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Molecular profile of BRCA-mutated biliary tract cancers

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ABSTRACT

Introduction Prognosis of biliary tract cancers (BTC) remains dismal and novel treatment strategies are needed to improve survival. *BRCA* mutations are known to occur in BTC but their frequency and the molecular landscape in which they are observed in distinct sites of BTC remain unknown.

Material and methods Tumour samples from 1292 patients with BTC, comprising intrahepatic cholangiocarcinoma (IHC, n=746), extrahepatic cholangiocarcinoma (EHC, n=189) and gallbladder cancer (GBC, n=353), were analysed using next-generation sequencing (NGS). Tumour mutational burden (TMB) was calculated based on somatic non-synonymous missense mutations. Determination of tumour mismatch repair (MMR) or microsatellite instability (MSI) status was done by fragment analysis, immunohistochemistry and the evaluation of known microsatellite loci by NGS. Programmed death ligand 1 expression was analysed using immunohistochemistry.

Results Overall, *BRCA* mutations were detected in 3.6% (n=46) of samples (*BRCA1:* 0.6%, *BRCA2:* 3%) with no significant difference in frequency observed based on tumour site. In GBC and IHC, *BRCA2* mutations (4.0% and 2.7%) were more frequent than *BRCA1* (0.3% and 0.4%, p<0.05) while in EHC, similar frequency was observed (2.6% for *BRCA2* vs 2.1% for *BRCA1*). BRCA mutations were associated with a higher rate in subjects with MSI-H/deficient mismatch repair (19.5% vs 1.7%, p<0.0001) and tumours with higher TMB, regardless of the MMR or MSI status (p<0.05).

Conclusions *BRCA* mutations are found in a subgroup of patients with BTC and are characterised by a distinct molecular profile. These data provide a rationale testing poly(ADP-ribose)polymeraseinhibitors and other targeted therapies in patients with *BRCA*-mutant BTC.

Key questions

What is already known on this subject?

Patients with advanced biliary tract cancer have a dismal prognosis with few treatment options available. Thus, establishment of innovative treatment strategies by identification of novel molecular targets is urgently needed to improve survival in these patients. *BRCA* mutations are known to occur in biliary tract cancers but their prevalence and molecular landscape in distinct sites of the biliary tract are unknown. Moreover, the impact of *BRCA* mutations on predictive immuno-related biomarkers remains elusive.

What dose this study adds?

We here present the largest study investigating the molecular landscape of patients with *BRCA*-mutated biliary tract cancer, which are characterised by a unique molecular profile. In addition, *BRCA*-mutated cancers are related to immunotherapy-associated biomarkers such as tumour mismatch repair or microsatellite instability status and programmed death ligand 1 overexpression.

How might this impact on clinical practice?

When considering the promising results of the recently presented POLO (Pancreas Cancer Olaparib Ongoing) trial performed in patients with *BRCA*-mutant pancreatic cancer, it is tempting to speculate that patients with *BRCA*-mutant biliary tract cancer might also benefit from treatment with poly(ADP-ribose) polymerase inhibitors. Finally, our data suggest that a therapy of drugs targeting the DNA-damage repair pathway in combination with checkpoint inhibitors may also be considered for clinical trials in advanced biliary tract cancers.

INTRODUCTION

Biliary tract cancers (BTC) are rare neoplasms originating from different anatomic sites of the biliary tree and include gallbladder cancer (GBC), as well as extrahepatic cholangiocarcinomas and intrahepatic cholangiocarcinomas (EHC and IHC) BTC.[1] The frequency of BTC is rather low accounting for 3% of all gastrointestinal (GI) malignancies[2] but its prevalence is increasing globally.[3] The 5-year overall survival rate is generally less than 5%[4] and cure can be achieved only with radical surgery in the setting of early stage disease. Different risk factors have been associated with the development of BTC. These include primary sclerosing cholangitis, bile duct adenomas, hepato- and cholelithiasis,

exposure to chemical carcinogens such as nitrosamines, chronic viral hepatitis, cirrhosis and obesity.[5] Besides these risk factors, a genetic predisposition for developing BTC in individuals with germline mutations in genes associated with Lynch syndrome or mutations in *BRCA1/2* genes is also well known. *BRCA2* mutation carriers have a lifetime risk of nearly 5% for developing BTC.[6] Moreover, the *BRCA* mutations characterised in BTC to date are frequently of somatic origin and less frequently associated with germline mutations.

Systemic treatment for BTC usually includes chemotherapy with cytotoxic agents such as platinum compounds, gemcitabine and 5-fluorouracil or capecitabine.[7] Recent data from the BILCAP trial have shown an improvement of 17 months in overall survival when adjuvant treatment with capecitabine was used after radical surgery.[8] In first-line treatment of advanced disease, the ABC-02 trial led to improved survival with the combination of gemcitabine and cisplatin over gemcitabine alone.[9] In contrast to other malignancies of the GI tract, no effective targeted therapies have been approved for BTC so far. However, recent genomic analyses of BTC showed that several potentially targetable genetic alterations are observed in nearly 40% of patients with BTC,[10] including *BRCA1/2* mutations. *BRCA1/2* mutated cells accumulate DNA double-strand breaks and exhibit genomic instability with an increased predisposition to malignant transformation.[11] Germline or somatic *BRCA* mutations are being increasingly described in BTC[12] and these mutations have been shown to result in defective repair mechanisms via homologous recombination (HR) for double-strand DNA breaks.[13] For this reason, *BRCA1/2* mutated carriers have a specific clinical phenotype, which in other tumour types has been associated with an increased sensibility to DNA damaging therapies.[14 15] Poly(ADP-ribose)polymerase (PARP) inhibitors have been described to selectively kill *BRCA*-mutated tumour cells in vitro[16] and are increasingly being used in *BRCA*-mutated malignancies such as ovarian, breast, prostate and, more recently, pancreatic cancer.[17] There are single case reports of the efficacy of PARP inhibitors in patients with *BRCA*-mutated advanced BTC[6 18] with overall survival ranging from 11 to 65 months. Thus, similar to *BRCA*-mutated pancreatic cancer, *BRCA*-mutated BTC appears to delineate as a distinct subgroup, which may benefit from a personalised treatment approach. However, it is still unknown whether and to what extent the molecular profile of *BRCA*-mutated BTC differs from *BRCA*-wild-type (WT) BTC. For these reasons, we aimed to comprehensively characterise the molecular landscape of *BRCA*-mutated BTC. Furthermore, we investigated the association of *BRCA*-mutated BTC with predictive biomarkers of response to immune checkpoint inhibitors, including mismatch repair (MMR)/microsatellite instability (MSI) status, tumour mutational burden (TMB) and programmed death ligand 1 (PD-L1) overexpression.

MATERIAL AND METHODS

Samples characterisation

BTC specimens of 1292 patients were submitted to Caris Life Sciences between June 2014 and January 2019. These cases were retrospectively reviewed, and gene sequencing, amplification and protein expression data evaluated. The pathology report was included with the specimens and H&E slides were prepared for each tumour sample to be reviewed by board-certified pathologists to confirm the diagnosis of BTC. Tumours with a histological diagnosis that was not concordant with the diagnosis of BTC were excluded from this analysis. During the recruitment period, tests have varied since there were different requests by the treating physicians and the testing technologies continuously evolved over time. The next-generation sequencing (NGS) platform for tumours tested in 2015 or earlier used the MiSeq platform (45 genes included) while those tested after 2015 were sequenced with the NextSeq platform (592 genes included). In keeping with 45 CFR 46.101(b), this study was performed using retrospective, deidentified clinical data. Therefore, this study is considered IRB exempt and no patient consent was necessary from the subject. Thus, only basic demographic information was available. Patients were stratified into *BRCA*-mutated and *BRCA*-WT cases. *BRCA* mutations included only pathogenic or presumed pathogenic mutations. Tumours with benign, presumed benign *BRCA* mutation or *BRCA* variants of unknown significance were categorised as *BRCA* WT. For *BRCA1*, there were 48 tumours carrying variants of unknown significance (1.8%) and for *BRCA2*, 89 tumours carrying variants of unknown significance (3.4%). Germline testing could not be performed due to the lack of access to germline DNA.

Analyses performed

Immunohistochemistry (ImHC) was performed on 1258 tumour samples on formalin-fixed paraffin-embedded (FFPE) sections on glass slides. 4 µm sections mounted on slides were stained using an automated system (Benchmark, Ventana Medical Systems, Tucson, Arizona, USA; Autostainer, DAKO, Carpinteria, California) according to manufacturer's instructions, and were optimised and validated per CLIA/CAO and ISO requirements. All proteins of interest were evaluated on tumour cells. An intensity score (0=no staining; 1+=weak staining; 2+=moderate staining; 3+=strong staining) and a proportion score to determine the percentage of cells staining positive (0%–100%) was used. The primary antibody used to detect PD-L1 expression was SP142 (Spring Biosciences). The staining was deemed positive if its intensity on the membrane of the tumour cells was ≥2+ and the percentage of positively stained cells was ≥5%. Results were divided in positive or negative by using previously defined thresholds specific to each marker, based on published clinical literature that associates biomarker status to specific treatment response. The primary antibody used for PD-1 testing was MRQ-22 (Ventana) and staining was scored as positive if the number of PD-1 positive cells was >1 cell per

high power field. A board-certified pathologist evaluated immunohistochemical results independently.

NGS was performed on 1292 tumour samples with genomic DNA isolated from FFPE tumour samples using either the MiSeq (n=202) or the NextSeq (n=1090) platform (Illumina, San Diego, California). For tumours tested with MiSeq, specific regions of the genome were amplified using the Illumina TruSeq Amplicon Cancer Hotspot panel. For tumours tested with NextSeq, a custom-designed SureSelect XT assay was used to enrich 592 whole-gene targets (Agilent Technologies, Santa Clara, California). All variants were detected with >99% confidence based on allele frequency and amplicon coverage with an average sequencing depth of coverage of >500 and with an analytic sensitivity of 5%. Genetic variants identified were interpreted by board-certified molecular geneticists and categorised as 'pathogenic', 'presumed pathogenic', 'variant of unknown significance', 'presumed benign' or 'benign', according to the American College of Medical Genetics and Genomics standards. When assessing mutation frequencies of individual genes, pathogenic, and presumed pathogenic were defined as mutations while benign or presumed benign variants and variants of unknown significance were excluded.

A combination of multiple test platforms was used to determine the MSI or MMR status of the tumours profiled, including fragment analysis (FA, Promega, Madison, Wisconsin), ImHC (MLH1, M1 antibody; MSH2, G2191129 antibody; MSH6, 44 antibody; and PMS2, EPR3947 antibody (Ventana Medical Systems)) and NGS (for tumours tested with NextSeq platform, 7000 target microsatellite loci were examined and compared with the reference genome hg19 from the University of California). FA was done on a total of 102 tumours tested from August of 2015 to September of 2018 and the tumour was determined MSI-high (MSI-H) if two or more mononucleotide out of the five markers included in the assay were abnormal; ImHC was done on 397 tumours tested from August of 2015 to January of 2019 and the tumour was considered mismatch repair deficient (dMMR) if complete absence of protein expression of any of the four proteins was observed; NGS was done on 1046 tumours tested from March of 2017 to January of 2019 and the threshold used to determine MSI-H was 46 or more altered loci per tumour. The three platforms generated highly concordant results as previously reported[19] and in the rare cases of discordant results, the MSI or MMR status of the tumour was determined in the order of FA, ImHC and NGS.

Statistics

Statistical comparisons were performed with the χ^2 test and the Mann-Whitney U test. A $p<0.05$ was considered as statistically significant.

RESULTS

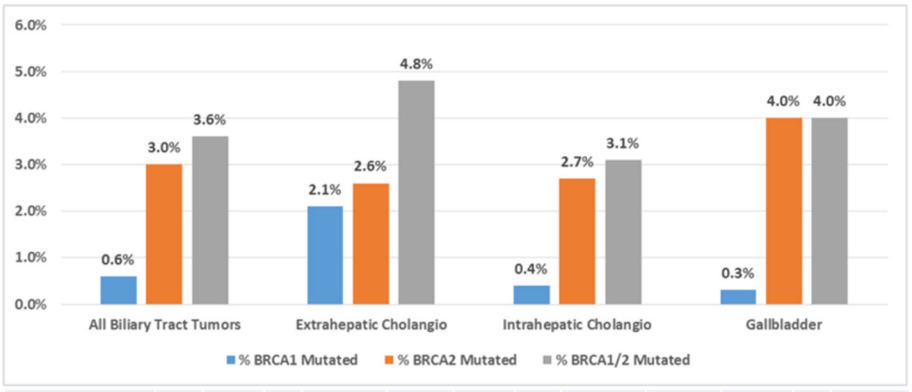
Characteristics of patients with BTC with BRCA mutations

BRCA mutations were detected in 3.6% (n=46) of 1292 BTC samples (*BRCA1* 0.6%, *BRCA2* 3%). Patients with BTC and *BRCA* mutations did not differ in terms of age compared with patients with BRCA-WT tumours. The median age of *BRCA*-mutant patients compared with *BRCA*-WT patients was 64.1 and 62.7 years, respectively (table 1). No significant gender differences in the *BRCA* mutation frequency were observed (3.7% in male and 3.4% in female patients). *BRCA* mutation frequency varies with the BTC subtypes, as EHC had the highest prevalence of *BRCA* mutations (4.8%) compared with the IHC (3.1%) and GBCs (4.0%). The anatomical site of four patients was unclear. In general, *BRCA2* mutations were more frequent, particularly in GBC where *BRCA2* mutations represented 93% (n=14 of 15 mutations) of all *BRCA* mutations detected, but also in IHC (87%, n=20 of 23 mutations) and EHC (56%, n=5 of 9 mutations). Variants of BRCA mutations are listed in the online supplementary table 1.

Table 1 Characteristics of BRCA mutant and wild-type biliary tract cancer

	BRCA1/2 mutant	BRCA1 mutant	BRCA2 mutant	BRCA wild type	
	Number (%)	Number (%)	Number (%)	Number (%)	P value
All cases (n=1292)	46 (3.6)	8 (0.6)	39 (3.0)	1246 (96.4)	
Age median (years)	64.1	57.9	65.3	62.7	NS
Sex					
Male	22 (3.7)	3 (0.5)	19 (3.2)	568 (44.0)	NS
Female	24 (3.4)	5 (0.7)	19 (2.7)	678 (52.5)	
Tumour origin					
Intrahepatic	23 (3.0)	3 (0.4)	20 (2.6)	746 (57.8)	
Extrahepatic	9 (4.5)	4 (2.0)	5 (2.5)	189 (14.6)	
Gallbladder	15 (4.1)	1 (0.3)	14 (3.8)	353 (27.3)	

P values were calculated by Benjamini and Hochberg χ^2 analysis.
NS, not significant.



Cancer Type	BRCA1 Wild Type	BRCA1 Mutated	Total	% BRCA1 Mutated	BRCA2 Wild Type	BRCA2 Mutated	Total	% BRCA2 Mutated	BRCA1/2 Wild Type	BRCA 1/2 Mutated	Total	% BRCA1/2 Mutated
All Biliary Tract Tumors	1295	8	1303	0.6%	1260	39	1299	3.0%	1246	46	1292	3.6%
Extrahepatic Cholangio	185	4	189	2.1%	184	5	189	2.6%	180	9	189	4.8%
Intrahepatic Cholangio	752	3	755	0.4%	733	20	753	2.7%	723	23	746	3.1%
Gallbladder	354	1	355	0.3%	339	14	353	4.0%	339	14	353	4.0%

Figure 1 Mutation rates of BRCA1 and BRCA2 in biliary tract tumours. Four biliary tract tumours with uncertain specific tumour location were included in the 'entire cohort'.

Association of molecular alterations with BRCA mutations

BRCA1/2 mutations were seen in 4.8%, 3.1% and 4.0% of EHC, IHC and gallbladder tumours, respectively. BRCA2 mutations are seen significantly more frequently than BRCA1 mutations (p<0.0001; figure 1). In *BRCA*-mutant BTC, the most frequently mutated genes were *TP53* (55.6%), *ARID1A* (52.2%) and *KRAS* (26.1%), followed by *KMT2D* (20.0%), *KMT2C* (13.3%) and *CDKN2A* (13.2%; figure 2, online supplementary table 2). Additionally, *RB1* (8.9%), *PTEN* (8.9%) and *KDM6A* (6.3%) mutations along with *FGFR1* amplifications were observed significantly more often in *BRCA*-mutated tumours (p<0.05). Median TMB level was 10.0 and 6.0 mutations per megabase for *BRCA*-mutant and *BRCA*-WT BTC, respectively (p<0.0001; figure 3). *BRCA1/2* mutations are associated with increased TMB in IHC (mutated: 10.5 mt/MB vs WT: 6.0 mt/MB, p<0.0001) and EHC (mutated: 10.0 mt/MB vs 6.0 mt/MB, p<0.0015), whereas no significant difference was observed in GBC (9.0 vs 7.0, p=0.19).

Phenotype of BRCA1-mutant and BRCA2-mutant BTC are related to MSI-H/dMMR but not PD-L1 expression

MSI-H/dMMR was observed in 2.4% tumours (online supplementary table 3) and was seen more frequently in patients with BTC harbouring a *BRCA* mutation. *BRCA*-mutated tumours showed MSI-H/dMMR status in 17.9% of patients, whereas *BRCA*-WT samples showed MSI-H/dMMR status in 1.8% of patients (p=0.0001; figure 4). On the other hand, no significant association was found with PD-L1 expression. Within the MSI-H/dMMR cohort, *BRCA*-mutant BTC showed a significant higher median TMB value (29.0 mt/MB in MSI-H/dMMR/*BRCA* mutant) compared with *BRCA*-WT (22.5 mt/MB in MSI-H/dMMR/BRCA-WT BTC; p=0.04); while in the MSS/pMMR cohort, a similar significant association was also seen (median of 9 mt/MB in *BRCA*-MT vs 6.0 mt/MB in *BRCA*-WT, p=0.0003).

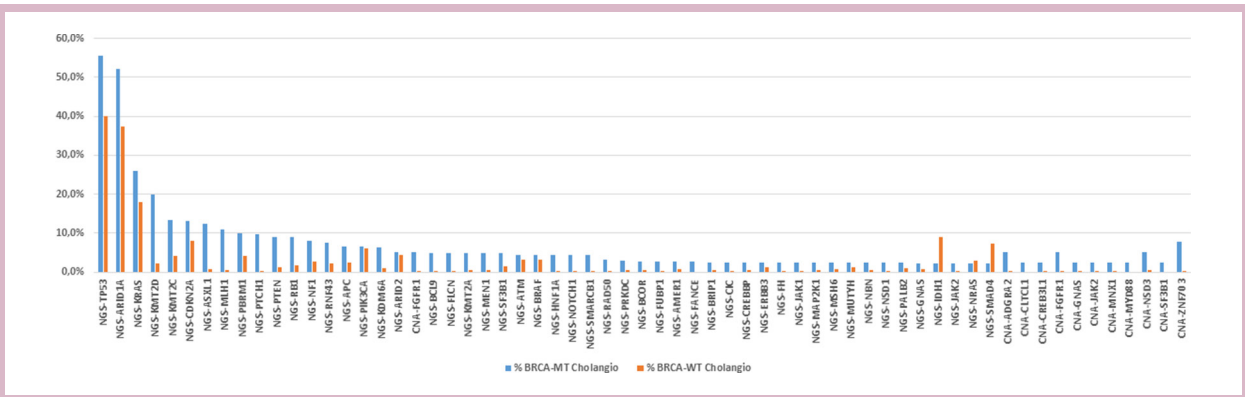


Figure 2 Gene mutation frequencies in BRCA-mutated (MT) and wild types (WTs) biliary tract tumours (see also online supplementary table).

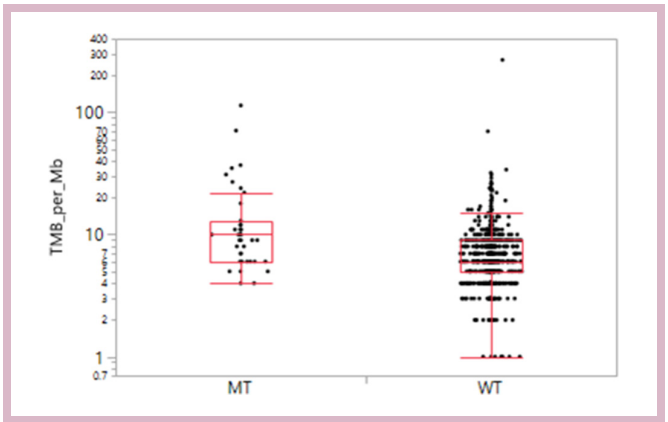
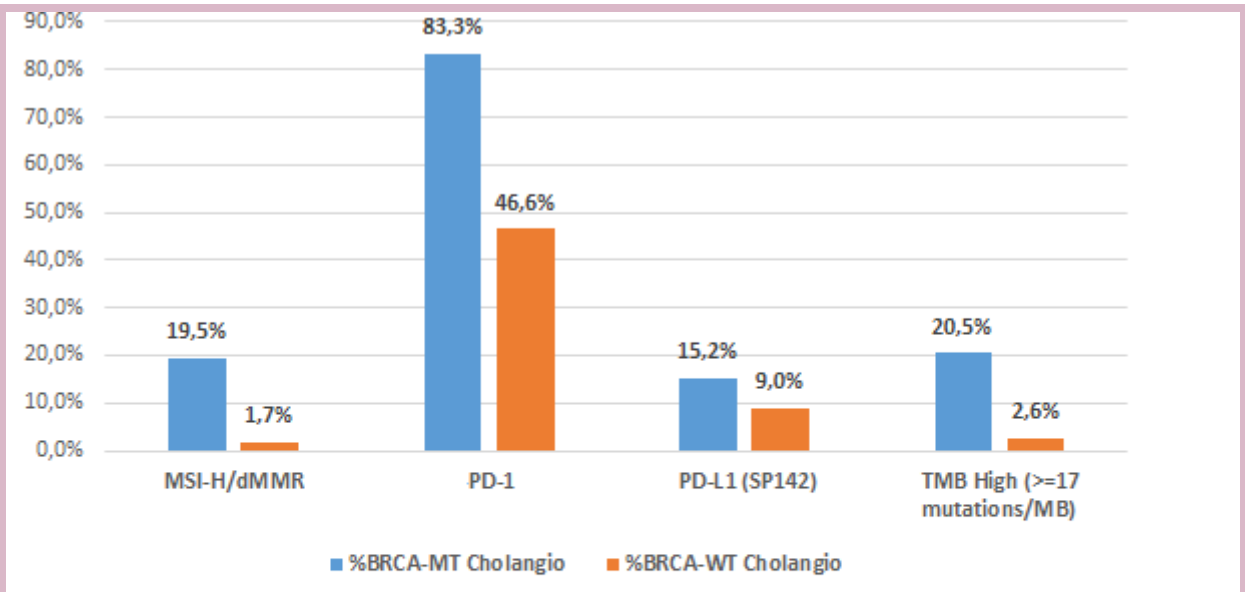


Figure 3 Tumour mutational burden in BRCA-mutated (MT) and wild-type (WT) cohorts.

DISCUSSION

This is the largest study to date describing the association of *BRCA*-mutational status with TMB in BTC. TMB is an emerging biomarker that measures the number of mutations per megabase (mt/mb) and was already shown to be associated with response to immune checkpoint inhibitors such as the combination of ipilimumab and nivolumab in patients with lung cancer[20] and other cancers.[21] Moreover, in a survey analysis of patients with 27 types of cancer, there was a linear correlation of higher TMB with the likelihood of response to anti-PD(L)1 therapy.[22] Even though the clinical significance is still controversial, ESMO guidelines for NSCLC (non-small cell lung cancer) used TMB to select patients for checkpoint inhibition with nivolumab and ipilimumab. The median number of mt/mb that we found in *BRCA*-mutant BTC was 10.0 mt/mb. Thus, *BRCA*-mutant BTC represents one of the tumours with a high median TMB, similar to what has been reported in patients with melanoma.[23] Of note, we found a correlation of *BRCA*-mutant BTC with MSI-H/dMMR, which might represent an additional predictive marker for response to checkpoint inhibition.[24] Recently, the FDA granted approval for pembrolizumab for MSI-H/dMMR[25] tumours irrespective of cancer site. Considering patients with *BRCA*-mutant BTC with MSI-H/dMMR, we observed a median TMB of 29 mt/mb. This is particularly relevant, since it was shown that patients with colorectal cancer with either MSI-H/dMMR or high TMB may respond in a high percentage to anti-PD1 antibodies.[26] Hence, patients with BRCA-mutant BTC might benefit from immune checkpoint inhibition and new trials are being designed for this purpose.

MSI-H is usually associated with a high TMB. On other hand, only a minority of high TMB samples were associated with MSI-H.[23 27] In line with these data, we observed that TMB was significantly higher in patients with *BRCA*



	% BRCA-MT BTC			% BRCA-WT BTC				
Test	Positive	Total	%BRCA-MT Cholangio	Positive	Total	%BRCA-WT Cholangio	P value	Q value
MSI-H/dMMR	8	41	19.5%	18	1039	1.7%	3.22E-13	1.95E-11
PD-1	5	6	83.3%	109	234	46.6%	0.075067	0.193256
PD-L1 (SP142)	7	46	15.2%	107	1190	9.0%	0.152177	0.306891
TMB High (>=17 mutations/MB)	8	39	20.5%	26	999	2.6%	7.06E-10	1.22E-08

Figure 4 Prevalence of immune checkpoint inhibitor-associated predictive biomarkers in BRCA-mutated (MT) and wild-type (WT) biliary tract cancer (BTC). dMMR, deficient mismatch repair; MSI-H, microsatellite instability high; PD-L1, programmed death ligand 1; TMB, tumour mutational burden.

mutations regardless of the microsatellite status. It has to be mentioned that *BRCA* mutations may be a secondary effect of a high TMB, because TMB reflects a higher load of a variance of mutations in cancer cells.[22]

Looking at the impressive results of the POLO (Pancreas Cancer Olaparib Ongoing) trial, where patients with germline *BRCA*-mutant pancreatic cancer were successfully treated with the PARP inhibitor olaparib,[17] it is worthwhile to test the hypothesis that patients with *BRCA*-mutant BTC might benefit from a similar approach.

Moreover, one must take into account that with greater understanding of the importance of currently unclassified *BRCA* variants, that the percentage of *BRCA*-mutant cases might significantly increase in the near future since an additional 5.2% of patients showed *BRCA* variants of undetermined significance. In fact, for some types of *BRCA* gene variants, additional information may be necessary before a variant can definitely be classified. As described by the 5-tier classification model,[28] the probability of *BRCA* variants of undetermined significance to be pathogenic ranges from 5% to 95%. With overall increase in *BRCA* testing of different cancer entities, we suppose that in the next years an integrated estimation taking more available evidence into account will permit a final pathogenic classification for many variants that are currently of undetermined significance.[29] Additionally, a proper differentiation of germline *BRCA* mutations from somatic *BRCA* mutations will also be necessary in patients with BTC to better evaluate the response behaviour to specific treatments in future trials. In other tumours such as ovarian cancer, PARP inhibitors have initially been tested in germline mutation carriers only but recent trials tend to also include patients with somatic *BRCA* mutations[30] or even those with *BRCA* WT carcinomas with high genomic loss of heterozygosity, a potential marker of HR deficiency and thus PARP inhibitor activity.[31]

Limitations in our study need to be mentioned as well. First, selection bias because of the retrospective study design cannot be excluded. Second, no clinical data, such as survival and treatments, are available. Third, we were not able to differentiate between germline and somatic mutations because no healthy tissue was available. As such, we are not able to analyse the prognostic and predictive value of *BRCA* mutations in GBC.

CONCLUSION

Taken together, to the best of our knowledge, this is the largest study to date mapping the molecular landscape of patients with BTC. Using this approach, we were able to provide a detailed characterisation of *BRCA* mutations in BTC and identified this molecular subgroup to be candidate for precision oncology trials validating treatment strategies focusing on the DNA-damage repair pathway (eg, PARP inhibitors) as monotherapy or in combination with immune checkpoint inhibitors.

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