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Mismatch Repair Deficiency in Biliary Tract Cancer: Prognostic Implications and Correlation with Histology

Caterina Vivaldi[a, b] Virginia Genovesi[b] Clara Ugolini[c] Laura Bernardini[b] Andrea Casadei-Gardini[d] Vincenzo Formica[e] Francesca Salani[b, f] Giulia Orsi[g] Valentina Massa[b] Andrea Cacciato-Insilla[h] Miriam Caccese[b] Silvia Cesario[b] Kalliopi Andrikou[i] Jessica Graziani[b] Daniela Campani[c] Enrico Vasile[b] Gabriella Fontanini[c] Lorenzo Fornaro[b] Gianluca Masi[a, b]

[a]Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy; [b]Unit of Medical Oncology 2, Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy; [c]Department of Surgical, Medical, Molecular Pathology and Critical Area, Division of Pathology, University of Pisa, Pisa, Italy; [d]Vita-Salute San Raffaele, University of Medicine, Milan, Italy; [e]Medical Oncology Unit, Tor Vergata University Hospital, Rome, Italy; [f]Interdisciplinary Research Center "Health Science", Scuola Superiore Sant'Anna, Pisa, Italy; [g]Department of Medical Oncology, Vita-Salute University, San Raffaele Scientific Institute, Milan, Italy; [h]SOC Anatomia Patologica Empoli e Prato, Ospedale San Giuseppe, Milano, Italy; [i]IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", Meldola, Italy

Keywords
Biliary tract cancer · Cholangiocarcinoma · Mismatch repair deficiency · Prognosis

Abstract
Introduction: Mismatch repair (MMR) deficiency represents a biomarker and therapeutic target in various neoplasms, but its role in biliary tract cancers (BTCs) remains misunderstood. ***Methods:*** MMR status was retrospectively assessed using immunohistochemistry in 163-BTCs patients. We identified MMR proficiency (pMMR)/deficiency (dMMR) according to the loss of MMR proteins (MLH1, PMS2, MSH2, MSH6). The primary objective of the study was to assess the incidence of dMMR in BTCs; the secondary purpose was to explore its association with prognosis and clinical features. ***Results:*** dMMR was recorded in 9 patients, and it was strongly associated with mucinous histology ($p < 0.01$). Regarding the prognostic effect, in 122-radically resected patients, disease-free survival (DFS) resulted significantly shorter in dMMR patients compared to pMMR patients (10.7 vs. 31.3 months, $p = 0.025$) and so did nodal status (48.2 vs. 15.3 months in N0 vs. N+) ($p < 0.01$). Moreover, dMMR confirmed its prognostic role in terms of DFS at multivariate analysis ($p = 0.03$), together with nodal status ($p = 0.01$), and resection margin ($p = 0.03$). In 103 M+ patients (encompassing 41 metastatic de novo and 62 recurred after surgery patients) there were not differences between dMMR and pMMR regarding survival analyses. ***Conclusions:*** dMMR status is strongly correlated with mucinous histology and represents an independent prognostic factor in terms of disease relapse in patients

karger@karger.com
www.karger.com/ocl

Correspondence to:
Caterina Vivaldi, caterinavivaldi@gmail.com

Karger

with resected BTC. ***Implications for Practice:*** MMR may play an independent role in promoting an aggressive behaviour in patients with radically resected BTC. These results could be useful in improving the selection of patients after resection and, above all, should justify the evaluation of MMR status as a therapeutic target in BTC, especially in patients with atypical histology.

Introduction

Biliary tract cancers (BTCs) are rare tumours, accounting for approximately 3% of gastrointestinal tract neoplasms, but their incidence is slowly increasing worldwide. These anatomically and molecularly heterogeneous neoplasms are characterized by aggressive behaviour and unfavourable prognosis with 5-year survival rates of 5–15% [1].

In patients with BTCs, surgery represents the only potentially curative therapeutic option but is burdened by a high rate of both local and systemic relapse, even with the use of capecitabine for adjuvant purposes [2]. Moreover, about two-thirds of BTCs are diagnosed in the advanced stage and are therefore unresectable: unfortunately, although the benefit of systemic chemotherapy has been widely demonstrated, the first-line treatment of choice with cisplatin plus gemcitabine allows only a modest improvement in survival as compared with gemcitabine alone [3]. For these reasons, a wide variety of biomarkers with prognostic and predictive values have been identified [4–6] and, in order to improve outcomes, attention has increasingly focused on the role of target therapy. Among the identified molecular features/fingerprints, there is a growing interest in microsatellite instability (MSI) [1] in a wide spectrum of solid tumours, including colorectal, endometrial, gastric [7], and last but not least BTCs.

The DNA mismatch repair (MMR) system is designated to correct errors in the DNA double helix and strictly depends on four key genes: *MLH1*, *PMS2*, MSH2, and *MSH6*. When one or more of the encoded proteins are not expressed or are dysfunctional due to germinal (as in Lynch syndrome [8]) or sporadic mutations, this status is called MMR deficient (dMMR) or MSI phenotype. MMR status can be assessed by polymerase chain reaction, to diagnose MSI, or by immunohistochemistry (IHC) to diagnose the lack of expression of proteins encoded by one of the 4 MMR genes above. Given the high concordance rate (about 90%) between IHC and MSI analysis and the high sensitivity (>95%) and specificity (>90%) of both systems [9–11], ESMO recommendations identify IHC using antibodies recognizing the four MMR proteins as the first test of choice to determine MSI status, reserving the use of confirmatory polymerase chain reaction in case of doubtful IHC [12].

Genomic instability typical of these tumours increases their immunogenicity and is a positive predictive factor of response to immunotherapy: this evidence was firstly reported in the pivotal study presented by Le et al. [13, 14] who explored the efficacy of the anti-programmed death-receptor-1 (PD-1) antibody pembrolizumab in patients with dMMR/MSI-high treatment-refractory progressive neoplasms, and by the phase II KEYNOTE-158 study, in which 233 patients with non-colorectal dMMR tumours received pembrolizumab: among the 22 enrolled patients with dMMR BTC, 2 achieved complete response and 7 partial response, with an overall response rate of 40.9%, and median overall survival (mOS) of 24.3 months [15].

Following these findings, pembrolizumab was the first drug approved by the Food and Drug Administration (FDA) in patients with MSI-high or MMR deficient neoplasms without specific regard to tumour type [16]. In August 2021, the FDA granted another tissue-agnostic approval to the anti-PD-1 antibody dostarlimab for any relapsed/refractory dMMR solid tumour, based on a preliminary report of 106 patients with dMMR non-endometrial solid tumours treated in the GARNET trial: in the study, 93 per cent of patients had gastrointestinal tract neoplasms, and 2 patients with BTC achieved a complete response at the data cutoff [17].

Treatment results for anti-PD-1 drugs in a variety of malignancies, along with the integration of immunotherapy in the context of the other available therapeutic options for advanced disease, led to exploring the efficacy of immunotherapy in BTC also in association with chemotherapy in patients not selected by microsatellite status: the TOPAZ-1 randomized phase III trial, recently presented at 2022 ASCO Gastrointestinal Cancer Symposium, showed a clinically meaningful overall survival (OS) benefit at a predefined interim analysis of the anti-programmed death-ligand 1 inhibitor durvalumab in combination with chemotherapy versus chemotherapy alone for patients with advanced BTC [18]. Moreover, the KEYNOTE-966 trial comparing pembrolizumab plus gemcitabine/cisplatin versus placebo plus gemcitabine/cisplatin for the first-line advanced and/or unresectable BTC, reached its primary endpoint demonstrating a significant improvement in OS with the combination of chemotherapy and pembrolizumab [19].

dMMR in BTC is a quite rare alteration [13], and it seems significantly reduced in case studies focussing on advanced or metastatic disease only [14, 19]. Data regarding its prognostic role are controversial [20–22], and need further investigation. The aim of the study was to

assess the incidence of dMMR in BTCs, its correlation with certain clinical and pathological features and to explore its prognosis role both in early and advanced stage in a multicenter case series.

Materials and Methods

Patients Selection

We retrospectively identified a cohort of 163 patients with a diagnosis of BTC (stage I–IV) followed in 4 Italian tertiary centres between 2010 and 2019. Main inclusion criteria were: age >18; cytologically or histologically confirmed diagnosis of intrahepatic cholangiocarcinoma (ICC) or extrahepatic cholangiocarcinoma (ECC), gallbladder carcinoma, or ampullary carcinoma; availability of paraffin-embedded tumour sample; availability of tumour assessment during follow-up; access to clinical information, pathological features, and survival data. Patients were treated and monitored as clinical practice. Written informed consent from the patients for research use of data was obtained before the investigation. Analyses were carried out according to clinical practice. Data have been collected and analysed assuring patient anonymization. MMR protein expression was evaluated by IHC in all samples.

MMR IHC Analyses

IHC analyses were performed with automatic method on BenchMark ULTRA machines, using the Ventana proprietary kit, according to the instructions provided by the manufacturer (Ventana Medical System, Inc). IHC staining for MLH1, PMS2, and MSH2 was performed by mouse specific primary monoclonal antibody; in particular, clone M1 (anti-MLH1), clone A16-4 (anti-PMS2), and clone G219-1129 (anti-MSH2) were used. Rabbit-specific primary monoclonal antibody, clone SP93, was used for MSH6 identification. These specific antibodies were conjugated with an enzyme (usually a peroxidase) which transforms its substrate (diaminobenzidine, DAB), colouring the tissue brown. All IHC stains were performed on 3-micron thick slices obtained from previously processed material embedded in paraffin in which a quantity of at least 50 neoplastic cells was present, as per guidelines. A positive control tissue (normal intestinal mucosa), obtained from the archive, was inserted on each slide. IHC staining was considered positive in the presence of specific brown nuclear staining, regardless of the intensity of the stain; only the complete absence of nuclear staining was considered to be truly negative. IHC expression of all 4 proteins of the MMR was identified with the MMR-proficient (pMMR) status, while the lack of expression of one or more proteins was identified with the dMMR status.

Statistical Analyses

The primary objective of the study was to assess the status of microsatellites and determine the percentage of patients with BTC in which the neoplasm is associated with dMMR phenotype. The secondary objective of the study was to evaluate the association of dMMR phenotype with clinical and biological features; another secondary endpoint was to evaluate the prognostic and predictive role of the dMMR-phenotype in terms of disease-free survival (DFS) and OS in patients with radically resected disease, and progression-free survival (PFS) and OS in patients with advanced disease (defined as metastatic or relapsed after surgery). DFS was measured from the date of surgery to the date of disease recurrence or death, whichever occurred first. OS was measured from the date of diagnosis (date of surgery for patients who had undergone radical resection or diagnosis of metastatic disease) to the date of death or the last follow-up visit. PFS was measured from the date of diagnosis of advanced disease (metastatic or relapsed after surgery) to the date of disease progression.

Firstly, we analysed the correlation between dMMR status and the main clinical-pathological parameters relating to individual patients. This association was evaluated using contingency tables analysed with the χ^2 test: significance was set at a level of $p < 0.05$.

DFS, PFS, and OS were estimated using the Kaplan-Meier product-limit method. Median values with corresponding 95% confidence intervals (95% CIs) were reported. The association of the parameters under study with survival (DFS, PFS, and OS) was evaluated through the log-rank test, considering a value of $p < 0.05$ as significant. The following factors were included in the univariate analysis for DFS, PFS, and OS: gender (male vs. female), age at diagnosis (≥65 years vs. <65 years), Eastern Cooperative Group (ECOG) Performance Status (PS) (0 vs. 1–2), tumour location (ICC vs. ECC vs. gallbladder vs. ampullary vs. Klatskin), T stage (T1 vs. T2 vs. T3 vs. T4 vs. Tx), N stage (N0 vs. N1 vs. N2 vs. Nx), resection margin (R0 vs. R1 vs. R2), tumour grading (G1 vs. G2 vs. G3 vs. G4), stage at diagnosis (1 vs. 2 vs. 3 vs. 4), surgery on T and N (yes vs. no), adjuvant chemotherapy (yes vs. no), histology (adenocarcinoma vs. mucinous adenocarcinoma vs. squamous cell carcinoma vs. other), MSI status (low vs. high).

The factors that were significant in the univariate analysis were subsequently tested in a multivariate analysis according to the Cox proportional model, considering a $p < 0.05$ to be significant. The statistical analyses were carried out using the SPSS Statistics software (IBM, Chicago, IL, USA) v20.

Results

Patient Characteristics

One-hundred sixty-three patients who met the eligibility criteria were included in the study. Baseline characteristics are reported in Table 1.

MMR Analyses

The dMMR phenotype was recorded in 9 patients (5.5%); 6 patients showed a loss of MLH1 and PMS2 protein, 2 patients a loss of MSH6 and 1 patient had a loss of MLH1, PMS2, and MSH6 (online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000533406). In the other 154 patients, the evaluation of MMR status led to the identification of an pMMR phenotype.

Association between dMMR Phenotype and Clinical and Pathological Features

MMR phenotype significantly correlated with histology ($p < 0.01$); in particular, dMMR status was documented in 57.1% (4/7) of patients with mucinous adenocarcinoma versus 3.4% (5/146) of patients with

Table 1. Baseline patients' characteristics

Patients' characteristics	Number	Percentage
Gender		
Male	94	57.7
Female	69	42.3
Age at diagnosis, median (range), years	67 (29–87)	
ECOG[1] PS		
0	88	54
1–2	75	46
Tumour site		
ICC[2]	78	47.8
ECC[3]	19	11.6
Gallbladder	29	17.8
Ampullary	17	10.4
Klatskin	20	12.4
Stage at diagnosis		
I	36	22.2
II	44	27
III	25	15.3
IV	54	33.1
NA	4	2.4
Surgery		
Yes	122	74.8
No	41	25.2
Resection margin		
R0	107	87.7
R1-R2	10	8.1
Histology		
Adenocarcinoma	146	89.6
Mucinous adenocarcinoma	7	4.3
Squamocellular carcinoma	2	1.2
Other	8	4.8
Grading		
G1	9	7
G2	62	48.4
G3-G4	55–2	44.6
T (tumour extension)		
Tx	1	0.8
T1	33	26.6
T2	56	45.1
T3	29	23.3
T4	5	4
N (nodal status)		
Nx	22	17.6
N0	63	50.4
N1	36	28.8
N2	4	3.2

[1]ECOG PS, Eastern Cooperative Oncology Group Performance Status. [2]ICC, intrahepatic cholangiocarcinoma. [3]ECC, extrahepatic cholangiocarcinoma.

adenocarcinoma. No case of MSI was reported in other histological types. We did not find a significant correlation with any of the other examined clinical and pathological features, in particular with tumour site (p = 0.33), grade (p = 0.75), and stage (p = 0.67) (details reported in Table 2).

Survival Analyses

Survival analyses were conducted after a median follow-up of 25.3 months (95% CI 21.7–28.9). We report the analyses conducted in the two settings of radically operated disease and locally advanced or metastatic disease.

Table 2. Association between MMR status and clinical-pathological features

Features	MSS proficient	MSS deficient	*p* value
Histology			
Adenocarcinoma	141	5	<0.01
Mucinous adenocarcinoma	3	4	
Squamous cell carcinoma	2	0	
Other	8	0	
Gender			
Male	88	6	0.57
Female	66	3	
Age at diagnosis			
≥65 years	91	4	0.43
<65 years	37	3	
Site of primary tumour			
ICC[1]	71	7	0.33
ECC[2]	19	0	
Gallbladder	29	0	
Ampullary	16	1	
Klatskin	19	1	
Surgery			
Yes	115	7	0.83
No	39	2	
Stage at diagnosis			
Stage I	35	1	0.67
Stage II	42	2	
Stage III	23	2	
Stage IV	50	4	
NA	4	0	
Grading (G)			
G1	8	1	0.75
G2	59	3	
G3	50	5	
G4	2	0	

[1]ICC, intrahepatic cholangiocarcinoma. [2]ECC, extrahepatic cholangiocarcinoma.

Radically Resected Disease

Of the 122 patients who underwent radical resection, 62 experienced disease progression and 29 died during follow-up. Median DFS and OS were 27 months (95% CI 17.6–34.4) and 59.5 months (95% CI 50.5–68.4), respectively. dMMR resulted significantly associated with risk of recurrence, with a median DFS in pMMR patients of 31.1 months (95% CI 20.1–42.1) versus 10.7 months in patients with dMMR phenotype (95% CI 0–26.9) ($p = 0.025$) (Fig. 1).

Only nodal status was the other parameter significantly associated with DFS. In patients without nodal involvement, DFS was 48.2 months (95% CI 21.8–74.6) versus 15.3 months in patients with 1 or more positive nodes (95% CI 8.4–22.2) ($p < 0.01$). Two other parameters were close to statistical significance: histology (mucinous vs. other) and status of resection margins with a *p* value, respectively, of 0.051 and 0.06 (online suppl. Table 2).

A multivariate analysis was conducted including the four aforementioned parameters: N stage, MMR status, histology, and resection margin. MMR status ($p = 0.03$), together with nodal status ($p = 0.01$), and resection margin ($p = 0.03$) confirmed their independent prognostic value. The results of the multivariate analysis are reported in Table 3.

Moreover, we explored the prognostic role of MMR phenotype in terms of DFS according to nodal status. Interestingly, we observed a statistically significant correlation between DFS and MMR status in patients with nodal involvement ($p = 0.01$); in fact, in 3/33 node-positive dMMR patients, median DFS was 2.5 months (95% CI 1.86–3.14) versus 17.5 months (95% CI 12.7–22.3) of pMMR group (30/33 patients) (Fig. 2a). In patients without nodal involvement, we did not observe any difference in DFS according to MMR status (2/63 dMMR and 61/63 pMMR).

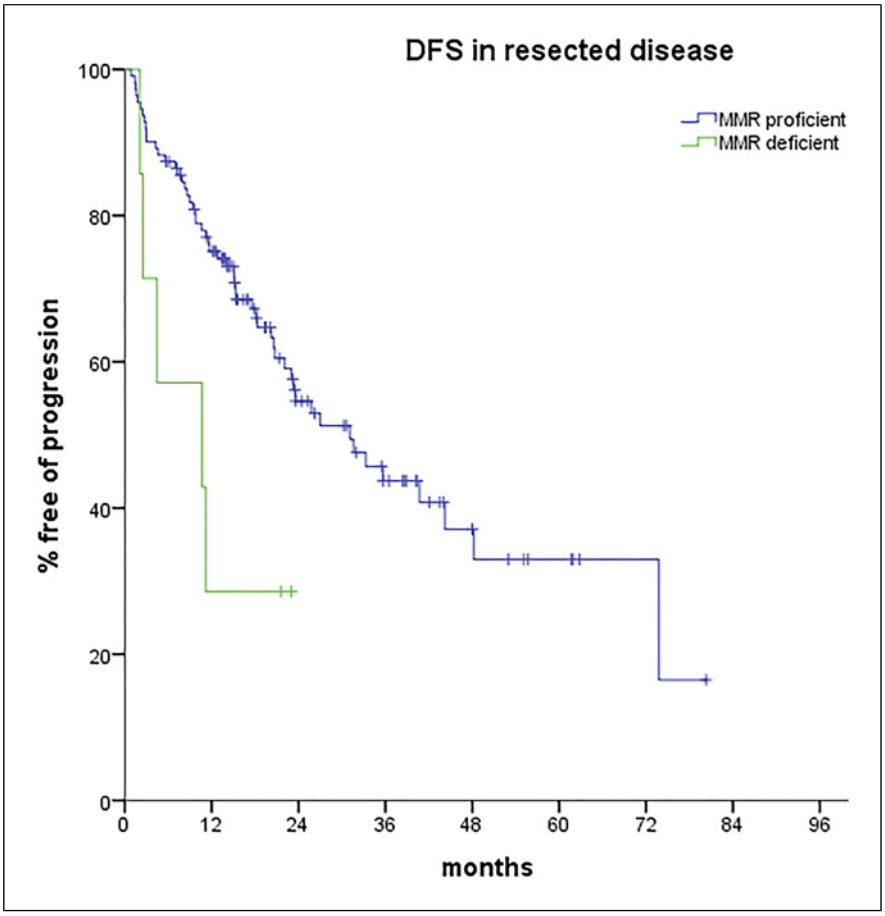

Fig. 1. Disease-free survival in resected patients according to MMR status.

Table 3. Multivariate analyses for DFS and OS in resected disease

Parameter	Disease-free survival		Overall survival	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Microsatellite status				
MMR[1] proficient	1	0.027	–	–
MMR deficient	3.51 (1.16–10.6)		–	
Nodal status				
N0	1	0.001	1	0.012
N+	2.87 (1.57–5.27)		3.2 (1.3–7.9)	
Histology				
Other	1	0.23	–	–
Mucinous adenocarcinoma	2.18 (0.61–7.8)		–	
Resection margin (R)				
R0	1	0.03	1	0.005
R1	0.31 (0.11–0.91)		0.08 (0.01–0.47)	

[1]MMR, mismatch repair.

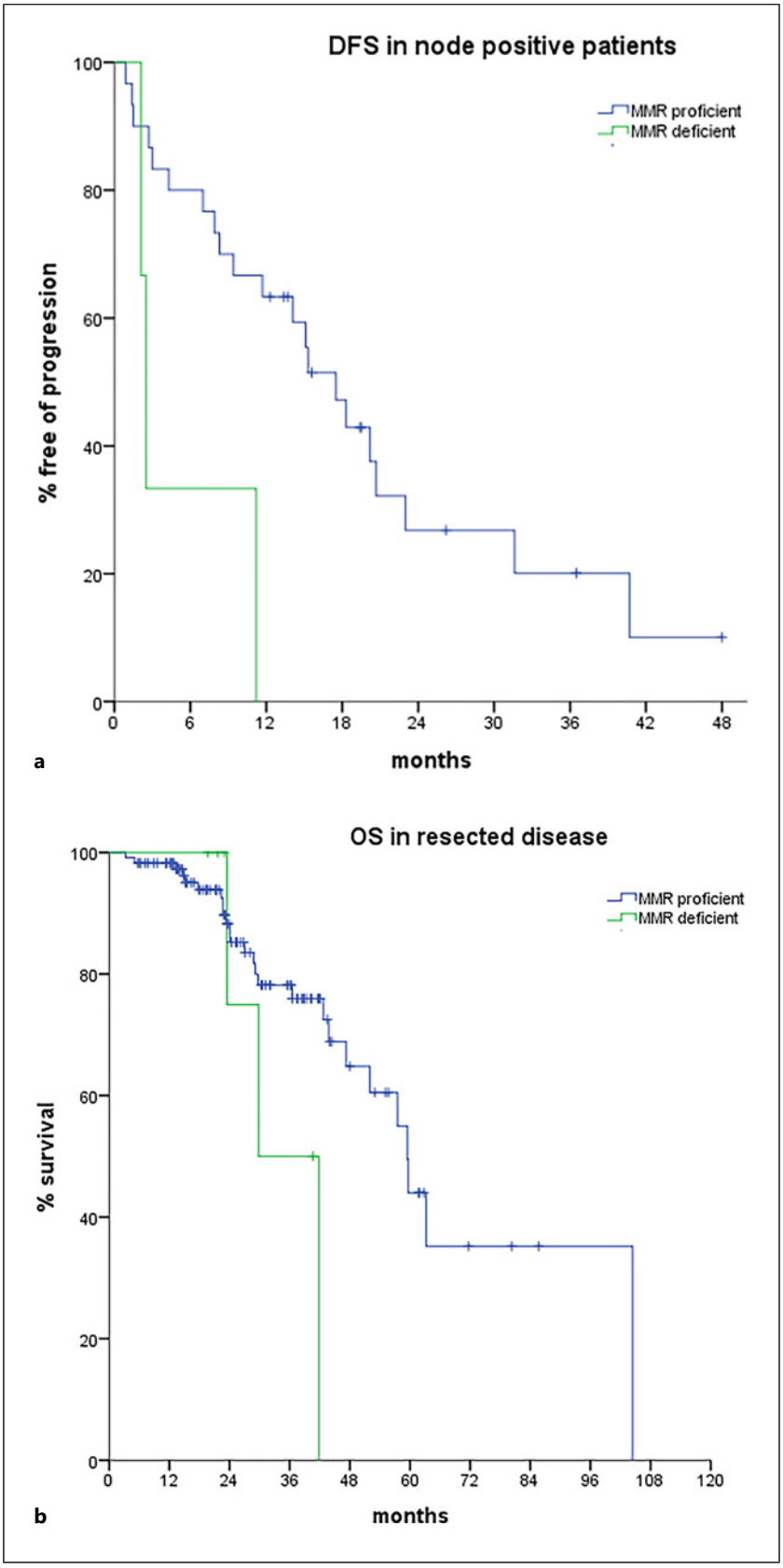

Fig. 2. Disease-free survival according to MMR status in resected patients with nodal involvement (**a**) and overall survival in resected patients according to MMR status (**b**).

As regards OS, in patients with dMMR, mOS was 29.8 months (95% CI 17.8–41.7) compared to 59.5 months in patients harbouring pMMR (95% CI 55.9–63.1), but this difference did not reach statistical significance ($p = 0.101$) (Fig. 2b). The following factors resulted associated with poor OS: nodal status ($p = 0.007$) and resection margin ($p = 0.006$) (online suppl. Table 2). In the OS multivariate analysis, nodal status and resection margin confirm their prognostic meaning with a p value, respectively, of 0.012 and 0.005 (Table 3).

Advanced Disease

In 103 patients with advanced disease (respectively, 41 metastatic de novo and 62 relapsed after surgery), mPFS and mOS were, respectively, 9.4 months (95% CI 7.5–11.3) and 20.6 months (95% CI 17.6–23.6). The incidence of dMMR was 6.8% (7/103). We did not observe any difference in terms of OS and PFS according to MMR status; in particular, mOS was 33.0 (95% CI 7.9–58.1) in dMMR group and 20.0 (95% CI 16.5–23.4) in pMMR cohort ($p = 0.57$) (Fig. 3a), while mPFS results in 10.1 months in dMMR subgroup (95% CI 8.3–11.8) and 8.5 months in microsatellite stable patients (95% CI 6.6–10.4) ($p = 0.92$) (Fig. 3b).

Discussion

BTC represents a group of rare and heterogenic cancers with a dismal outcome. Historically, surgery and chemotherapy have represented the cornerstones of treatment, but in the last years knowledge about this disease has quickly evolved, and its management, especially concerning advanced disease, has rapidly changed. In particular, improvement of molecular characterization has become crucial for the identification of potential therapeutic targets in patients progressing to chemotherapy and different studying are investigating tailored strategies also in the first-line treatment [20]. Furthermore, recently immunotherapy has also shown a clinically and statistically significant long-term benefit in addition to the first-line chemotherapy with cisplatin and gemcitabine [18].

On the other side, as regards resectable disease the recurrence rate after curative resection is still very high, and to date, waiting for phase III ongoing studies results [21], a more intensive chemotherapy treatment seems not to show any advantage over monotherapy, even considering only patients at higher risk of relapse [22]. Several studies described the incidence of MSI in BTC, but the clinical role of such alteration is not so clear.

Among 163 BTC patients, we reported an incidence of dMMR of 5.5%, in line with data reported by other authors [23], as in other series the prevalence of dMMR in BTC ranges from approximately 3% as reported by Le and colleagues [13] to 5% for gallbladder cancer and ECC, to 10% for ICC and ampullary carcinoma in the analysis shown by Silva and colleagues [8].

As our analyses were performed as per clinical practice, in line with ESMO guidelines we performed IHC analyses [12]. Our report confirms that dMMR is not only a prerogative of ICC, but it can also be found in a non-negligible percentage of the other BTC subtypes. Interestingly, in line with results from the study by Goeppert et al. [24], from our analysis emerged that the majority of patients with MSI present a neoplasm with an atypical histology that is mucinous adenocarcinoma. In addition to the well-established predictive role for immunotherapy benefits in advanced disease, the prognostic value of MMR status in earlier stages has not yet been clarified unlike in other neoplasms like colorectal and gastric cancer.

In our series, dMMR resulted significantly associated with a significantly increased risk of recurrence, with a median DFS of 10.7 versus 31.1 months in dMMR and pMMR phenotype, respectively ($p = 0.025$) and the aforementioned results were confirmed in multivariate analysis. Only nodal involvement and radical resection confirmed their independent prognostic value as previously reported by our and other experiences [5, 25].

To deeply explore the role of MMR status in early stage BTC, we analysed patients with and without nodal involvement separately. Interestingly, although the numbers are small, we did not find any difference in DFS according to MMR status in patients without nodal involvement while in N+ dMMR retained its negative prognostic value. Although we observed a numerically difference in median OS between pMMR and dMMR resected patients (59.5 vs. 29.8 months, respectively), this parameter did not reach statistical significance ($p = 0.101$), as well as the outcome of advanced patients, probably due to heterogeneity in clinical presentation, treatment administered in the metastatic setting and other unknown putative molecular alterations that could have influenced patients' prognosis.

Our result partially disagrees with what emerges in colorectal cancer, where dMMR represents a well-known positive prognostic factor in stage II [26], while its role in stages III and IV is still controversial [27]. As we have learnt over the years, some molecular alterations do not have the same prognostic impact in different diseases (for example, *BRAF* mutations in melanoma and in colorectal cancer) [28], for this reason is important to investigate these

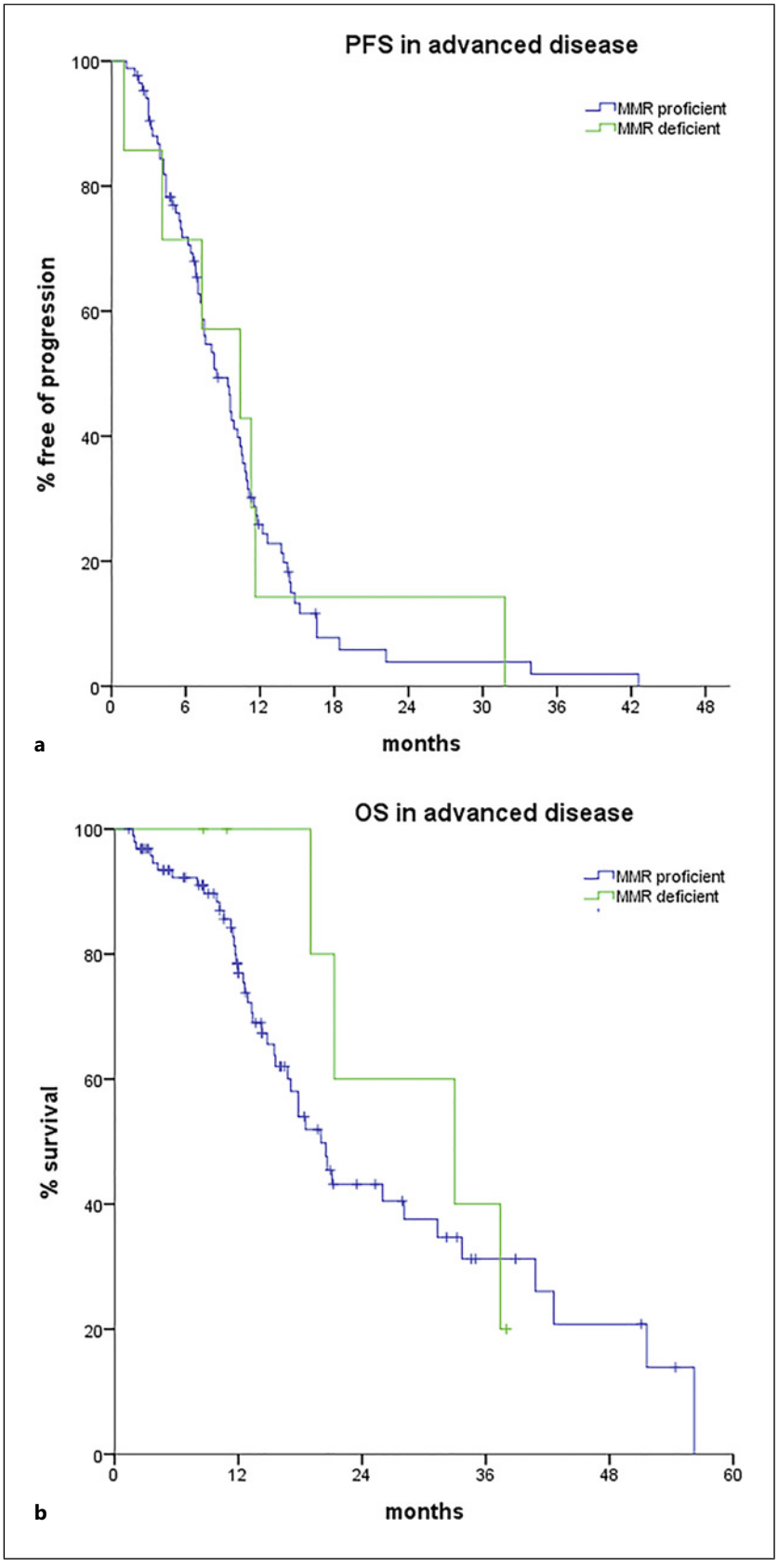

Fig. 3. Progression-free survival (**a**) and overall survival (**b**) in advanced disease according to MMR status.

molecular features in specific tumour to find out whether the effect of the alteration is superimposed on those known in other cancers. In several studies about BTC, dMMR seems to be associated with improved prognosis [29, 30] in line with other tumours with the same phenotype. On the contrary, Nakamura et al. [31] interestingly showed that patients with hypermutated BTCs and elevation in the expression of immune checkpoint molecules had the worst prognosis, suggesting an unclear prognostic role in these neoplasms and that is an incentive to explore more in deeper this subset of patients.

Although our study has objective limits, including its retrospective nature, the use of IHC and the limited number of dMMR patients, to our knowledge this is the first that hypothesizes the prognostic impact of MMR in resectable BTC. Considering the rapid evolution of the molecular scenario of BTC and the increasing availability of extensive molecular tests, if this result were confirmed on larger case series, it could have significant implications in improving the therapeutic strategy of BTC patients, for example, by thinking about a more intensive adjuvant chemotherapy treatment in MMR deficient patients or adding immunotherapy in a neoadjuvant/perioperative setting.

Conclusions

In conclusion, dMMR could be found in a not-negligible percentage of BTC, especially with mucinous histology, regardless of the site of origin, and seems to be an independent prognostic factor in radically resected disease. To date, considering the implications that MMR can have in predicting a significant benefit of immunotherapy treatment, it should be a test to be proposed to all patients diagnosed with BTC, especially with atypical histology such as mucinous adenocarcinoma.

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Statement of Ethics

Written informed consent from the patients for research use of data was obtained before the investigation. The study protocol was reviewed and approved by the Local Ethics Committee. In particular, the protocol was first approved by the Comitato Etico Area Vasta Nord Ovest (CEAVNO) for the coordinating centre (No. ESR-21-21387, date of decision 28-Apr-2022) and subsequently approved by the remaining Ethics Committee. A written informed consent was obtained according to the Ethics Committee's recommendations. This study was performed in line with the principles of the Declaration of Helsinki. Written informed consent for treatment was obtained for all patients.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Author Contributions

Conceptualization: C.V., L.F., E.V., and G.M.; methodology: C.V., L.F., and E.V.; software: C.V.; validation, writing – review and editing, visualization, and investigation: all authors (C.V., V.G., C.U., L.B., A.C.-G., V.F., F.S., G.O., V.M., A.C.-I., M.C., S.C., K.A., J.G., D.C., E.V., G.F., L.F., and G.M.); formal analysis and project administration: C.V. and V.G.; data curation: C.V., V.G, C.U., A.C.-G., and V.F.; writing – original draft preparation, C.V., V.G., and L.B.; supervision: G.M. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data are available upon request from the authors. Data are not publicly available due to ethical reasons. Further enquiries can be directed to the corresponding author.

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