DNA/RNA FFPE kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. If extracted DNA/RNA quality checks were lower than the established cut-off, alternative methods were adopted: Qiamp DNA Micro Kit (Qiagen, Hilden, Germany); RNeasy DSP FFPE Kit (Qiagen, Hilden, Germany). DNA and RNA concentrations were measured on a Qubit 2.0 Fluorometer (ThermoScientific, Paisley, UK) using the Qubit dsDNA High Sensitivity and RNA High Sensitivity assay kits, respectively. The percentage of fragments > 200 bp (DV200) was assessed for RNA samples using the Agilent RNA ScreenTape kit on the TapeStation 4200 platform (Agilent Technologies, CA, USA). DNA was evaluated using the Infinium HD FFPE quality control (QC) Assay Protocol (Illumina, Cambridge, UK) on the CFX Connect Real-Time PCR Detection System instrument (Biorad). Quality cut-off assessing fragmentation of nucleic acids was established as follows: RNA with DV200 ≥ 20%; DNA with Delta Cq value ≤ 5.

From April 2022 to June 2024, a total of n = 59 iCCA patients were included in the FPG500 program. Interestingly, DNA and RNA from 37 of 59 specimens (62.7%) met the quality specifications required for CGP and were successfully analyzed. For 17 samples falling due to low input that ranges 10 ng/μl < DNA < 40 ng/μl and 10 ng/μl < RNA < 90 ng/μl) OPA assay (Thermo Fisher Scientific) was used to simultaneously evaluate RNA fusions and DNA variants.

In 5 cases, molecular profiling was partially carried out or failed: 2 of 5 patients underwent DNA analysis with the *TSO500HT* virtual panel (reporting only *IDH1* mutations) while RNA analysis failed; 3 out of 5 patients underwent orthogonal DNA profiling and RNA analysis showed uninterpretable data.

In line with literature, 4 of 37 (10.8%) CGP samples highlighted *FGFR2* in-frame rearrangements eligible to target treatment confirmed by Archer™ FUSIONPlex™ Sarcoma v2 panel (Archer) assay (Tab. V).

Table V. Real life experience of referral institution to test FGFR2 on routine samples.

Samples	NC%	RNA aberrations	Reads count	Functional Consequence
Case #1	80	**FGFR2** FGFR2-EFCAB14 F17:E4	112	Inframe
Case #2	35	**FGFR2** FGFR2-DBP F17:D4	1325	Inframe
Case #3	70	**FGFR2** FGFR2-KHDRBS1 F17:K2	1015	Inframe
Case #4	20	**FGFR2** FGFR2-GOLGA6C F17:G1	200	Inframe

Abbreviations: *DBP* (D-Box Binding PAR BZIP Transcription Factor); *EFCAB14* (EF-Hand Calcium Binding Domain 14); *FGFR2* (Fibroblast Growth Factor Receptor 2); *GOLGA6C* (Golgin A6 Family Member C); *KHDRBS1* (KH RNA Binding Domain Containing, Signal Transduction Associated 1); *NC%* (Neoplastic cells percentage); *RNA* (Ribonucleic acid).

Discussion

With the advent of the genomic era, the clinical paradigm for iCCA patients has radically shifted [22]. In this context, *FGFR2* aberrant transcripts emerged as pivotal predictive target to select the best therapeutical option for iCCA patients [23]. As a consequence, *FGFR2* molecular testing has become essential in the diagnostic routine practice of molecular laboratories but the lack of optimized procedures may hinder the clinical stratification of iCCA patients [24]. We assessed the real-world proficiency of *FGFR2* molecular testing in 8 Italian laboratories using an artificial reference mimicking iCCA diagnostic routine samples. Interestingly, both DNA and RNA were simultaneously tested in 6 of 8 (75.0%) participating institutions to detect *FGFR2* clinically relevant aberrant transcripts. FISH historically represents the gold standard strategy to detect RNA fusions but scant reference range and high interlaboratory variability are opening challenges [25]. Alternative techniques, mostly NGS-based, are commonly adopted for targeting *FGFR2* aberrant transcripts in the routine practice of iCCA patients [8,25-27]. A series of 10 reference samples (4 positive and 6 negative *FGFR2* cases from iCCA patients) was tested in two consecutive round robin tests involving 21 participating institutions. Interestingly, *FGFR2* aberrant transcripts were heterogeneously investigated adopting RNA based NGS assays demonstrating a low agreement rate (37.5%) on uncommon *FGFR2* fusion partners [*FGFR2*(NM_000141.4)::*ATE1*(NM_007041.3) dependent on missing primers mapping *ATE* [17]. In this scenario, the wide technical landscape of NGS assays designed to target *FGFR2* aberrant rearrangements significantly impacts the detection rate of clinically relevant *FGFR2* transcripts in clinical practice. Comparing DNA- and RNA-based NGS strategies and break-apart FISH analysis on a series of 226 iCCA samples a total agreement of 95.1% was achieved. Of note, a combined DNA-RNA based strategy was able to both identify novel fusion partners and assess a positivity rate of 10.1% [28]. Notably, NGS assays able to cover both kinase domain usually and unknown partners in *FGFR2* aberrant transcripts, are recommended by ESMO guidelines as upfront testing strategy [12]. Accordingly, all institutions achieved a complete agreement in *FGFR3(17)-TACC3(11)* detection, irrespective of the technical specifications of NGS assays (amplicon-based vs hybridization-based NGS panels) [29]. In addition, *FGFR2(3)-CD44(1)* was successfully detected on RNA of standard reference samples in three institutions (ID#2, ID#4, ID#7) adopting a CGP assay (sensitivity 100.0% versus 12.5% of target NGS assays, p = 0.002). Conversely, *FGFR2* CNV signals were detected by all institutions on DNA samples demonstrating that both target and CGP assays can identify unbalanced *FGFR2* on DNA samples whereas RNA based *FGFR2(3)-CD44(1)* aberrant rearrangement was detected by CGP assays (3 out of 4 centers) thanks to reference range covering common and uncommon fusion partners. Noteworthy, CGP assays highlighted higher technical performance in detecting *FGFR2* aberrant transcripts compared to target NGS assays [30]. Our data provides evidence that CGP panels should be preferentially adopted in *FGFR2* molecular analysis for optimizing clinical stratification of iCCA patients [29]. ID#2 successfully detected *FGFR2(3)-CD44(1)* adopting CGP panel after a previous target NGS analysis on actionable genes (Tab. III). Supporting data derived from standard reference samples demonstrated higher analytical performance of CGP strategies compared with targeted NGS panels revealing *FGFR2* clinically relevant rearrangements in diagnostic routine iCCA patients [31]. To date, RNA represents the most insightful source to detect aberrant transcripts, but the lack of harmonized procedures should suggest that integrating RNA and DNA molecular analysis to identify iCCA patients eligible to target therapy may represent the best technical strategy [32]. These data were also confirmed detecting *FGFR2(17)-BICC1(3)* aberrant transcripts in the second round of standard reference sample during validation step. Inter-

estingly, all participating laboratories clearly detect *FGFR2* breakpoints. RNA from slide sets and cell pellets were successfully investigated confirming *FGFR2(17)-BICC1(3)* aberrant fusion. Moreover, optimized analytical procedures are also fundamental for generating technically robust and clinically relevant reports. In our study, read-counts supporting *FGFR2* CNV from DNA-based analysis of standard reference samples were heterogeneous, because of different pre-analytical and analytical handling procedures. Standardized technical cut-offs defined by scientific societies are required to increase concordance rate for molecular testing.

Small tissue biopsies are the conventional diagnostic samples available to perform molecular analysis of predictive biomarkers in iCCA patients [23]. Consequently, a non-negligible percentage of iCCA patients may not benefit from target treatments due to insufficient tumor tissue [19]. In this regard, harmonized pre-analytical procedures play a crucial role in sparing diagnostic tissue for molecular analysis [24]. We found that nucleic acids from SNU16 and RT112 suspensions yielded a higher recovery rate compared with matching cell blocks. Furthermore, DNA and RNA fragmentation profiles also revealed a significant variation between cell line resuspension and cell blocks, further demonstrating the impact of pre-analytical handling processes on successful rates of molecular testing. In addition, technical scenarios of nucleic acis isolation procedures confirmed the high variability of recovering DNA and RNA, but all participants successfully carried out molecular analysis. At the sight of these critical points, a diagnostic workflow integrating orthogonal technologies is fundamental to successfully manage iCCA patients [14]. In our study, ID#1 confirmed *FGFR2(3)-CD44(1)* aberrant transcript using RT-PCR assay revealed that diagnostic algorithms built on complementary technical approaches are essential in optimizing turnaround times of molecular testing [33,34]. Despite several insights, our study has limitations. Firstly, the standard reference sample, developed to mimic diagnostic routine samples of iCCA patients covering *FGFR* aberrant transcripts, was affected by heterogeneous neoplastic cell abundance among the sections. Secondly, this study focused on *FGFR2* molecular testing and did not cover *IDH1* actionable alterations to evaluate the technical performance of diagnostically available solutions on *FGFR2* clinically relevant rearrangements. Further harmonization trials on dedicated standard reference specimens should be designed to comprehensively investigate clinically relevant biomarkers in iCCA patients. Interestingly, 18-plex Seraseq Fusion RNA Mix v4 reference standard sample (LGC Seracare, Milford USA), containing 18 RNA targets (including *FGFR2*) quantified by dCPR system during manufacturing procedures (0.2 µg/µL) was compared with an orthogonal pyrophosphorolysis based assay to evaluate the technical performance on the detection of clinically relevant aberrant rearrangements across several tumor types. In terms of technical performance, 6 molecules per 6 µL target volume was the minimum request to yield a perfect match in molecular profiling of RNA rearrangements between the two technical approaches [35]. Despite these promising results, the lack of technical harmonization trials specifically focusing on *FGFR* analysis in iCCA patients paves the way for a novel diagnostic workflow (spanning from internal/external control to critical inspection of quality metrics supporting *FGFR* aberrant transcripts by NGS analysis) and fill a gap in diagnostic setting by developing an accurate, reproducible, and technically affordable testing strategy for *FGFR* analysis in iCCA [36].

Abbreviations list:

ARID1A: AT-Rich Interaction Domain 1A
BAP1: BRCA1 Associated Deubiquitinase 1
BICC1: BicC Family RNA Binding Protein
CCA: Cholangiocarcinoma
CD44: CD44 Molecule
CGP: Comprehensive Genomic profiling
CNV: Copy Number Variation
DCR: Disease Control Rate
DIN: DNA Integrity Number
DNA: DeoxyriboNucleic Acid
dPCR: Digital Polymerase Chain Reaction
eCCA: extrahepatic Cholangiocarcinoma
FFPE: Formalin Fixed Paraffin Embedded
FGFR1-3: Fibroblast Growth Factor Receptor1-3
FISH: Fluorescent in Situ Hybridization
KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog
iCCA: intrahepatic Cholangiocarcinoma
IDH1/2: Isocitrate Dehydrogenase (NADP(+)) 1/2
NGS: Next Generation Sequencing
OCA: Oncomine comprehensive assay
OPA: Oncomine precision assay
PBRM1: Polybromo 1
PBS: Phosphate Buffered Saline
PFS: Progression-Free Survival
PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
RIN: RNA Integrity Number
RNA: RiboNucleic Acid
RT-PCR: Real Time Polymerase Chain Reaction
TACC3: Transforming Acidic Coiled-Coil Containing Protein 3

TAT: Turnaround Time
TK: Tyrosine Kinase
TKI: Tyrosine Kinase Inhibithor
TP53: Tumor Protein P53

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Conflict of interest statement

Francesco Pepe has received personal fees as speaker bureau from Menarini, Roche for work performed outside of the current study. Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen and Bayer, unrelated to the current work. Matteo Fassan has received personal fees as speaker bureau from Amgen, Astellas, Astra Zeneca, BMS, Diapath, Eli Lilly, GSK, Incyte, IQvia, Janssen Pharma, MSD, Novartis, Pierre Fabre, Roche, Thermofisher, Sanofi, Pfeizer unrelated to the current work.

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Authors contributions

Conceptualization, Francesco Pepe, Gianluca Russo, Giancarlo Troncone and Umberto Malapelle.; methodology, all the authors; software, Francesco Pepe, Gianluca Russo; validation, all the authors; formal analysis,, all the authors; data curation, Francesco Pepe, Gianluca Russo and Umberto Malapelle.; writing—original draft preparation, Francesco Pepe, Gianluca Russo; writing—review and editing, Giancarlo Troncone and Umberto Malapelle.; visualization all the authors; supervision, Giancarlo Troncone, and Umberto Malapelle.; project administration, Giancarlo Troncone and Umberto Malapelle All authors have read and agreed to the published version of the manuscript.

Ethics consideration

IRB approval is not required.

Data availability statement

Data are available on request to the corresponding author. All data relevant to the study are included in the article or uploaded as supplementary information All data that are publicly available and used in the writing of this article in the text and the reference list.

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