Low prevalence of biliary tract cancer with defective mismatch repair genes in a Japanese hospital-based population

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Abstract. Recent studies have reported that immune checkpoint inhibitors are effective against various defective mismatch repair (dMMR)/microsatellite instability-high (MSI-H) cancers. A limited number of reports are available on the frequency of dMMR/MSI-H carcinoma in biliary tract cancer (BTC), describing its clinicopathological characteristics and prognosis. The latter carcinoma is also associated with Lynch syndrome (LS). The present study was performed to investigate the frequency of patients with dMMR/MSI-H in BTC and the clinical characteristics of BTC with dMMR/MSI-H in a single institution in Japan. A total of 116 patients with BTC who underwent curative surgical resection at Kagawa University Hospital between January 2008 and December 2017 were included. The protein expression levels of the mismatch repair (MMR) genes [mutL homolog 1 (MLH1), mismatch repair endonuclease PMS2 (PMS2), MutS homolog (MSH)2 and MSH6] were assessed by immunohistochemistry (IHC) using formalin-fixed paraffin-embedded tissue specimens. Subsequently, MSI testing was performed on patients who exhibited loss of MMR protein expression. Loss of expression of one or more proteins was detected in five cases (4.3%). Loss of MLH1/PMS2 expression was observed in one case of intrahepatic cholangiocarcinoma, whereas loss of PMS2 expression was noted in one case of perihilar cholangiocarcinoma. Loss of MSH2/MSH6 and MSH6 expression was noted in two cases of distal cholangiocarcinoma and loss of PMS2 expression in one case of ampullary carcinoma. Out of the five patients, two demonstrated MSI-H. Microsatellite stability was observed in two cases and for one case, no data were available. Two MSI-H cases were patients with loss of expression of MLH1/PMS2 and MSH2/MSH6. None of the five patients exhibited a past medical history or family history of suspected LS. The frequency of dMMR in BTC was ~5%, which was similar to that reported by similar studies performed in other countries. In the present study, IHC appeared to be more useful than MSI testing for detecting MMR abnormalities with regards to the detection rate. Furthermore, there may only be a limited number of patients with BTCs who are likely to benefit from the therapeutic effects of treatment with immune checkpoint inhibitors.

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Abbreviations: dMMR, defective mismatch repair; MSI, microsatellite instability; LS, Lynch syndrome; IHC, immunohistochemistry; UICC, Union for International Cancer Control; CCA, cholangiocarcinoma; iCCA, intrahepatic CCA; pCCA, perihilar CCA; dCCA, distal CCA; GBCA, gallbladder carcinoma; ampCA, ampullary carcinoma; PD-1, programmed death-1

Key words: biliary tract cancer, defective mismatch repair, microsatellite instability-high, immunohistochemistry, Lynch syndrome

Introduction

Malignant tumors derived from the biliary tract exhibit a variety of clinicopathological characteristics. These characteristics may be considered as epidemiological risk factors and may affect the diversity of the genetic architecture and the tissue microenvironment (1). Defective mismatch repair (dMMR) tumors are caused by loss of function of MMR proteins due to genetic (2) or epigenetic (3) events. This pathogenic variant in the germline of MMR genes [mutL homolog 1 (MLH1), MutS homolog (MSH)2, MSH6 and mismatch repair endonuclease PMS2 (PMS2)] is known to be the cause of Lynch syndrome (LS), a hereditary disease with a high incidence of colorectal and endometrial cancers. Numerous studies have reported on the expression of MMR proteins and on the incidence of microsatellite instability (MSI) status in biliary tract cancer (BTC), which is considered an LS-associated tumor (4-18). The approximate frequency of MSI-high (MSI-H) BTC is 5% for gallbladder carcinoma (GBCA), perihilar cholangiocarcinoma (pCCA) and distal CCA (dCCA), whereas it is estimated to be 10% for intrahepatic CCA (iCCA) and ampullary carcinoma (ampCA) (15).

In general, the treatment of BTC requires extended surgery, such as hepatectomy or pancreaticoduodenectomy, but the disease may be unresectable, depending on the patient's condition. Furthermore, BTC has poor prognosis due to the limited availability of effective chemotherapy options (19-22). Previous studies performed in recent years have reported that the immune checkpoint inhibitor anti-programmed death-1 (PD-1) antibody has a high response rate in patients with MSI-H. The latter is a genetic characteristic of certain solid tumors, is caused by dMMR and contributes to the overall survival of these patients (7,23,24). It has been previously reported that certain cases of MSI-H BTC may be successfully treated with pembrolizumab (25,26). Therefore, it is necessary to evaluate MMR protein expression and/or the MSI status in tumors in order to select patients who may be expected to benefit from treatment with anti-PD-1 antibodies.

In the case of colorectal (27) and endometrial (28) cancers, the concordance rate between immunohistochemistry (IHC) and MSI results is >90%, whereas such comparisons have not yet been made in patients with BTC. IHC is cost-effective and widely available in several facilities compared with MSI testing. Furthermore, IHC may be performed to determine the expression levels of the MMR genes that are likely to exhibit genetic/epigenetic changes (29,30).

The present study was performed to investigate the frequency of dMMR tumors and the clinical factors in dMMR cases using IHC. The aim was to determine the extent to which dMMR/MSI-H cases may be detected in BTCs resected at a single institution in Japan.

Patients and methods

Patients. The present study retrospectively enrolled patients who were diagnosed with BTC and underwent surgery between January 2008 and December 2017 at Kagawa University Hospital (Kagawa, Japan). A total of 116 patients were included in the present study. A total of 73 (62.9%) males and 43 (37.1%) females participated in the study. The demographic and clinicopathological data and the personal/family history of the patients were obtained from medical charts. Pathological tumor-node-metastasis staging was performed using the international system for BTC staging adopted by the American Joint Committee on Cancer and the Union for International Cancer Control, 8th edition (31). Table I presents the clinicopathological characteristics. The entire series consisted of iCCA (n=14, 12.1%), pCCA (n=17, 14.6%), dCCA (n=32, 27.6%), GBCA (n=30, 25.9%) and ampCA (n=23, 19.8%). The median age at diagnosis was 73 years (range, 38-93 years) and the majority of the included patients were aged ≥70 years (n=76; 65.5%).

IHC analysis for MMR protein detection. IHC was performed in order to assess the expression levels of four MMR proteins (MLH1, MSH2, MSH6 and PMS2) using a VENTANA MMR IHC Panel (Ventana Medical Systems, Inc.; Roche Diagnostics), according to the manufacturer's protocol. The samples were prepared from 4-μm formalin-fixed paraffin-embedded (FFPE) sections. The following primary antibodies were used for the detection of human (h) MMR proteins: Anti-hMLH1 antibody [cat. no. 518-114336;

Table I. Clinicopathological characteristics of the patient cohort.

Variable	Value
Sex	
Male	73 (62.9)
Female	43 (37.1)
Age at diagnosis, years	73 (38-93)
<70	40 (34.5)
≥70	76 (65.5)
Localization	
iCCA	14 (12.1)
pCCA	17 (14.6)
dCCA	32 (27.6)
GBCA	30 (25.9)
ampCA	23 (19.8)
History of LS-associated diseases	
Yes	18 (15.5)
No	98 (84.5)
Family history of LS-associated diseases	
Yes	8 (6.9)
No	108 (93.1)
Carcinoembryonic antigen, ng/ml[a]	2.6 (0.8-35)
Normal	96 (84.2)
Abnormal	18 (15.8)
Carbohydrate antigen 19-9, U/ml[a]	32.5 (2-63143)
Normal	61 (53.5)
Abnormal	53 (46.5)
Main differentiation	
Papillary	20 (17.3)
Well	48 (41.4)
Moderate	31 (26.7)
Poor	10 (8.6)
Adenosquamous	7 (6)
Lymph node metastasis	
0	73 (62.9)
1	43 (37.1)
Resection margin	
0	104 (89.7)
1	12 (10.3)
UICC stage	
1	31 (27)
2	48 (41.7)
3	30 (26.1)
4	6 (5.2)
Invasion into lymphatic vessels	
0	38 (36.5)
1	66 (63.5)
Invasion into veins	
0	42 (36.8)
1	72 (63.2)

Table I. Continued.

Variable	Value
Perineural invasion	
0	47 (45.6)
1	56 (54.4)

Values are expressed as n (%) or the median (range). [a]The normal reference ranges of carcinoembryonic antigen and carbohydrate antigen 19-9 are 0-5 U/ml and 0-37 ng/ml, respectively. LS, Lynch syndrome; UICC, Union for International Cancer Control; CCA, cholangiocarcinoma; iCCA, intrahepatic CCA; pCCA, perihilar CCA; dCCA, distal CCA; GBCA, gallbladder carcinoma; ampCA, ampullary carcinoma.

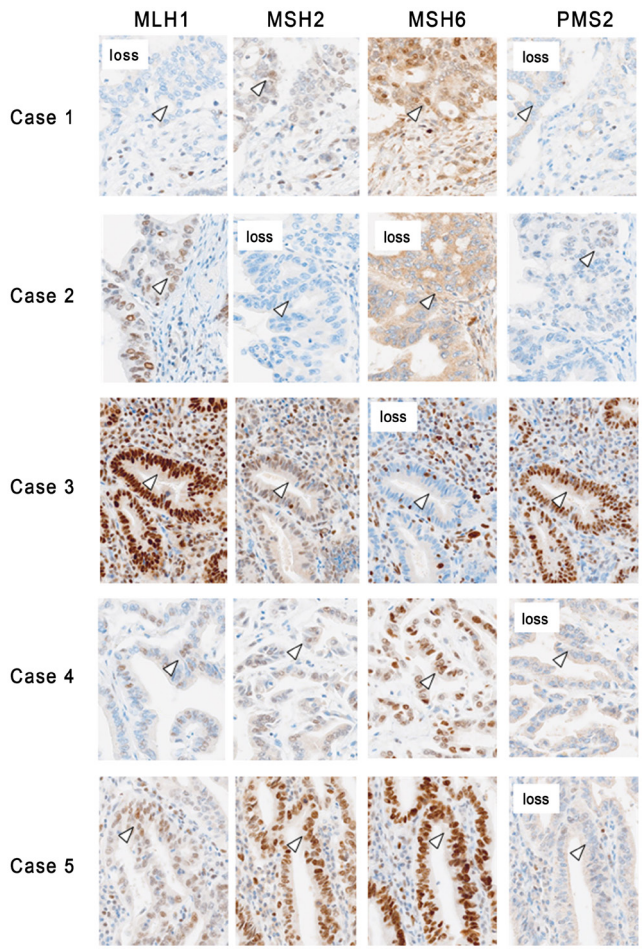

Figure 1. Expression pattern of MMR proteins in tissues from patients with biliary tract cancer and defective MMR. In case 1, loss of MLH1 and PMS2 was observed in the nuclei of cancer cells, while loss of MSH2 and MSH6 was observed in case 2. In case 3, loss of MSH6 was observed in the nuclear region of the cancer cells, while loss of PMS2 was observed in cases 4 and 5. The arrowheads indicate cancer cells (magnification, x200). MMR, mismatch repair; MLH1, mutL homolog 1; PMS2, mismatch repair endonuclease PMS2; MSH, MutS homolog.

anti-MLH1 (M1) mouse monoclonal primary antibody], anti-hMSH2 antibody (cat. no. G219-1129; anti-MSH2 mouse monoclonal primary antibody), anti-hMSH6 antibody (cat. no. SP93; anti-MSH6 rabbit monoclonal primary antibody) and anti-hPMS2 antibody (cat. no. A16-4; anti-PMS2 mouse monoclonal primary antibody); all ready-to-use from Ventana Medical Systems, Inc.; Roche Diagnostics. The normal staining patterns for MLH1, MSH2, MSH6 and PMS2 were nuclear. The absence of nuclear staining in the tumor cells in the presence of nuclear staining of non-neoplastic cells, such as normal epithelial cells, lymphocytes and stromal cells, was considered to represent an abnormal pattern (29). The staining results were evaluated by consensus between two independent pathologists and surgeons, who were blinded to the clinical status of each patient.

DNA extraction. DNA was extracted from 4-μm FFPE sections of cancer tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen GmbH).

MSI testing. MSI testing was performed in patients with dMMR and in 8 patients with a family history of LS-associated cancers. MSI testing was performed using the MSI analysis system, version 1.2 (Promega Corporation), which evaluates the MSI status of the following five mononucleotide microsatellite markers: BAT25, BAT26, NR21, MONO-27 and NR24 (32). Following amplification of the marker genes, the products were subjected to fragment analysis using the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and GeneMapper software version 4.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). When two or more markers demonstrated altered numbers of repeats, the MSI status of the tumor was classified as MSI-H.

Statistical analysis. Descriptive statistics are reported as the median (range) for continuous variables and as frequency (%) for categorical variables. The overall survival was calculated as the time from surgery to the date of death (event) or the last follow-up date (censored) using the Kaplan-Meier method. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using JMP Pro version 14 (SAS Institute Inc.).

Results

IHC analysis. A total of 5 out of the 116 patients with BTC (4.3%) exhibited loss of expression of one or more proteins. Loss of MMR protein expression was observed for MLH1/PMS2 in one case of iCCA, for PMS2 in one case of pCCA, for MSH2/MSH6 and MSH6 in two cases of dCCA and for PMS2 in one case of ampCA (Fig. 1).

MSI assessment and clinicopathological and molecular characteristics of dMMR cases. MSI assessment was performed on five patients with dMMR as determined by IHC. A total of two patients (1.7%) exhibited MSI-H, with loss of MLH1/PMS2 (iCCA) loss of MSH2/MSH6 (dCCA) expression was noted in each of these two patients (Fig. 2). In one case with the loss of MSH6 (dCCA) expression, it was not possible to measure DNA amplification owing to suspected DNA degradation, whereas in two cases with the loss of PMS2 (pCCA) and PMS2 (ampCA) expression, MSI testing exhibited microsatellite stability (MSS). Table II indicates the clinicopathological characteristics of these patients with dMMR BTC. The patient age range was 55-93 years, with

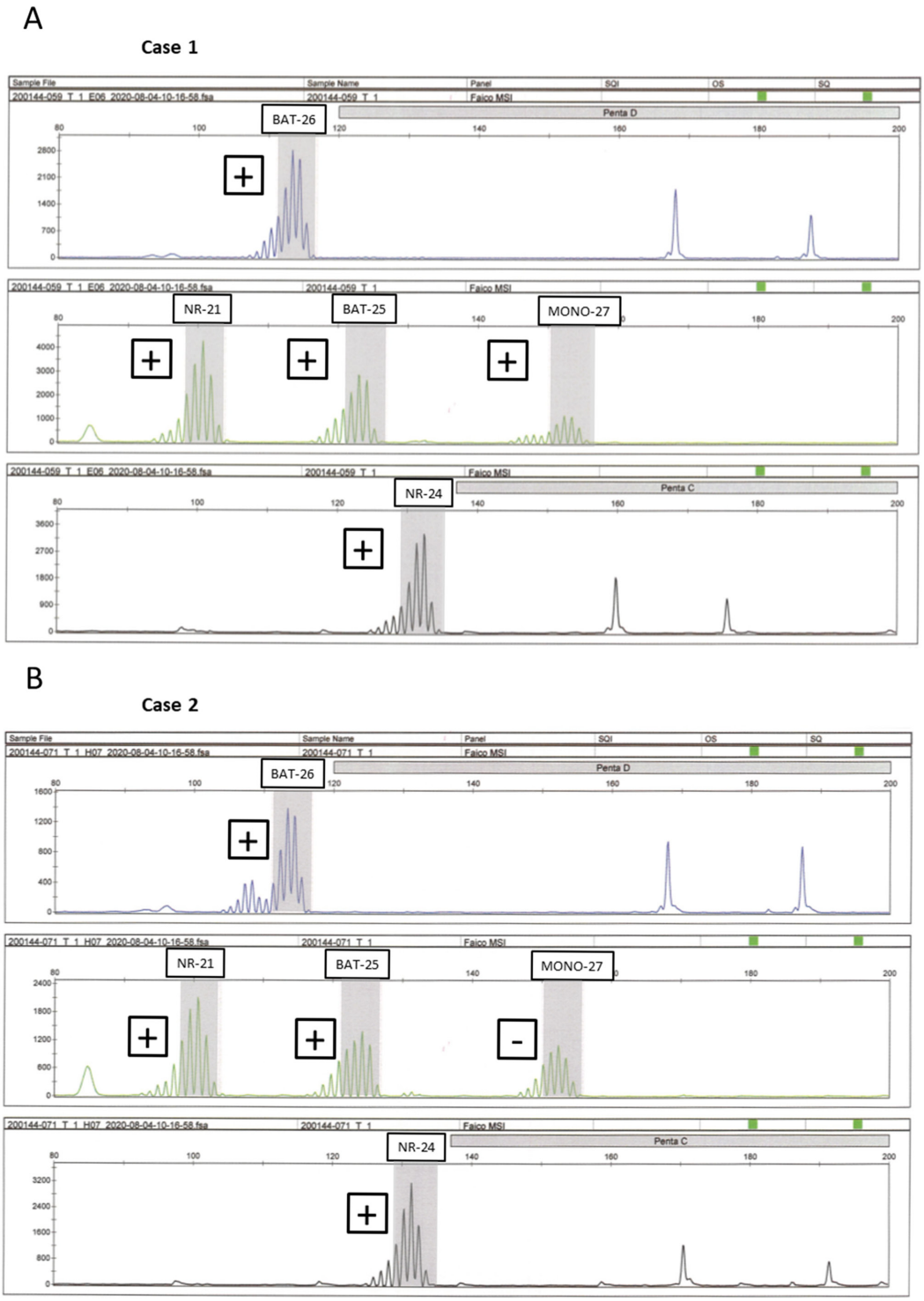

Figure 2. Representative electropherograms of the cancer tissues exhibiting MSI-H. The presence of MSI for (A) case 1 and (B) case 2 is indicated. MSI-H is present when the maximal peak at each marker is situated outside the quasi-monomorphic variation range (gray zone). In cases 1 and 2, different-sized peaks were observed other than a distinctive peak from an allele (plus). Different-sized peaks were described as 'positive' and the results that did not yield peaks as 'negative'. Five mononucleotide microsatellite markers: BAT25, BAT26, NR21, MONO-27 and NR24. MSI-H, MSI-high; MSI, microsatellite instability.

3 (60%) males and 2 (40%) females, and none of the 5 patients had any history of cancer or family history of LS-associated disease. None of the patients survived; two succumbed to the original disease due to recurrence and the remaining three did not survive due to the presence of other conditions. Among 8 patients with a family history of LS-associated cancers, all cases exhibited both proficient MMR and MSS.

Discussion

The present study was performed at a single institution in Japan. The frequency of dMMR in BTC was 4.3% and that of MSI-H in BTC was 1.7%. In addition to the commonly performed evaluation of dMMR, which includes IHC analysis, MSI assessment was performed. In a case series of BTC, a

Table II. Clinicopathological and molecular characteristics of five cases of defective mismatch repair in biliary cancer.

Item	Case 1	Case 2	Case 3	Case 4	Case 5
Age, years	55	93	60	62	76
Sex	Male	Female	Female	Male	Male
Location	iCCA	dCCA	dCCA	pCCA	ampCA
Previous history of cancer	None	None	None	None	None
Family history	None	None	None	None	None
Immunohistochemistry	dMLH1/PMS2	dMSH2/MSH6	dMSH6	dPMS2	dPMS2
MSS status	MSI-H	MSI-H	Unmeasurable	MSS	MSS
BAT-25	+	+		-	-
BAT-26	+	+		-	-
NR-21	+	+		-	-
MONO-27	+	-		-	-
NR-24	+	+		-	-
Main differentiation	Well	Moderate	Poor	Well	Moderate
UICC stage	2	3A	1	3C	3A
Resection margin	0	0	1	0	0
Recurrence (location)	+ (Lymph node)	-	-	+ (Pulmonary)	-
Overall survival, months	17	6	48	37	34
Circumstance of death	DOD	DID	DID	DOD	DID

UICC, Union for International Cancer Control; CCA, cholangiocarcinoma; iCCA, intrahepatic CCA; pCCA, perihilar CCA; dCCA, distal CCA; ampCA, ampullary carcinoma; d, defective; MLH1, mutL homolog 1; PMS2, mismatch repair endonuclease PMS2; MSH, MutS homolog; MSI-H, microsatellite instability-high; MSS, microsatellite stability; DOD, died of disease; DID, died with intercurrent disease.

small number of dMMR/MSI-H BTC was reported. Previous studies have reported that the prevalence of dMMR in BTCs is 0-9.4% (4,10,12,14,18). A large study, which used next-generation sequencing, reported a frequency of ~2% in BTCs (7). Previous studies that investigated the prevalence of MSI-H BTCs reported a frequency of 0-18% (5,6,8-14). Silva *et al* (15) reported that MSI-H accounted for ~5% of GBCA and extrahepatic CCA and for ~10% of iCCA and ampCA. Comparison of previous studies performed in Asia and Japan (6,8-11,16,33) with those from Western countries (4,5,12-14,34) indicated that the incidence of BTC with dMMR/MSI-H was almost the same in both Asian and Western countries (3.1 and 4.4%, respectively). Of note, the results of the present study were observed to be similar to those of the previous reports. However, while there were no MSI cases among patients with GBCA in the present cohort study, the frequency of dMMR/MSI-H cases in GBCA was ~5% in previous reports (15). In previous studies from Japan, Yoshida *et al* (35) determined a frequency of 0% (0/30), Yanagisawa *et al* (36) detected 6% (1/17), Nagahashi *et al* (37) reported 42% (8/19) and Akagi *et al* (33) 1.5% (3/200). The incidence of GBCA with dMMR/MSI-H was inconsistent even in Japan. The result of the present study showing that no MSI cases were observed in GBCA may be due to differences in population.

Several studies have reported concordant and discordant findings regarding the prevalence of dMMR and MSI-H in BTC. Previous studies on colorectal cancer have reported that IHC is also useful for the detection of dMMR with a sensitivity of 92% and for preventing MSI with a specificity of 99% (38-40). These results are different from those reported in the present study. Of the five cases with loss of MMR protein expression, two exhibited MSI-H. The prevalence of MSI-H BTC was lower than that of the dMMR tumors and discordant results were observed between IHC and PCR-based techniques. One case of MSH6 was reported in the current study, which was dMMR as assessed by IHC, but unmeasurable according to MSI testing. The resection specimen of this case was a paraffin-embedded section of an old specimen collected 9 years previously and therefore, it was hypothesized that the IHC staining result may have been compromised due to damage caused to the fixed tissue. The other two cases were deficient in PMS2 and the sensitivities of the MSI and IHC tests for detection of PMS2 mutations were 67 and 75%, respectively; thus, MSI testing was comparatively less sensitive (41,42). As mentioned earlier, inconsistencies have been reported in the results obtained between the two aforementioned methods (IHC and MSI testing). Therefore, these methods should be considered complementary. Specific differences in sensitivity have been reported depending on the mutated pathogenic gene. Therefore, it is considered that the therapeutic effect of anti-PD-1 antibodies may be expected if either dMMR or MSI-H is recognized.

In the present study, dMMR/MSI-H was observed in one case of MLH1/PMS2 and in one case of MSH2/MSH6. In general, the MLH1/PMS2 expression pattern suggested hypermethylation of *MLH1* (43). It is considered that methylation is the cause of loss of MLH1 expression, which is observed

in colorectal and uterine cancers, whereas loss of MSH2 expression may be due to somatic mutations in both alleles in addition to methylation. Tajima *et al* (44) reported that somatic mutations were also detected in both of these alleles in uterine carcinoma. In the present study, the five patients with loss of MMR protein expression did not survive and it was therefore impossible to perform an additional detailed genetic analysis. However, it was hypothesized that methylation and somatic alterations may have been associated with loss of MLH1 or MSH2 expression in the cases with dMMR/MSH-H, since these patients did not have any past history or family history of cancer. A study from Thailand (45) reported that CCA derived from liver fluke infection had a high incidence of MSI-H (9/13 cases, 69%). Although the pathogenesis of BTC in Japan is expected to be different from that in Thailand, the study will contribute to identify the mechanisms of alteration to MSI-H, leading to an increased understanding of the development of BTC.

BTC is also known as one of the important LS-associated tumors, as described in the revised Bethesda guidelines (46). Patients with colon and uterine cancers, which have a high frequency of dMMR/MSI-H, may be screened for the incidence of LS. In colon cancer, LS is diagnosed in ~3% of sporadic cases (47,48). Although eight patients had a family history of LS-associated cancers, they exhibited both proficient MMR and MSS. In the present study, none of the BTC patients with dMMR had a past medical history or family history of LS-associated cancers. Therefore, universal screening for all patients with newly diagnosed BTC may be an effective screening method, similar to that used for colorectal and uterine cancers.

A relatively large proportion of patients with BTC are diagnosed as unresectable (49) and even those who undergo curative resection have a high recurrence rate (50,51). A comparable treatment regimen to gemcitabine or cisplatin is yet to be established. The 5-year survival rate is 5-15% for all patients with BTC (19-21). In recent years, anti-PD-1 antibodies and immune checkpoint inhibitors have been reported to be effective in the treatment of various types of MSI-H solid tumors (23,52). It is expected for this therapeutic approach to improve the overall survival of patients with non-colorectal cancer who exhibit loss of MMR protein expression and/or MSI-H (53,54). Therefore, evaluation of MMR protein expression and/or MSI may be necessary to select patients with cancer who may benefit from treatment with anti-PD-1 antibodies. In the present study, the incidence of BTC with dMMR/MSI-H was ~5%, which is in agreement with that reported in previous studies (4,10,12,14,18). Based on these findings, it is suggested that screening should be routinely performed, since the prognosis of patients with BTC and dMMR/MSI-H was considerably poor.

There were certain limitations to the present study. First, it was performed at a single institution in the area of Shikoku, Japan. Therefore, the possibility of a regional selection bias among patients with BTC should be considered. Furthermore, all five patients with loss of MMR protein expression had already succumbed to the disease and it was not possible to perform any detailed genetic analysis on them. Finally, the difference between patients with pMMR and dMMR was not analyzed due to the low number of dMMR cases. In the future, additional data from other institutions must be collected in order to determine the exact prevalence of dMMR among patients with BTC.

The present study was a comprehensive study performed to examine the incidence of dMMR BTC in a hospital-based population in Japan. The data revealed a relatively low prevalence of dMMR cases. In the present study, IHC was indicated to be more useful than MSI testing for detecting MMR abnormalities with regards to the detection rate. Consequently, selected patients with BTC may benefit from the therapeutic effects of immune checkpoint inhibitors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YA devised and designed the study. YA and KK analyzed and interpreted the patient data regarding biliary tract disease. HKa, AM, HKo and TsM contributed by providing medical preoperative diagnoses. RI, ToM and RH performed IHC and histopathological evaluations. KK, YS and KO were involved in drafting the manuscript and revising it critically for important intellectual content. YA, KK, HM, HS, MO, YS and KO designed the treatment plan and performed the surgeries. KK and KO confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Committee of Kagawa University (approval no. 2019-231). It conformed to the provisions of the Declaration of Helsinki. Written informed consent was obtained from the subjects in the form of an informative or disclosure document.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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