Unexpected finding of a rare pathogenic germline BRCA1 variant in an intrahepatic cholangiocarcinoma using the Oncomine Focus DNA assay: clinical and diagnostic implications

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Abstract

Background

Cholangiocarcinoma (CCA) is a malignant tumor arising from the epithelial cells of the bile ducts and is the second most common liver cancer after hepatocellular carcinoma.

Recently, our Institution launched a Comprehensive Genomic Profiling (CGP) program (named FPG500 program), set up to provide a complete molecular characterization through the TruSight Oncology 500 High Throughput (TSO500HT) solution and samples that do not reach pre-set sample quantity and/or quality thresholds required for TSO500HT, are addressed to Oncomine Focus DNA Assay (OFA) and the Archer's FusionPlex Lung Panel (AFL).

Methods and results

Here we report the case of a patient with iCCA enrolled in the FPG500 program and screened by the orthogonal workflow (OFA/AFL). Although BRCA1 is not among the genes declared in the OFA panel, we unexpectedly detected a pathogenic variant in this gene (c.5278-2del, rs878853285).

Conclusions

This case highlights the diagnostic capabilities of CGP, now widely used in both clinical practice and academic setting. The incidental involvement of BRCA1 focuses attention on the role of BRCA genes in biliary tract cancers. Finally, as an orthogonal test confirmed the germline origin of BRCA1 c.5278-2del variant, the germline implications of CGP need to be considered.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor arising from the epithelial cells of the bile ducts and is the second most common liver cancer after hepatocellular carcinoma [1, 2]. Based on anatomical location of the primary tumor, CCA is classified into intrahepatic (iCCA) and extrahepatic (eCCA).

The most frequent genomic alterations found in iCCA involve mutations in the isocitrate dehydrogenases (NADP+) 1/2 (IDH1/2) gene, rearrangements and fusions of the fibroblast growth factor receptor 2 (FGFR2) gene, and amplification of the human epidermal growth factor receptor 2 (ERBB2) gene [3].

Other alterations such as the presence of microsatellite instability (MSI), BRAF mutations, neurotrophin receptor tyrosine kinase (NRTK) fusions, and KRAS mutations have been reported, potentially enlarging the therapeutic options for iCCA patients. Additionally, pathogenic germline variants of cancer susceptibility genes such as BRCA1/2, RAD51D, mutL homolog 1 (MLH1), or mutS homolog 2 (MSH2) are also detected in iCCA patients [4].
Recently, our Institution (FPG: Fondazione Policlinico Universitario “Agostino Gemelli” IRCCS) launched a Comprehensive Genomic Profiling (CGP) program (named FPG500 program: Ethical committee approval number 3837) enrolling 10 different cancer types [5].

Herein, we report the case of a patient with iCCA enrolled in the FPG500 program. CGP profiling was set up to provide a complete molecular characterization through the TruSight Oncology 500 High Throughput (TSO500HT, Illumina) solution [6]. According to our FPG500 workflow (Fig. 1), samples that do not reach pre-set sample quantity and/or quality thresholds (DNA ≥ 3.5ng/µL and RNA ≥ 8.5ng/µL) required for the assessment of Next Generation Sequencing (NGS) via TSO500HT, are addressed to orthogonal NGS molecular analysis approaches. In these cases, the Oncomine Focus DNA Assay (OFA) (Thermo Fisher, Waltham, MA, USA) and the Archer's FusionPlex Lung Panel (AFL) (Archer, Boulder, CO, USA) were adopted for DNA and RNA evaluation, respectively.

The iCCA case here described was addressed to orthogonal evaluation for both DNA and RNA analyses. Despite not comprised among the genes tested in the OFA panel, we unexpectedly detected a pathogenic variant in the BRCA1 gene (c.5278-2del, rs878853285, NM_007294.4).

The germline role of BRCA1/2 genes in iCCA patients, as well as the predictive and diagnostic capabilities of CGP that are now widely utilized in both clinical practice and academic settings, are here discussed. Finally, as an orthogonal assay confirmed the germline origin of the c.5278-2 variant, germline implications of somatic mutational profiling is also discussed.

Materials and methods

Case Presentation

An early 50s smoker (20–30 cigarettes/day) man presented with weight loss of about 7 kg/3 months and jaundice. An abdomen ultrasound (US) and subsequent hepatic magnetic resonance imaging (MRI) showed a coarse mass at the hepatic hilum infiltrating both hepatic ducts and determining marked dilatation of intrahepatic bile ducts (IBD). An internal biliary stent placement and liver biopsy were carried out. Chest computed tomography (CT) was negative for metastases. Histological examination of liver biopsy documented an adenocarcinoma with positive expression at immunohistochemistry (IHC) assay for cytokeratin (CK)-7, -19 and -20, and caudal-type homeobox transcription factor 2 (CDX2), compatible with CCA (Klatskin IIIb).

In relation to histology and disease stage, patient underwent first-line chemotherapy with the association of Cisplatin (25 mg/m² ev) and Gemcitabine (1000 mg/m² ev) for a total of eight cycles. Treatment was complicated by platelet count decrease (grade 2 for CTCAE 5.0) and anemia (grade 2 for CTCAE 5.0). Hepatic MRI after 4 and 8 cycles both documented a minimal dimensional reduction (< 20%) of the hepatic mass, configuring a stable disease (SD) according to RECIST 1.1 criteria. No additional pulmonary or abdominal lesions appeared on CT scan.
To explore the presence of potential molecular biomarkers, patient was addressed to a CGP. A written informed consent was obtained and tumor specimen was addressed to FPG500 program. In Fig. 2, the patient's clinical history is shown.

**DNA/RNA isolation and qualification**

After an informed consent was obtained, DNA/RNA were extracted from 2 × 5-µm formalin-fixed paraffin-embedded (FFPE) scrolls using AllPrep® DNA/RNA FFPE kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

DNA and RNA concentrations were measured on a Qubit 2.0 Fluorometer (Thermo Scientific, Paisley, UK) using the Qubit dsDNA High Sensitivity and RNA High Sensitivity assay kits, respectively. Nucleic acid purity was assessed by NanoPhotometer P-Class (Implen), evaluating the ratio of the absorbance at 260 nm and 280 nm and 260 nm and 230 nm. DNA quality was determined by the Infinium HD FFPE quality control (QC) Assay Protocol (Illumina, Cambridge, UK). The percentage of fragments > 200 nucleotides in size (DV200) was assessed for RNA using TapeStation 4200 in association with the Agilent RNA ScreenTape kit (Agilent Technologies, Santa Clara, CA, USA). At least 20% tumor cell content was required.

**DNA/RNA library preparation, sequencing and data analysis**

The OFA (Thermo Fisher Scientific, Inc.) was used to generate sequencing libraries to identify single nucleotide variants (SNVs), **indels** and copy number variations (CNVs) in the following genes: AKT1, ALK, AR, BRAF, CDK4, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ERBB4, ESR1, FGFR2, FGFR3, GNA11, GNAQ, HRAS, IDH1, IDH2, JAK1, JAK2, JAK3, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, PDGFRA, PIK3CA, RAF1, RET, ROS1 and SMO.

Library preparation, amplification and ligation steps were performed in line with the OFA protocol. Sequencing was performed using semiconductor sequencing technology (Ion S5 Prime sequencer, Thermo Fisher Scientific). Analysis was carried out using Ion Torrent Suite™ Browser version 5.0 and Ion Reporter™ version 5.0.

The AFL NGS assay is designed to detect key fusions in 17 genes, skipping events in EGFR vIII and MET exon 14 skipping, and select point mutations in 14 key lung cancer-associated genes. The AFL uses Archer's Anchor Multiplex PCR chemistry to target regions of interest. Reagent preparation and DNA synthesis, ligation and amplification are performed according to the official assay protocol. Libraries were multiplexed for sequencing on an Illumina MiSeq. Data were analyzed with the Archer Analysis software.

**BRCA1 (c.5278-2del, rs878853285) variant identification: orthogonal assay and confirmatory test on blood**

The BRCA1 variant (c.5278-2del, rs878853285, NM_007294.4) was confirmed by an orthogonal assay. In particular, the Deyvser BRCA kit (Deyvser AB, Stockholm, Sweden) was used according to our well-established workflow [7]. In addition, as variant allele frequency (VAF) (44%) raised suspicion of a likely
germline origin, a blood sample from the patient was requested. After blood DNA extraction, targeted Sanger sequencing for the \textit{BRCA1} variant was performed, as previously reported [8].

**Results**

**Mutation analysis**

The AFL NGS assay did not identify any oncogenic/likely oncogenic fusion event.

Instead, after applying stringent parameters for reliable variant calling (coverage depth > 500X; VAF > 5%) the Ion Torrent Suite™ Browser called an oncogenic variant (VAF = 44%) in \textit{BRCA1} gene. Integrative Genomics Viewer (IGV) inspection of this alteration is shown in Fig. 3A. \textit{BRCA1 c.5278-2delA} (known as \textit{IVS20-2delA} in the literature) affects an acceptor splice site in intron 20 of the gene. It is expected to disrupt RNA splicing and likely results in an absent or disrupted protein product. This variant has been observed in several individuals with a personal or family history of breast or ovarian cancer and experimental studies have shown that this variant disrupts mRNA splicing [9]. For these reasons, this variant has been classified as pathogenic. To date, this variant does not have a GnomAD frequency.

Considering that \textit{BRCA1} is not declared in the OFA genes panel, we investigated the underlying reason for the sequencing of this specific region. As shown, in the OFA Browser Extensible Data (BED) file, which describes genomic coordinates starting from sequencing data, this \textit{BRCA1} region (exon 21) and also 4 additionally gene regions are effectively sequenced. In details, these regions are considered amplification control regions for CNV calling.

Additional NGS Deyvser \textit{BRCA} (Devyser AB, Stockholm, Sweden) test confirmed the presence of \textit{BRCA1} variant on tissue specimen (Fig. 3B), while the targeted Sanger for the \textit{BRCA1} variant, performed on peripheral blood highlighted the germline origin of the variant (Fig. 3C).

**Discussion**

OFA is a highly accurate and reproducible platform for the detection of hotspot mutations in 35 cancer genes, using low input FFPE derived DNA. OFA is also able to identify CNVs in 19 genes of the panel. In this context, some amplicons are sequenced only for the purpose of creating a sample-specific baseline, as reported in the BED file, that will be used by the software to calculate the normal ploidy of the sample. In details, 5 amplicons map to different highly conservative \textit{BRCA1} regions and all the generated reads are used in the CNV pipeline.

Another BED file, called the \textit{hotspot} BED file, provides the instructions on the location of the hotspot mutations. By default, the OFA analysis workflow not only calls canonical hotspot mutations, but also all variants that fall within target regions, so that mutations outside the gene list can be seen. This was the reason for the unexpected identification of the \textit{BRCA1 c.5278-2delA} variant in the iCCA patient, as here described.
This variant was first confirmed by an orthogonal assay and then highlighted in the patient's blood.

The \textit{BRCA1} incidental finding is of great interest in the context of iCCA, focusing on the role of \textit{BRCA} genes in biliary tract cancers (BTCs).

Genomic studies have highlighted the molecular heterogeneity of BTCs at different locations of the biliary tree and revealed that up to 40\% of iCCA patients harbor actionable molecular alterations. Among these, the most prevalent are \textit{FGFR2} fusions and \textit{IDH1} mutations and, with lower frequencies, alterations affecting \textit{BRAF}, \textit{ERBB2}, or members of the DNA damage repair (DDR) pathway, most notably \textit{BRCA} genes\cite{10}.

\textit{BRCA} are the most well-studied DDR pathway genes and their involvement emerged from CGP analysis of patients with BTC. From the molecular evaluation of 75 patients with CCA, Churi \textit{et al.} \cite{11} described as 16\% of iCCA and 40\% of eCCA cases were carriers of alterations affecting genes associated with DNA DDR pathways. In addition, a comprehensive analysis published by Nakamura \textit{et al.} \cite{12} reported a high rate (38.9\%) of potentially targetable somatic genetic alterations in analyzed CCA cases, including the \textit{BRCA} genes.

Interesting, Spizzo \textit{et al.} \cite{14} reported that \textit{BRCA} mutations were detected in 3.6\% (\(n = 46\)) of all BTC samples (\textit{BRCA1}: 0.6\%, \textit{BRCA2}: 3\%). In a recent study, \textit{BRCA} genes have proved to be mutated in 209 iCCA patients, with a predominance of \textit{BRCA2} (2.4\%) over \textit{BRCA1} (1.0\%) \cite{13}. Curiously, \textit{BRCA} mutations in BTC have been observed more frequently as somatic rather than germline mutations. Moreover, BTC patients with \textit{BRCA} mutations do not differ in terms of age from BTC patients with \textit{BRCA}-wt tumors \cite{13}. Conversely, data from the Breast Cancer Linkage Consortium suggested that \textit{BRCA2}-carriers have higher relative risk of developing BTC than patients affected by infection with liver parasites, hepatitis C virus, and hepatitis B virus \cite{14}.

Germline or somatic \textit{BRCA} variants open the possibility to identify a distinct subgroup of BTC patients that may benefit from a personalized treatment strategy \cite{15-17}. In fact, although these mutations generally correlate with poor response to standard treatments, some reports about \textit{BRCA} mutated BTC suggest sensibility to platinum salts and poly (adenosine diphosphate-ribose) polymerase inhibitors (PARPi) \cite{18}, accordingly to good response to cisplatin-based therapy observed in our patient. Clinical trials are now ongoing to establish PARPi as a viable treatment option for \textit{BRCA}-mutated CCA and other solid tumors (NCT03565991; NTC04123366).

This case highlights the effective possibility of somatic gene profiling to reveal underlying germline alterations. Since the purpose of tumour profiling is the identification of somatic mutations, the discovery of germline variants is usually ancillary to the primary purpose of the test. However, we emphasise that while most patients undergoing tumour profiling have advanced disease and the identification of an inherited susceptibility to cancer is unlikely to benefit them, the information is certainly useful to their family members for the cascade screening program.
To date, there is an ongoing debate about the obligation to actively search for germline mutations as part of cancer mutation profiling. Although traditional pre- and post-test counselling may not be necessary or feasible in the context of somatic testing, physicians should be aware of the potential germline risks of somatic testing and honour patients' preferences regarding the receipt of incidental and secondary germline results.

The OFA DNA panel provides a profile of somatic hot spot mutations with the aim of cataloguing somatic changes. Thus, for the genes included in the panel, it may not have been necessary to communicate the potential for incidental and secondary germline information specific to the test offered.

Unexpectedly, in the case of our patient, the test revealed the BRCA1 c.5278-2delA variant, which then resulted of germinal origin. BRCA1, however, is not a gene explicitly reported in the panel and, consequently, the finding of its alteration represents the unique aspect of the presented case.

Therefore, this case highlights the importance of understanding the test offered and whether or not its results may have germline consequences, so that patients can be better informed of any results that may suggest an inherited predisposition to cancer. In this context, the American Society of Clinical Oncology (ASCO) calls for continuing research to develop best practices regarding the provision of incidental and secondary germline results and supports research to improve understanding of patient preferences, develop optimal pre-test information and support informed consent [19].

In this scenario, it is important that healthcare professionals and patients understand which tests do not include germline testing, which should be performed independently if there is a family history that suggests an inherited predisposition to cancer.

Finally, continued commitment of multiple stakeholders will be crucial to refine guidelines and best practices to advance the field of precision medicine for the benefit of cancer patients and their families.

**Conclusions**

CGP programs are allowing further understanding of cancer molecular alterations, opening new scenarios in both therapeutic and prevention terms.

No robust data are available to assess the therapeutic role of targeted agents such as PARPi in iCCA but CGP programs might provide evidences and support hypothesis for future trials.

Finally, for germline alterations of cancer predisposition genes, cascade testing in relatives may imply life-saving surveillance and risk reduction interventions for other family members.

**Abbreviations**

CCA: Cholangiocarcinoma; iCCA: Intrahepatic Cholangiocarcinoma; eCCA: extrahepatic Cholangiocarcinoma; IDH1/2: Isocitrate Dehydrogenases (NADP+) 1/2; FGFR2: fibroblast growth factor
receptor 2; **ERBB2**: epidermal growth factor receptor; **MSI**: microsatellite instability; **NRTK**: neurotrophin receptor tyrosine kinase; **MLH1**: mutL homolog 1; **MSH2**: mutS homolog 2; **FPG**: Fondazione Policlinico Universitario; **CGP**: Comprehensive Genomic Profiling; **NGS**: Next Generation Sequencing; **OFA**: Oncomine Focus Assay; **AFL**: Archer’s FusionPlex Lung;

**US**: abdomen ultrasound; **MRI**: magnetic resonance imaging; **IBD**: intrahepatic bile ducts; **CT**: computed tomography; **IHC**: immunohistochemistry; **CK**: cytokeratin; **CDX2**: caudal-type homeobox transcription factor 2; **SD**: stable disease; **FFPE**: formalin-fixed paraffin-embedded; **QC**: quality control; **SNVs**: single nucleotide variants; **CNVs**: copy number variations; **VAF**: variant allele frequency; **IGV**: Integrative Genomics Viewer; **BED**: Browser Extensible Data; **BTCs**: biliary tract cancers; **DDR**: DNA damage repair; **PARPi**: poly (adenosine diphosphate-ribose) polymerase inhibitors; **ASCO**: American Society of Clinical Oncology.

### Declarations

#### Contributors

Conceptualisation: AM, NN, MDB, FM. Methodology: MDB, AM, EDP. Data analysis: MDB, NN, LG. Data curation: AM, LG, MDB. Writing (original draft preparation): AM, MDB, CN, EDP. Writing, review and editing: all the authors.

#### Compliance with ethical standards

#### Conflict of interest

The authors declared no potential conflicts of interest.

#### Ethical approval

This study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki and was certified by the Committee of the Applicable Institution of the Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome (ID: FPG500, ethical committee approval number 3837).

#### Consent to participate

The patient signed written informed consent before genetic tests.

#### Consent to publish

All authors have given their consent to participate in this report and submit it to Molecular Biology Reports.

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Data availability

The data that supports this study is available upon reasonable request.

References


Figures
Figure 1. FPG500 workflow. Key and decision steps of the process are in bold.

See image above for figure legend.
Figure 2

Patient’s clinical history
Figure 3

IGV visualization and electropherogram image of the BRCA1 c.5278-2delA variant. A. IGV by NGS OFA. B. IGV by NGS Devyser BRCA (Devyser AB, Stockholm, Sweden). As previously reported, Devyser BRCA assay confirmed the presence of BRCA1 variant on tissue specimen (black arrow). C. Electropherogram image of the BRCA1 c.5278-2delA variant (black arrow). A targeted Sanger of the c.5278-2delA variant performed on peripheral blood highlighted the germline origin of the variant.