Response to the Comments from Reviewers and Editors (Ms. No. 82233)

Dear Editor:

Thank you very much for us to know the important comments from the reviewers. We respond to each comment as follows.

Response to the comments from reviewers
Reviewer #1:
Comment 1:
Perhaps, authors should state why CYP2E1 was tested, as there are other human CYPs that could be carcinogen-activating enzyme e.g. CYP4B1.

Response: Thank you for the important comment. When we initiated our study, we anticipated that nitrosamines in food might also be involved in cholangiocarcinogenesis as reported by Petcharin et al. [Ref. 4, 1991]. In addition, we previously reported that consumption of alcoholic beverages may contribute to CCA risk [Ref. 5, 2005]. Moreover, a literature search revealed that alcohol consumption can lead to the overexpression of *CYP2E1*, implying that CYP2E1 is the most plausible enzyme by which nitrosamines are metabolized at very low concentrations during the development of hepatocellular carcinoma [Ref. 23 Tsutsumi et al. 1993]. Therefore, we studied polymorphisms of *CYP2E1*." Thus, we now cite the following two articles as Refs. 22 and 23 in the last sentence of the Discussion to clarify the purpose. Also, as you suggested, we added the following 'limitations' sentences at the end of the Discussion.

"**Limitations and future work**
Because the numbers of cases and controls were not **large**, the **conclusions** from this work should be confirmed in a future study with more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions."

[Reference 23]
Tsutsumi M, Matsuda Y, Takada A. Role of ethanol-inducible cytochrome P-450 2E1 in the development of hepatocellular carcinoma by the chemical

carcinogen, N-nitrosodimethylamine. *Hepatology* 1993; **18**(6): 1483-1489 [PMID: 8244274]

[Reference 24]
Chowdhury G, Calcutt MW, Nagy LD, Guengerich FP. Oxidation of methyl and ethyl nitrosamines by cytochrome P450 2E1 and 2B1. *Biochemistry* 2012; **51**(50):9995-10007. [PMID: 23186213 PMCID: PMC3525961 DOI: 10.1021/bi301092c]

Comment 2:
I am not well-versed with case study manuscript, but it would be good if authors could provide the conclusion after the discussion section, limitations of your study and future work.

Response:
The **main conclusion is** presented in the Abstract, and therefore we added text concerning "limitations and future work" at the end of the revised Discussion in response to your Comment 1.

"**Limitations and future work**
Because the numbers of cases and controls were not large, the conclusions from this work should be confirmed in a future study with more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions."

Reviewer 2:
Comment 1:
1. The methodology was comprehensively described in the manuscript, however, the associated with each of the inflammation genes and the well-known metabolizing enzymes were highly due to the small sample size in the study.

Response:
We agree that the sample size was not large. Therefore, the conclusion of our study should be confirmed by future work. Thus, at the end of the revised Discussion, we have added text to describe the limitations of our study and

that future work must be carried out, as follows.
“**Limitations**
Because the numbers of cases and controls were not large, the conclusions from this work should be confirmed in a future study with more cases and controls. In addition, genes encoding other drug-metabolizing enzymes should also be tested with respect to gene-gene interactions.”

Comment 2:
2. If the statement or inclusion criteria of normal alpha fetoprotein are not the main contributor variable in the study, please remove. Else, there is no point mentioning in the manuscript.

Response:
Based on your recommendation, we deleted the following description from the Materials and Methods. “and a normal alpha fetoprotein level (<20 ng/ml), although the latter was not considered obligatory for inclusion of a case in our study.”

Comment 3:
3. Were the blood samples stored at -80C before DNA extraction? Why was it not extracted immediately after? Or was the DNA that was stored at -80C?

Response:
Because the Ubon Cancer Center, Ubon Ratchathani, Thailand, where the blood samples were taken, did not have a –80°C freezer, the blood samples, which were frozen immediately after collection, were transferred to the National Cancer Center, Bangkok, and subsequently transferred in dry-ice to The University of Tsukuba (the institution for one of the co-authors) where they were stored at –80°C. Then, the blood samples were transferred in dry-ice to the Nagahama Institute of Bio-Science and Technology where they were stored at –80°C before DNA was extracted.
Thus, the sentence was modified as “Blood samples were frozen and stored at –80°C.”

Comment 4:
4. Please emphasis the basis of assessing the inflammation genes

polymorphisms with CCA. And the link between the carcinogen infection was unclear to correlate it with CCA and the inflammation markers. Need to justify the purpose.

Response:
Thank you for the important comment. Elevated plasma IL-6 has been reported to be associated with CCA risk [Sripa et al. 2012]. Thus, we added the following sentence to the Introduction (line 76), **immediately after** the sentence, "----carcinogenesis is reported to be involved in CCA risk [10]." "Also, elevated plasma IL-6 was associated with increased risk of CCA in patients infected with OV [11]. Thus, maintenance of ----".

[Reference 11]
Sripa B, Thinkhamrop B, Mairiang E, Laha T, Kaewkes S, Sithithaworn P, Periago MV, Bhudhisawasdi V, Yonglitthipagon P, Mulvenna J, Brindley PJ, Loukas A, Bethonu JM. Elevated plasma IL-6 associates with increased risk of advanced fibrosis infected by Opisthorchis viverrini. *PLOS Negl Trop Dis* 2012; **6**(5): e1654 [PMID: 22629477 DOI: 10.1371/journal.pntd.0001654]

Comments by Science editor:
The manuscript has been peer-reviewed, and it' s ready for the first decision.
Language Quality: Grade B (Minor language polishing)
Scientific Quality: Grade C (Good)

Response:
Language was finally polished by BiomEditor keeping our original meaning of the manuscript although the certificate document has not been sent at the moment. It will have been uploaded once it will have been sent to us. We have asked the BiomEditor to dispatch the certificate as soon as possible.

Comments by Company Editor-in-Chief
Before final acceptance, when revising the manuscript, the author must supplement and improve the highlights of the latest cutting-edge research results, thereby further improving the content of the manuscript.

Response:
Thank you for the important comment. The following “ARTICLE HIGHLIGHTS” was added as a separate section before Acknowledgements. We also removed **Mini abstract** and inserted **the core tip** after key words.

Core tip
Cholangiocarcinoma (CCA) is an intractable cancer, and its prevalence in northeastern Thailand is the highest worldwide.
An inflammatory condition produced by infection with the liver fluke *Opisthorchis viverrini* (OV) has been associated with CCA risk, but the susceptibility of individuals has not been fully examined.
Our study revealed that persons with the *GSTT1* wild-type and *CYP2E1* c1/c2+c2/c2 genotype had an increased risk for developing CCA (OR = 3.33 (95% CI :1.23–9.00). Therefore, both gene-gene interactions and OV infection should be considered as risk factors for cholangiocarcinogenesis.

ARTICLE HIGHLIGHTS