Peer Review File

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Reviewer A

Lynch syndrome is a condition of widespread interest in terms of cancer risk and treatment, but clinical information in biliary tract cancer is lacking. This is therefore a very important report. I would like to make a few minor comments.

Comment 1:
Page 6 line 17
It is recommended to use internationally widely used terms for the names of each organ of Biliary tract cancer, referring to WHO classification, etc. For example, intrahepatic bile duct, extrahepatic bile duct, amulla of Vater, etc.
Reply 1:
We would like to thank the reviewer for the suggestion. As your suggestion, we agreed to use WHO classification. We have modified our text as advised (see Page 6, line 12-13).

Comment 2:
Two patients with biliary tract cancer associated with Lynch had synchronous and metachronous BTC. This is very interesting and should be explored further. It would be interesting if you could mention if other precancerous lesions such as BilIN or IPMB were present in the biliary epithelium.

Reply 2:
We are glad that the reviewer is interested in these 2 patients. We pathologically explored biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPMB), and found out that the 2 patients didn't show other precancerous lesions such as BilIN or IPMB. We added the clinical data as advised (see Page 9 line 17-18).

Comment 3
You described, "All available BTC lesions (n = 7) showed high-frequency of microsatellite instability (MSI-H)." (P3L15-16).
However, "Probands underwent genetic testing if they presented with high-frequency of microsatellite instability (MSI-H) tumors or met a Modified Amsterdam II criteria...." (P6, L10-11).
If the current study subjects are all patients who have been diagnosed with Lynch syndrome by the former procedure, the MSI-H positivity rate would be meaningless.
Therefore, we would like a flow diagram of the selection of patients for this study to be illustrated.p
Reply 3:

We would like to thank the reviewer for the suggestion. As we mentioned, the utility of MSI testing still remains unclear for screening to detect LS from BTCs. Therefore, we also tested MSI analysis for patients with primary BTCs (21 lesions of BTCs) who underwent surgical resection between July 2008 and May 2014 at our institute. We believe that this MSI testing is interesting because all sporadic BTCs (15 lesions) revealed MSS or MSI-L while 6 lesions of BTCs in 5 individuals with LS showed MSI-H. As your suggestion, we added the flow diagram for MSI testing (Figure S1, see page 8 line 1-2).

Comment 4:
You mention the degree of differentiation of the pathology findings. It would be interesting if you could also mention the presence or absence of findings of medullary carcinoma, which is sometimes reported in pancreatic cancer associated with Lynch syndrome, and the presence or absence of lymphocytic infiltration.
Reply 4:
We would like to thank the reviewer for option. We are also interested in unique pathological feature of Lynch syndrome associated colorectal cancers such as Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, and medullary growth pattern as you suggested. Poor differentiated carcinoma (por) used to include medullary carcinoma in colorectal cancers. In this study, the initial BTC in the case 3 showed majority of tub2 (as shown table 3) and partly por. Then, a pathologist re-checked the specimens and didn't detect the medullary carcinoma in the specimens. Then, we also didn't detect medullary carcinoma in our cases (except case 6). Unfortunately, tumor-infiltrating lymphocytes were not observed in this study due to higher demand of regular pathological works. (See page 9 line 18, page 10 line 1-2).

Comment 5:
J Hepatobiliary Pancreat Sci. 2021 Oct 24. may also be compared and discussed as a report from the same Japan.
Reply 5:
We would like to thank the reviewer for telling us the interesting report. We have modified our text as advised (see Page 12, line 14)".

Reviewer B

This study aims to investigate the clinical features of BTC in individuals with LS, and assess the utility of MSI testing for LS identification in individuals with BTC. This is an important study with some valuable data and reasonable conclusions (surveillance for BTC in MLH1-carriers in high-prevalent areas, and that MSI is appropriate for screening). However, there are some missing points that should be supplemented or clarified.
Major
1. Clinical data is missing regarding the development of BTC cases: were these cases were prevalent on incident (developed during surveillance for BTC)? This is especially interesting for those who were diagnosed before with LS.
Reply 1:
We would like to thank the reviewer for the suggestion. Currently, no standardized surveillance

method for BTC on patients with LS has been established. However, we have been able to diagnose 7 asymptomatic BTCs among 3 patients by CT scan and blood biochemical tests performed during postoperative follow-up for colorectal or biliary tract cancers. Three asymptomatic patients with BTCs showed earlier clinical stages and better clinical outcomes than other 3 symptomatic patients. We added the clinical data as advised (see Page 14 line 1-5).

2. Also, clinical data regarding family history of BTC in the case is missing. Other potential risk factors (age, gender, gene mutation, FH) should be evaluated in uni/multivariate analysis, compared with the other LS-carriers.

Reply 2:

We would like to thank the reviewer for important suggestion. As the suggestion, we evaluated the risk factors (age, gender, gene variant and family history of BTC) using univariate analysis. Unfortunately, as shown below, we could not detect the significant risk factors due to some limitation we shown in discussion (page 14 line 8-9). Then, we would like to plan prospective study with multiple center design in the future to evaluate in uni/multivariate analysis (see page 14 line 12-15).

Variable	BTC + (n=6)		BTC- (n=86)		p value (Univariate analysis)
Sex					
male	4	66.7(%)	38	44.2(%)	0.406
female	2	33.3(%)	48	55.8(%)	
Age at last follow up					
<50	2	33.3(%)	34	39.5(%)	0.226
50≦	4	66.7(%)	52	60.5(%)	
Gene variant					
MLH1	6	100(%)	69	80.2(%)	0.589
Others	0	0(%)	17	19.8(%)	
Amsterdam II					
met	3	50(%)	39	45.3(%)	1
unmet	3	50(%)	47	54.7(%)	
Family history of BTC					
yes	2	33.3(%)	29	33.7(%)	1
no	4	66.7(%)	57	66.3(%)	

3. As mentioned in the introduction, survival benefit is proved in CRC. If data is available in the 15/21 sporadic BTC, it would be preferable to compare survival in the two groups.

Reply3:

We would like to thank the reviewer for important opinion. Unfortunately, it is very hard to match the clinical background between individuals with LS and individuals with non-LS. We are thinking to make a plan prospective study with multiple center design in the future to understand this survival benefit in detail.

Minor

1. Discussion page 12: I wouldn't put a statement that "Therefore, MSI analysis could be an efficient screening tool for BTC to identify LS when patients with BTC have tumor associate family history and medical history". Instead, it's more appropriate to say that BTC should be screened for LS in the appropriate clinical setting (fulfillment of Bethesda / Amsterdam criteria).

Reply1:

In general, Revised Bethesda Guideline is used for testing colorectal tumors for MSI testing. Then, Amsterdam criteria Amsterdam is widely used to identify patients and families at risk for Lynch syndrome. However, BTC doesn't include in the criteria. Therefore, we explored the

utility of MSI for BTC as colorectal cancer. Then, we found out BTCs with LS showed MSI-H while sporadic BTCs showed MSS/MSI-L. Of course, the sample number is small but we believe that the results are interesting and can be useful screening tool for BTC as well.

2. Table 4 and Figure 2 can be omitted. They do not add valuable data on top of that written in the manuscript.

Reply 2:

We would like to thank the reviewer for the suggestion. We deleted Table 4 and Figure 2 as your suggestion.

3. Results page 10: "The cumulative risk of MSH2 at 75 years of age was 0%." Since all patients with BTC were MLH1 carriers, this sentence is obvious and can be omitted (also from Figure 1).

Reply 3:

We would like to thank the reviewer for important suggestion. We omitted the sentence as advised (see Page 10, line 8-10).

4. If I understand correctly, the 6/21 patients with MSI-H in the BTC samples were the same 6 LS cases that are reported in the results? this point should be clarified. If not, these new cases can be added and be clinically / genetically described.

Reply 4:

We apologize the point for MSI analysis. To clarify MSI analysis, we added Figure S1 and contents in methods (see Page 8 line 1-2 and Figure S1).

5. Results page 9: "Among the 92 individuals, 6 had 10 BTCs". You should clarify that for one patient tissue and clinical tumor data was not available, before reporting the other 9 patients.

Reply 5:

We would like to thank the reviewer for the suggestion. We added Figure 1 to understand it easily. We have modified our text as advised (see Page 9, line 9-10)".

6. Discussion page 13: "hematological examinations" are too general. Liver enzymes and CA 19-9 is more appropriate.

Reply 6:

As we mentioned in page 13 line 13- page 14 line 1, hematological examination means liver and bile duct enzymes. We have modified our text as advised (see Page 13, line 14-18, Page 14, line 1)".

7. Discussion, page 13. The institutional practice should be clarified. Additionally, what is the recommended age for BTC surveillance in those MLH1-carriers without FH ?

Reply7:

We would like to thank the reviewer for the suggestion. We added the family history of BTC in Table 3. Then, we mentioned the recommendation about surveillance. We have modified our text as advised (see Page 13, line 2-9)".

Reviewer C

This is a great addition to the literature for MLH1-associated LS and for patients with LS of Japanese descent. I applaud the authors for this study and their attention to statistical analysis and to clinical application, such as recommendations for screening. I have the following observations/suggestions for revision.

Comment 1:
page 6, line 8. the authors state that a retrospective review of patients with diagnosed LS was conducted, but then describes a process for tumor testing (MSI) based on clinical criteria to make this diagnosis. Can you please clarify if the tumor and subsequent germline testing was done to identify these cases prior to this study? Were all BTC screened by MSI at this institution or are the authors looking at data from patients who already had LS and then pulling out the BTC information for these patients? If the later, this should be made clearer in the methods section.
Reply1:
We would like to thank the reviewer for important suggestion.
In this study, three patients were diagnosed to LS when they developed BTC and the remaining three were diagnosed to LS when they developed CRC. We added related information in Table 2.

Comment 2:
page 9, lines 13-19. The authors state that 10 BTC were diagnosed, but locations are listed out of an N=9 and the MSI-H testing was listed as N=7 in one place (page 10, line 3) but was described as 6 tumors in another location (page 10, line 14). Based on the tables, it seems some of this information was unknown or the tumor was not available for testing or the patient had multiple primary tumors and only one was included. Please clarify this in the text.
Reply 2:
We would like to thank the reviewer for important suggestion. The two lesions were too small to do MSI testing in case 3 (Table 3). We also added the information in our text (See page 10 line 3-4).

Comment 3:
Page 10, line 7, authors should clarify that this is cumulative risk of BTC for individuals with an MLH1 pathogenic variant.
Reply3:
We apologize the mistake. We have now corrected the mistake. (See Page 10, line 8-9).

Comment 4:
Page 10, line 15. It would be important to clarify if two of the lesions of case 3 did not have MSI-H pathology or to clarify if testing was only performed on two lesions. Page 10, line 7, please clarify if "figure 1" is the correct location to describe the characteristics of case 3.

Reply4:
We would like to thank the reviewer for important suggestion. As we mentioned in comment 2, the two lesions were too small to do MSI testing in case 3 (Table 3). We added this data (See Page 10 line 1-2).

Comment 5:
The discussion (page 10, line 18) states that MSI analysis is an effective screening tool for identifying LS, however only 2/70 tumors in the LS cohort had BTC as the initial tumor and the authors also state (page 11, line 18) that data is limited on MSI status in both sporadic and LS cases. This is also addressed a bit later (page 12, line 8) as being appropriate in conjunction with family history data. The authors should clarify if this and make sure it is consistent.
Reply 5:
We would like to thank the reviewer for the opinion. Compared to colorectal cancers, it is not often to detect BTC as initial cancers, but in this study, 2 patients developed 2 BTC as initial cancer. We admit BTC is less popular cancer than CRC and EC in LS. However, patients with LS who developed CRC or EC are not always accurately diagnosed with LS. We experienced a case that LS was diagnosed because of BTC that developed as the second cancer after CRC (Table3, Case 5). Therefore, we believe it is important to know whether the MSI testing for BTC can be a screening test for LS as well as for CRC and EC.

Comment 6:
Page 12, line 9 and Page 12, line 13. Authors state different numbers for cumulative risk for BTC from previous studies, please clarify if this is because the cumulative number was to age 70 vs. 75. Authors should consider combining these in the discussion for clarity.
Reply 6:
We would like to thank the reviewer for the suggestion. we have modified our text as advise (see page 12 line 11).

Comment 7:
Page 12, line 12. The authors should consider clarifying that the total LS cohort was majority patients with MLH1 pathogenic variants (page 9, line 5), so the pool of patients with BTC would also weight heavily towards MLH1, even if the incidence of BTC was present for other LS associated genes.
Reply 7:
We would like to thank the reviewer for the suggestion. In this study, we pointed the limitation in discussion (See Page 14 line 8-10).

Comment 8:
Page 13, lines 1-5 are confusing and should be clarified
Reply 8:
We would like to thank the reviewer for the suggestion. we have modified our text as advised (see Page 13, line 9-13).

Comment 9:

Page 13, line 13. The authors should comment on why they are extending this recommendation to all Asian-Pacific Islanders if the data is specific for their Japanese cohort.
Reply 9:
We would like to thank the reviewer for the suggestion. we have modified our text as advised (see Page 14, line 7).

Comment 10:
Discussion: the authors could consider commenting on what other factors they think increase the risk for BTC in the Japanese cohort with LS, but without primary sclerosing cholangitis. is there any literature to support why the risk might be significantly higher in this population? If there is literature to support this, does it extend to other Asian-Pacific Islanders which is why they are suggesting a screening protocol in that population?
Reply 10:
As some guidelines showed, BTC is one of the LS associated tumors (Revised Bethesda guideline and Japanese guideline (Int J Clin Oncol. 2021 Aug;26(8):1353-1419. doi: 10.1007/s10147-021-01881-4. Epub 2021 Jun 29.)). In Japan, there is a recent literature about LS associated BTCs (J Hepatobiliary Pancreat Sci. 2022 Mar;29(3):377-384. doi: 10.1002/jhbp.1063. Epub 2021 Nov 9.). We have modified our text as advised (see Page 14, line 7).

Comment 11:
Discussion: LS screening is often based on family history of a LS-associated rare cancer. Is there any family history data for these 6 patients? If yes, it would be interesting to note if there was any BTC family history reported (other than the included proband).
Reply11:
We would like to thank the reviewer for the suggestion. We added the data in Table 3 as advised.

Reviewer D

In this manuscript, the authors reported a descriptive study on the characteristics of biliary tract cancer (BTC) in Japanese individual with Lynch syndrome (LS). The topic is of interest since there are not so many studies reported biliary tract cancer and it is considered rare comparing to the other LS associated cancers. The authors may consider these following points to improve the presentation.

1. Why the cancer of ampulla of Vator included in BTC in this manuscript? Generally, this cancer is quite different from cholangiocarcinoma or other types of biliary tract cancer, for example, in term of cell type origin, risk factor, etc.
Reply 1:
We would like to thank the reviewer for the suggestion. According to classification of BTCs by the Japanese Society of Hepato-Biliary-Pancreatic Surgery, cancer of ampulla of Vater is classified into BTC. Further, recent previous studies also included ampulla of Vater in BTC (J Hepatobiliary Pancreat Sci. 2022 Mar;29(3):377-384. doi: 10.1002/jhbp.1063. Epub 2021 Nov

9., J Gastrointest Cancer. 2018 Mar;49(1):93-96. doi: 10.1007/s12029-017-0040-9.). We believe this information is critical because we may have to monitor ampulla of Vater for individuals with LS by endoscopy.

2. There were some people developed more than 1 BTC tumors in the same subject. It would be better to report the information for individual tumor since readers may argue whether they were independently occurred or locally metastasized. The factors that might be associated with the greater tumor burden should be also analyzed.

Reply 2: We would like to thank the reviewer for the suggestion. In case 3 (Table 3), we realized one lesion as the second BTC before surgery. After that, we pathologically confirmed other small 2 lesions (not metastasis) although the 2 lesions were too small to be available for MSI testing. We also added the information in our text (See page 10 line 2-3).

3. Among all individuals with BTC, there only 2 who have BTC as an initial caner. The data of these subjects should be shown and discussed what could be the risk factors or precipitating factors for BTC carcinogenesis in these individual.

Reply3:

We would like to thank the reviewer for the suggestion. Of the two patients who developed BTC as their initial cancer, one developed BTC at 78 years old. Therefore, we think the patient developed BTC as her initial cancer because the patient did not develop colon or uterine cancer. Regarding risk factors for BTC, age, ethnicity, and geographical lesion are generally important. In patients with Lynch syndrome, *MLH1* variant is reported to be a risk facer to develop BTC (Moller, et al). Besides, family history of BTC was observed in 3 of the 6 presented cases, suggesting that a family history of BTC may also be a risk factor. Aside from development of BTC as an initial cancer, we believe it is important to detect asymptomatic BTCs (at early stage) by surveillance in order to improve the prognosis of patients with Lynch syndrome. We have added our text as advised (See Page 13, line 2-9).

4. The authors should also discuss the suggestions for further study, not just only acknowledged the limitations.

Reply4: We have added our text as advised (See Page 14, line 12-15).

Reviewer E

Abstract is very confusing making it a hard read. surveillance not well delineated and with only 7 cases hard to make any specific recommendations. multiple uses of moreover in the text was also confusing

Reply:

We would like to thank the reviewer for the suggestion and apologize for confusing abstract. We have modified our abstract (see Page 3). In Japan, it is very hard to collect a lot of patients with LS associated BTC and a recent study also showed only 8 patients with LS associated BTC (Clinical characteristics of pancreatic and biliary tract cancers associated with Lynch

syndrome. J Hepatobiliary Pancreat Sci. 2022 Mar;29(3):377-384. doi: 10.1002/jhbp.1063. Epub 2021 Nov 9.) We believe that it is very important to report one by one if disease is rare.