case reports

Acquired Resistance Mechanisms to PD-L1 Blockade in a Patient With Microsatellite Instability-High Extrahepatic Cholangiocarcinoma

Monica Niger, MD[1]; Federico Nichetti, MD[1,2]; Francesca Dell'Angelo, PhD[3]; Vera Cappelletti, PhD[3]; Chiara Pircher, MD[1]; Marta Vismara, PhD[3]; Christian Cotsoglou, MD[4]; Sherrie Bhoori, MD[5]; Andrea Vingiani, MD[6]; Maria Di Bartolomeo, MD[1]; Filippo Pietrantonio, MD[1]; Filippo de Braud, MD[1,7]; Giancarlo Pruneri, MD[6,7]; Maria Grazia Daidone, PhD[3]; and Vincenzo Mazzaferro, MD[5,7]

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Introduction

Cholangiocarcinoma (CCA) is an aggressive disease, with poor prognosis and limited therapeutic options. Standard chemotherapy (ChT) for advanced CCA is still unsatisfactory, with a median overall survival of < 12 months.[1-3] CCA is a molecularly heterogeneous disease, and several potential molecular targets are reported in up to 47% of patients.[4] Among these, microsatellite instability-high (MSI-H) status is found in < 1% of cases,[4] but it is relevant given the unprecedented benefit of MSI-H cancers from immune checkpoint inhibitors.[5] However, mechanisms of acquired resistance to immune checkpoint inhibition in MSI-H patients need to be elucidated.

Here, we present the clinical history and molecular characterization of a patient with advanced, MSI-H distal CCA (dCCA) treated with ChT, immunotherapy (IO), and repeated surgeries and longitudinally profiled in both tumor samples and liquid biopsies.

The patient provided informed consent for his case to be published and for various studies approved by the institutional ethics committee, allowing the use of clinical and molecular data.

Other than for his case to be published, the patient provided informed consent for the studies: "Exploratory evaluation of new potential prognostic factors in patients with gastrointestinal carcinomas and neuroendocrine neoplasms," "Monitoring of response to conventional or targeted anticancer therapies in patients with cholangiocarcinoma using circulating tumor DNA (ctDNA) analysis," and "An observational study on the feasibility and usefulness of an extensive molecular characterization using FoundationOne CDx in patients with biliopancreatic cancers." All these studies were approved by the institutional ethics committee (INT 117/15, INT 177/14, and INT 105/19, respectively) and allowed use of clinical and molecular data for research purposes.

Clinical Case

In June 2016, a 35-year-old man presented at our institution with right-sided abdominal pain associated with jaundice and cancer antigen 19.9 (CA19.9) increase (73.42 U/mL). He had a history of neonatal jaundice caused by a choledochal cyst and treated with a cyst-jejunum anastomosis (Roux en Y). Computed tomography imaging showed a 5-cm mass originating from the distal biliary tract and enlarged regional lymph nodes. After successful biliary decompression and confirmed histologic diagnosis of dCCA, the disease was deemed unresectable. After jaundice resolution, CA19.9 levels remained below the normality threshold (37 U/mL) throughout the disease course. Overall, the patient's history is depicted in Figure 1. He received upfront cisplatin plus gemcitabine for 6 months with a partial response (PR) that allowed us to perform pancreatoduodenectomy (ypT4N+). Multiplex polymerase chain reaction (PCR) using five quasimonomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-22, and NR-24) showed instability in all the markers, and thus, the cancer was classified as MSI-H. Germline mutations in *MLH1*, *MSH2*, *MSH6*, *EPCAM*, and *PMS2* were absent. Despite adjuvant capecitabine–based chemoradiation, early relapse with the appearance of peritoneal and liver metastases was observed. Therefore, the patient started atezolizumab within a clinical trial. The treatment was administered at the flat dose of 1,200 mg once every 3 weeks and achieved a PR (–34%), which was sustained for 14 cycles until liver, peritoneal, and nodal disease progression (Fig 2). Then, second-line ChT with fluoruracil plus oxaliplatin achieved a new PR, thus allowing a repeated surgical resection of the liver lesion, right hemicolectomy with removal of the peritoneal nodule, and regional lymphadenectomy in December 2018. Molecular profiling (FoundationOne CDx[6]) confirmed the MSI-H status, with a high tumor mutational burden (TMB, 48 Muts/Mb) and several gene alterations (Fig 3), including a subclonal mutation of *ATM* and a *BRCA2* deletion. No germline mutation in *BRCA1* and *BRCA2* was documented. After approximately 6 months, disease progression occurred and retreatment with cisplatin and

ASSOCIATED CONTENT

Author affiliations and support information (if applicable) appear at the end of this article.

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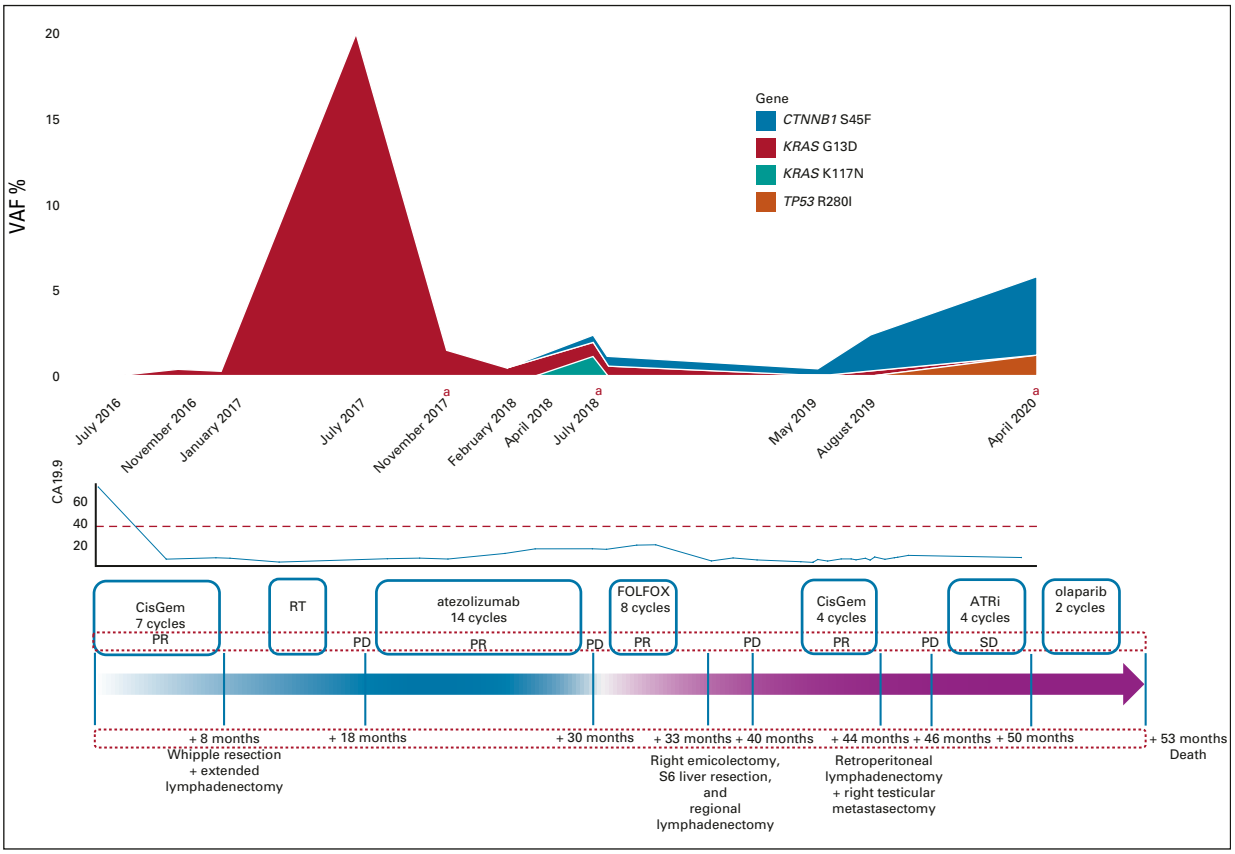

FIG 1. Patient's disease history and treatment sequence paired with ctDNA evaluation. The timeline displays sequential treatment that the patient received from diagnosis (June 2016) until death (October 2020). Reported VAF% refers to data obtained by ddPCR personalized assays. At selected times ([a]November 2017, July 2018, and April 2020 in the plot), plasma samples were subjected to direct sequencing with the Oncomine Pan-Cancer Cell-free assay, and the identified variants, confirmed by ddPCR, were tracked at subsequent timepoints. CA19.9 levels are reported, with the red dashed line indicating the normal value threshold (< 37 U/mL). ATRi, ATR serine/threonine kinase inhibitor; CA, cancer antigen; CisGem, cisplatin plus gemcitabine; ctDNA, circulating tumor DNA; ddPCR, digital droplet polymerase chain reaction; FOLFOX, fluoruracil plus calcium levofolinate plus oxaliplatin; PD, progressive disease; PR, partial response; RT, radiation therapy; SD, stable disease; VAF, variant allele fraction.

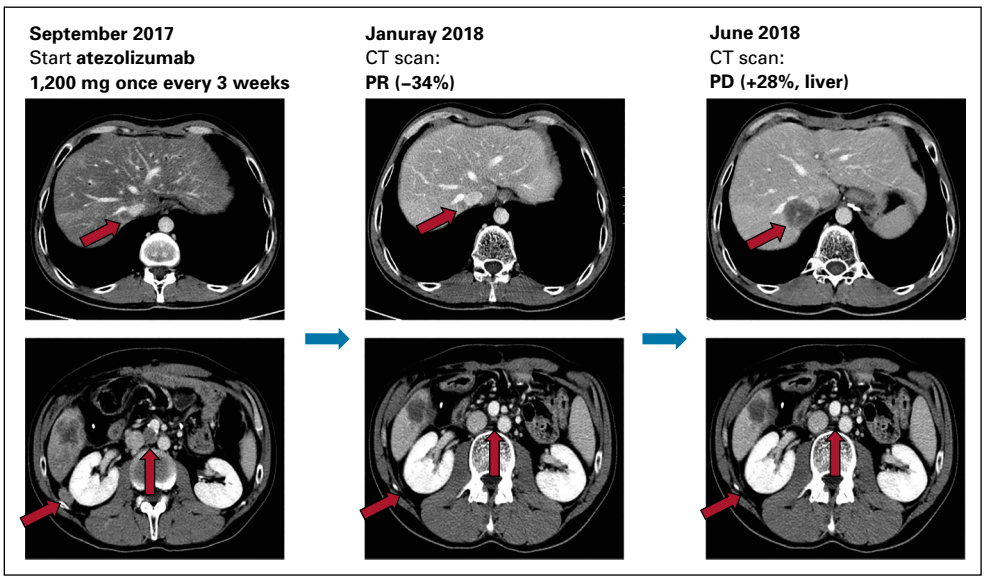

FIG 2. CT scan evaluations depicting immune checkpoint inhibitor treatment efficacy. At treatment start (first column on the left), the patient had disease relapse with liver metastases, retroperitoneal lymph nodes, and one peritoneal nodule. Treatment with atezolizumab lasted for 14 cycles, with significant PR (–34%). Disease progression was documented with enlargement of the liver lesions (+28%). CT, computed tomography; PD, progressive disease; PR, partial response.

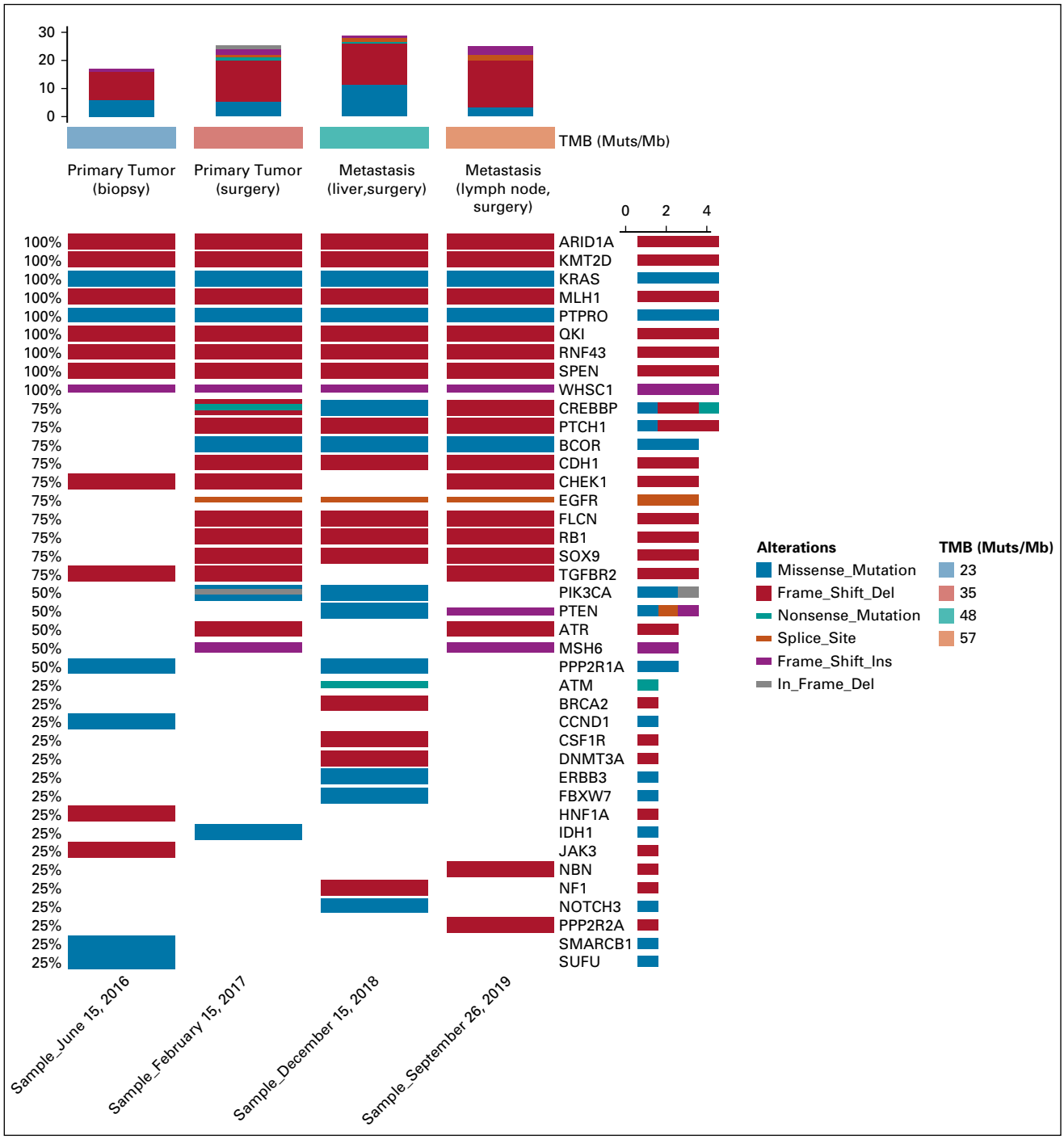

FIG 3. OncoPrint of genetic alterations detected in four sequential tumor samples with the FoundationOne CDx panel. The baseline biopsy tissue and subsequent three surgical specimens were sampled for the analysis. The third sample was sequenced while the patient was still alive, thus guiding therapeutic decisions, whereas others were analyzed a posteriori. FMI, FoundationOne CDx; TMB, tumor mutational burden.

gemcitabine achieved a new PR. A new surgical approach was performed, but retroperitoneal nodal disease progression occurred within two months. Therefore, the patient was treated at first in a phase I study evaluating an *ATR* serine/threonine kinase inhibitor and then with off-label use of olaparib, but the rapid clinical deterioration caused by a liver abscess did not allow him to receive continuous dosing. The patient deceased because of disease progression soon thereafter.

Molecular Profiling

The disease was monitored during treatments by serial liquid biopsies that combined tracking single-nucleotide variants (SNVs) identified at the tissue level and targeted

sequencing for identification of new SNVs, as shown in Figure 1. ctDNA was evaluated at baseline and at disease re-evaluations, whenever the patient accepted to undergo an additional blood sample. In detail, ctDNA was at first analyzed by digital droplet PCR (ddPCR) using a custom mutation-specific ddPCR assay targeted on the known driver mutation (*KRAS* G13D), designed using the Thermo Fisher custom SNP genotyping assay tool. At specific timepoints, the ctDNA fraction was evaluated a posteriori by direct sequencing using the 52-gene panel Oncomine Pan-Cancer Cell-Free Assay. All called variants were then validated using the corresponding ddPCR assay at all the timepoints.

ctDNA level variations mirrored radiologic disease evolution. Moreover, liquid biopsies were able to capture tumor clonal evolution: *KRAS* G13D mutation was detected in the early phase of patient history and allele fraction raised with disease progression, while shrinking with response to IO; conversely, *CTNNB1* S45F mutation appeared at IO progression, and allele fraction levels raised alongside the increase of tumor burden.

Comprehensive genomic profiling was obtained in all available tissue samples (Fig 3). The mutational landscape was characterized by a large number of alterations mostly represented by small deletions, as expected in MSI-H cases, and heterogeneity in the presence or absence of specific alterations across samples (eg, *CDH1* and *BRCA2*), consistent with tumor heterogeneity or subclonality. Notably, the TMB progressively increased in each sample analyzed, highlighting a very heterogeneous landscape or treatment-induced evolution of clonal diversity.

Pathologic Features

After retrospective histopathologic evaluation of surgical samples (Fig 4), specimen No. 1 (2017, pre-IO treatment) showed a predominantly solid, poorly differentiated adenocarcinoma, whereas samples No. 2 and No. 3 (2018 and 2019, respectively, both after IO) showed extensive extracellular mucin production and signet-ring cell features. Programmed death ligand-1 expression showed a slight increase across longitudinal samples (0%, 5%, and 10%, respectively). Both CD3 and CD8 showed a decreasing prevalence of tumor-infiltrating lymphocytes (TILs; 85, 38, and 35 CD3+ cells/high power field in No. 1, No. 2, and No. 3, respectively, and 43, 27, and 21 CD8+ cells/high power field in No. 1, No. 2, and No. 3, respectively). Of note, although in sample No. 1, we found a significant proportion of intraepithelial TILs, in No. 2 and No. 3, only scattered CD3+ and CD8+ T cells were found in direct contact with tumor cells. Remarkably, we found a heterogeneous expression of β-catenin in samples No. 1 and No. 2 (30% and 5% of immunoreactive cancer cells, respectively), whereas there was a diffuse β-catenin immunoreactivity (70%) in the last sample (No. 3), which was consistent with protein impaired degradation and accumulation because of prevalence of the subclone with *CTNNB1* gene mutation.

Immunohistochemical analysis of the DNA mismatch repair complex status revealed MLH1 and PMS2 loss across all the samples at the three timepoints, whereas MSH2 and MSH6 were retained (Fig 5).

Discussion

Our case is unique from both the clinical and molecular point of view. The longitudinal availability of blood and tumor tissue samples in a MSI-H patient treated with ChT and IO is a novelty, and it allows us to investigate secondary resistance mechanisms to immune checkpoint inhibitors in MSI-H dCCA, which are not established yet. In addition, it offers a proof of concept of the value of liquid biopsy in identifying resistance mechanisms missed by tissue molecular profiling.

Indeed, ctDNA analysis has potential clinical value for patients with CCA.[7,8] Specifically for dCCA, in which tissue biopsies are often a challenge, liquid biopsy provides a valid tool not only for treatment response monitoring but also for

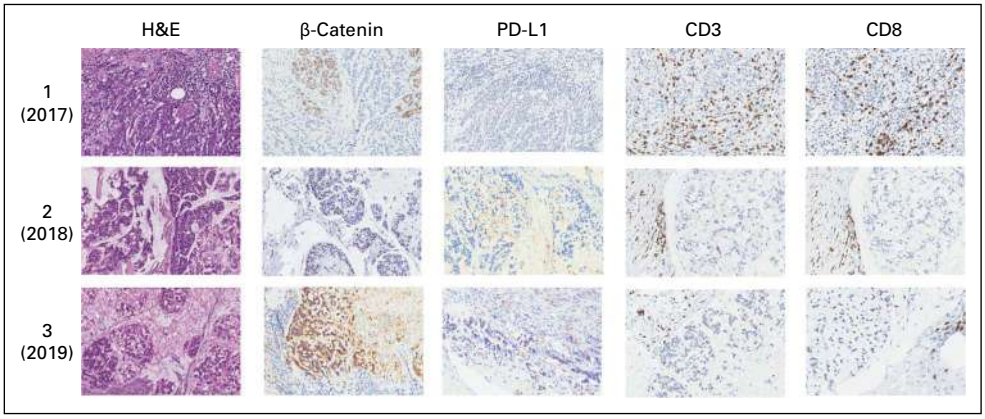

FIG 4. Pathologic features of the pretreatment and treatment-resistant tumors. Tumor CD3+ and CD8+ immune cells' prevalence, β-catenin expression, and PD-L1 expression were evaluated by immunohistochemistry through the GA503 (Dako, Santa Clara, CA), C8/144B (Dako), β-Catenin-1 (Dako), and the 22C3 pharmDx (Dako) antibody clones, respectively. CD3+ and CD8+ immune cell infiltration was quantitatively assessed by evaluating the average number of immunoreactive cells per high power field (400× magnifications). β-catenin was evaluated taking into account the prevalence of tumor cells showing membranous and cytoplasmatic immunoreactivity. PD-L1 positivity was reported as the percentage of immunoreactive cancer cells, adopting the Tumor Proportion Score. H&E, hematoxylin and eosin; PD-L1, programmed death ligand-1.

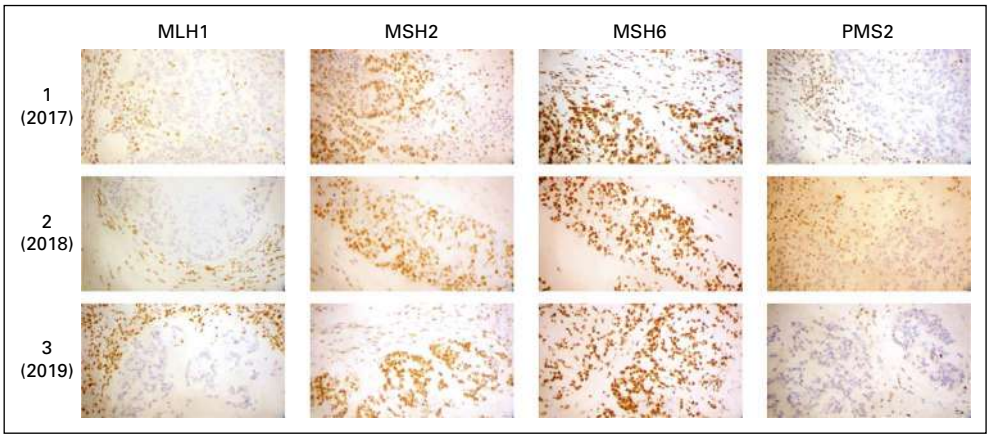

FIG 5. Immunohistochemical analysis of the DNA MMR complex status. DNA MMR complex status was assessed by MLH1, MSH2, MSH6, and PMS2 immunostains (Dako, Glostrup, Denmark). A tumor sample was considered MMR-defective in the complete absence of nuclear staining, in the presence of an unquestionable internal positive control represented by normal epithelial cells, stromal cells, muscle cells, or lymphocytes. MMR, mismatch repair.

an early detection of tumor evolution and for identifying new treatment targets. Our case shows well how ctDNA could help identifying emerging subclones resistant to ongoing treatments.

Notably, the *KRAS* G13D mutation spiked at the first relapse and then significantly reduced during treatment with IO, thus mirroring tumor burden. *CTNNB1* S45F emerged at acquired resistance to atezolizumab, whereas *TP53* R280I mutation progressively increased in the last 2 years, again mirroring chemoresistance[9] and gradual increase of tumor burden.

The appearance of *CTNNB1* S45F mutation in ctDNA is remarkable since aberrant WNT signaling is associated with impaired anticancer immunity and considered a key contributor to tumor progression and resistance to checkpoint inhibitors.[10-12] Indeed, tumor cell–intrinsic WNT/β-catenin signaling has been shown to prevent antitumor immunity in melanoma[13] and in hepatocarcinoma.[14,15] Furthermore, Luke et al[16] profiled the correlation of WNT/β-catenin signaling and the T-cell–inflamed tumor microenvironment across The Cancer Genome Atlas and showed a pan-cancer association of this signaling pathway with immune exclusion. The role of the Wnt/β-catenin pathway as the primary resistance mechanism in MSI-H gastrointestinal cancers was also suggested by Kwon et al,[17] who evaluated the genomic, immunologic, and clinical outcome heterogeneity within MSI-H gastric cancers treated with programmed cell death protein-1 blockade. We were able to track a specific genomic alteration in the WNT pathway that was initially undetectable and possibly drove acquired resistance to immune checkpoint inhibition. Failure to detect the *CTNNB1* mutation at tissue sequencings could be due to temporal and spatial heterogeneities, which are quite relevant in MSI-H cancers where thousands of frameshifts and SNV mutations mount up over time. However, we found a diffuse β-catenin immunoreactivity (70%) in the final surgical sample, consistent with protein-impaired degradation and accumulation because of prevailing of the subclone with *CTNNB1* gene mutation over time. In addition, the role of the Wnt/β-catenin pathway in immune exclusion is also supported by the decreasing prevalence of TILs in the tumor samples of our patient after IO, despite the increase of the TMB over time.

In conclusion, our report describes a unique case of a young patient affected by MSI-H dCCA, for whom the combination of aggressive surgery, ChT, and IO ultimately ended in a survival largely above the median overall survival reported in the literature for this disease. The complex mutational landscape of our case, and of CCA overall, highlights the utility of longitudinal and multimodal molecular profiling in these rare cancers.

AFFILIATIONS

[1]Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Milan, Italy
[2]Computational Oncology, Molecular Diagnostics Program, National Center for Tumor Diseases (NCT) and German Cancer Research Center (DKFZ), Im Neuenheimer Feld 280, Heidelberg, Germany
[3]Department of Applied Research and Technological Development, Biomarkers Unit, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Milan, Italy
[4]General Surgery Department, ASST-Vimercate, Vimercate, Italy
[5]Division of HPB, General Surgery and Liver Transplantation, Department of Surgery, Fondazione IRCCS Istituto Nazionale Tumori di Milano, Milan, Italy
[6]Pathology and Laboratory Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Milan, Italy
[7]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy

CORRESPONDING AUTHOR

Monica Niger, MD, Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori di Milano, Via Venezian 1, 20133 Milan, Italy; e-mail: Monica.niger@istitutotumori.mi.it.

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DATA SHARING STATEMENT

The authors declare that data supporting the findings of this study are available within the paper. Raw data of liquid biopsies will be made available upon reasonable request. Commercially available platforms were employed with results shown in the figures included.

AUTHOR CONTRIBUTIONS

Conception and design: Monica Niger
Financial support: Maria Grazia Daidone
Provision of study material or patients: Vera Cappelletti, Marta Vismara, Sherrie Bhoori, Andrea Vingiani, Giancarlo Pruneri, Vincenzo Mazzaferro
Collection and assembly of data: Monica Niger, Federico Nichetti, Chiara Pircher, Sherrie Bhoori, Maria Di Bartolomeo
Data analysis and interpretation: Monica Niger, Federico Nichetti, Francesca Dell'Angelo, Vera Cappelletti, Marta Vismara, Christian Cotsoglou, Andrea Vingiani, Filippo Pietrantonio, Filippo de Braud, Giancarlo Pruneri, Vincenzo Mazzaferro
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Monica Niger
Consulting or Advisory Role: Incyte, Basilea Pharmaceutica, EMD Serono, MSD/AstraZeneca
Travel, Accommodations, Expenses: Celgene

Sherrie Bhoori
Honoraria: Eisai, Ipsen, Boston Scientific
Consulting or Advisory Role: Eisai

Maria Di Bartolomeo
Honoraria: Lilly, MSD Oncology, Servier
Consulting or Advisory Role: Lilly, MSD Oncology
Research Funding: Lilly
Travel, Accommodations, Expenses: Roche, Sanofi

Filippo Pietrantonio
Honoraria: Servier, Bayer, AstraZeneca/MedImmune, Lilly, Sanofi, MSD Oncology, Amgen
Consulting or Advisory Role: Amgen, Servier, MSD Oncology, Merck
Research Funding: Bristol Myers Squibb (Inst), AstraZeneca (Inst)

Filippo de Braud
Honoraria: Roche, Pfizer, BMS, Merck, MSD, Servier, Sanofi, Amgen Astellas BioPharma, Incyte
Consulting or Advisory Role: Roche, Incyte, EMD SERONO, Bristol Myers Squibb, Nerviano Medical Sciences, Sanofi, Novartis Italy, NMS Medical Science, Menarini
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Giancarlo Pruneri
Honoraria: Novartis, Roche, Genomic Health
Consulting or Advisory Role: ADS Biotec
Research Funding: Roche, Roche Molecular Diagnostics

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REFERENCES

1. Valle J, Wasan H, Palmer DH, et al: Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. N Engl J Med 362:1273-1281, 2010
2. Lamarca A, Palmer DH, Wasan HS, et al: ABC-06|A randomised phase III, multi-centre, open-label study of active symptom control (ASC) alone or ASC with oxaliplatin/5-FU chemotherapy (ASC+mFOLFOX) for patients (pts) with locally advanced/metastatic biliary tract cancers (ABC) previously-treated with cisplatin/gemcitabine (CisGem) chemotherapy. J Clin Oncol 37, 2019 (abstr 4003)
3. Yoo C, Kim K-P, Kim I, et al: Liposomal irinotecan (nal-IRI) in combination with fluorouracil (5-FU) and leucovorin (LV) for patients with metastatic biliary tract cancer (BTC) after progression on gemcitabine plus cisplatin (GemCis): Multicenter comparative randomized phase 2b study (NIFTY). J Clin Oncol 39, 2021 (abstr 4006)
4. Lowery MA, Ptashkin R, Jordan E, et al: Comprehensive molecular profiling of intrahepatic and extrahepatic cholangiocarcinomas: Potential targets for intervention. Clin Cancer Res 24:4154-4161, 2018
5. Le DT, Durham JN, Smith KN, et al: Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357:409-413, 2017
6. FoundationOne CDX. 2018. https://www.foundation-medicine.com/genomic-testing/foundation-one-cdx
7. Mody K, Kasi PM, Yang J, et al: Circulating tumor DNA profiling of advanced biliary tract cancers. JCO Precis Oncol 10.1200/PO.18.00324
8. Ettrich TJ, Schwerdel D, Dolnik A, et al: Genotyping of circulating tumor DNA in cholangiocarcinoma reveals diagnostic and prognostic information. Sci Rep 9:13261, 2019
9. Li H, Li J, Gao W, et al: Systematic analysis of ovarian cancer platinum-resistance mechanisms via text mining. J Ovarian Res 13:27, 2020
10. Galluzzi L, Spranger S, Fuchs E, et al: WNT signaling in cancer immunosurveillance. Trends Cell Biol 29:44-65, 2019
11. Zhou Y, Xu J, Luo H, et al: Wnt signaling pathway in cancer immunotherapy. Cancer Lett 525:84-96, 2022

12. Ruiz de Galarreta M, Bresnahan E, Molina-Sanchez P, et al: Beta-catenin activation promotes immune escape and resistance to anti-PD-1 therapy in hepatocellular carcinoma. Cancer Discov 9:1124-1141, 2019
13. Spranger S, Bao R, Gajewski TF: Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. Nature 523:231-235, 2015
14. Sia D, Jiao Y, Martinez-Quetglas I, et al: Identification of an immune-specific class of hepatocellular carcinoma, based on molecular features. Gastroenterology 153:812-826, 2017
15. Harding JJ, Nandakumar S, Armenia J, et al: Prospective genotyping of hepatocellular carcinoma: Clinical implications of next-generation sequencing for matching patients to targeted and immune therapies. Clin Cancer Res 25:2116-2126, 2019
16. Luke JJ, Bao R, Sweis RF, et al: WNT/β-catenin pathway activation correlates with immune exclusion across human cancers. Clin Cancer Res 25:3074-3083, 2019
17. Kwon M, An M, Klempner SJ, et al: Determinants of response and intrinsic resistance to PD-1 blockade in microsatellite instability-high gastric cancer. Cancer Discov 11:2168-2185, 2021