Dear Editor,

Please find enclosed the revised manuscript in word format (file name "Revised manuscript").

Name of Journal: World Journal of Gastroenterology

Manuscript number: 47869

Column: Basic study

Title: Honokiol-enhanced cytotoxic T lymphocyte activity against cholangiocarcinoma cells mediated by dendritic cells pulsed with DAMP components-derived tumour cell lysates

Authors: Arunya Jiraviriyakul, Worawat Songjang, Pongsathorn Kaewthet, Phachsita Tanawatkitichai, Punyapat Bayan, Sutatip Pongcharoen

Thank you very much for your kind e-mail, which give us the possibility to revise our manuscript. We emended the paper according to the reviewers and editor comments. We hope this revision will make our manuscript better to be accepted in your journal.

Each comment has been answered accordingly in the manuscript and each text that has been altered was highlighted yellow in the revised manuscript.

We hope that the revised version will fulfill the requirements for publication in the World Journal of Gastroenterology.

Sincerely yours,
Sutatip Pongcharoen

Department of Medicine, Faculty of Medicine,

Naresuan University, Tapho District, Muang,

Phitsanulok 65000, Thailand. sutatipp@nu.ac.th

Telephone: +6689-562-8060

Answer to editor' comments:

First of all, accordingly to the general information of our manuscript. We would like to recheck the repeat and the missing name of the author as show below.

Step 1: Verify the accuracy of general information for your manuscript

Name of journal: World Journal of Gastroenterology

Manuscript NO.: 47869

Column: Basic Study

Title: Honokiol-enhanced cytotoxic T lymphocyte activity against cholangiocarcinoma cells mediated by dendritic cells pulsed with DAMP components-derived tumour cell lysates

Phachsita Tanawatkitichai

Authors: Arunya Jiraviriyakul, Worawat Songjang, Pongsathorn Kaewthet, Pongsathorn Kaewthet, Punyapat Bayan and Sutatip Pongcharoen

Correspondence to: Sutatip Pongcharoen, PhD, Associate Professor, Department of Medicine, Naresuan University, Department of Medicine, Faculty of Medicine, Naresuan University, Phitsanulok, Thailand. sutatipp@nu.ac.th, Meaung 65000, Phitsanulok, Thailand. sutatipp@nu.ac.th

Reviewer code: 00057659, 03291363, 02739495, 00291404, and 00681914

First decision: 2019-06-10

Science editor: Jia-Ping Yan

1. Please don't include abbreviations in the title, the title should no more than 12 words and a short running title should be no more than 6 words.

Answer The title already converted to "**Honokiol-enhanced cytotoxic T lymphocyte activity against cholangiocarcinoma cells mediated by dendritic cells pulsed with Damage-associated molecular pattern**" and a short running title converted to **"Effector T lymphocyte against cholangiocarcinoma"**

2. Please include the postcode

Answer We have followed reviewer's suggestion

3. You need to provide the grant application form(s) or certificate of funding agency for every grant, or we will delete the part of "**Supported by...**".

Answer We send the grant certificate from Thailand Research Fund (TRF, BRG6180010) and Naresuan University research grant (R2561B001) and prefer to delete R2559B102.

4. Please provide the approval file of Institutional review board, and state it on the title page

Answer We have followed reviewer's suggestion

5. Please provide the approval file of Institutional animal care and use committee, and state it on the title page.
Answer This research did not involve animal subject.

6. Please download the Conflict of Interest (PDF), fill it in, and then upload the completed PDF version to the system.
Answer We have followed reviewer's suggestion

7. Please offer the audio core tip
Answer The audio core tip has been supplemented.

8. Please upload the PDF version of a statement affirming that the statistical review of the study was performed by a biomedical statistician to the system

Answer The statistical review has been performed and the certificate has been uploaded with the revised manuscript.

9. Please write article highlights
Answer We have followed reviewer's suggestion

10. Please check and confirm that there are no repeated references and add PUBMED and DOI citation
Answer The references have been checked and there are no repeated references. The PUBMED and DOI has been cited correctly.

11. Regarding the figures.

Answer Figures have been re-checked and revised. The missing labeling has been added, and we provided the editable figures as PPT format.

Answer to reviewers' comments:

Name of Journal: World Journal of Gastroenterology

Manuscript number: 47869

Column: Basic study

Title: Honokiol-enhanced cytotoxic T lymphocyte activity against cholangiocarcinoma cells mediated by dendritic cells pulsed with DAMP components-derived tumour cell lysates

Authors: Arunya Jiraviriyakul, Worawat Songjang, Pongsathorn Kaewthet, Phachsita Tanawatkitichai, Punyapat Bayan, Sutatip Pongcharoen

The manuscript has been improved according to the suggestions of reviewers. We have carefully addressed the criticisms point -by-point, our response is indicated below in blue and highlighted in the updated version of the manuscript.

(1)Reviewer no 00681914

The conclusions and novel findings are well stated, and the essential data appropriately summarized. The authors **could better explain the future directions** of the topic described. The impact for clinical practice is clearly shown.

1. Page 3: The reviewer suggest to improve the statement of background from "Cell-based immunotherapy such as dendritic cells (DCs) would be beneficial as a combined treatment for the patients. However, pulsing DCs with whole tumor cell lysates may show low efficacy against cholangiocarcinoma" to "Since immunotherapy by dendritic cells (DC) may be beneficial for cholangiocarcinoma treatment but their efficacy against cholangiocarcinoma was low. We suggest how such anti-tumor activity can be increased using cell lysates derived from an honokiol-treated cholangiocarcinoma cell line (KKU-213L5).
Answer We have followed reviewer's suggestion.

2. Page 5: The reviewer suggests correcting the gramma.
"Therefore, improvement…" to "Therefore, an improvement…"
Answer We have followed reviewer's suggestion.

3. Page 5: The reviewer suggests correcting the gramma.
"CCA antigen immunogenicity for DC …" to "CCA antigen immunogenicity for a putative DC …"
Answer We have followed reviewer's suggestion.

4. Page 6: The reviewer suggests correcting the gramma.
"… the efficacy of DC cancer vaccines against CCA requires improvement and no reports are available…" to " …the efficacy of DC cancer vaccines against CCA requires improvement, but till now no reports are available…"
Answer We have followed reviewer's suggestion.

5. Page 6: The reviewer suggests giving more explanation about "*Opisthorchis viverrini*"
Answer A clarify sentence was added for giving the explanation.

6. Page 7: The reviewer suggests specifying the honokiol concentration.
Answer We have followed reviewer's suggestion.

7. Page 14: The reviewer suggests correcting the gramma.
"…because coculturing these T cells with human cholangiocyte…" to "…because coculturing of these T cells with the human cholangiocyte…"
Answer We have followed reviewer's suggestion.

(2)Reviewer no 00291404

1. The authors have used one human cancer cell line (KKU-213L5 cells) as the only target cells of study, and then some DCs and T lymphocytes from healthy donors. In both case, HLA types are unknown. The authors forget the very basic knowledge that for adaptive antitumor immunity, matched HLA types of immune cells and cancer cells are needed in order to elicit adaptive immune responses and mediate specific targeted cancer cell killing. From the data presented in Figure 6, it is so obvious that the cytotoxic effect was minimal, HLA-independent and non-specific. Therefore, the authors have to find some donors who match the HLA types of the target cells KKU-213L5 cells and re-do the whole experiments in Figure 6 with right controls.

Answer

Thank you for your value suggestions. We do agree with this comment that the best control of the experiment in figure 6 should be T lymphocyte that isolated from matched HLA donor with KKU-213L5. However according to the limited of cell harvesting and human ethic agreement that affect to our process of donor selection. Therefore, the random donors were used instead of the matched HLA donors. However, KKU213L5 was established from Thai CCA patients and the immune cells were also separated from Thai healthy donors, which the chance for their compatibility was high as HLA-A2. This was confirmed in previous studies that using of CCA cells test with PBMC from Thai donor that suitable by shown minimal toxic in control group [1, 2]. We already added this point in the discussion paragraph 4 as highlight in yellow.

1. Thepmalee C, Panya A, Junking M, Chieochansin T, Yenchitsomanus PT. Inhibition of IL-10 and TGF-beta receptors on dendritic cells enhances activation of effector T-cells to kill cholangiocarcinoma cells. Hum Vaccin Immunother. 2018;14(6):1423-31. Epub 2018/02/09. doi: 10.1080/21645515.2018.1431598. PubMed PMID: 29420117; PubMed Central PMCID: PMCPMC6037468.
2. Junking M, Grainok J, Thepmalee C, Wongkham S, Yenchitsomanus PT. Enhanced cytotoxic activity of effector T-cells against cholangiocarcinoma by dendritic cells pulsed with pooled mRNA. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2017;39(10):1010428317733367. Epub 2017/10/17. doi: 10.1177/1010428317733367. PubMed PMID: 29034817.

2. The study on immunogenic cell death. There are two types of signals derived from DAMPs (and PAMPs). One is called "find-me" signal, such as extracellular ATP and HMGB1; and the second type is called "eat-me" signal, such as ecto-CRT (calreticulin) now expressed on the cell surface. The ecto-expression of CRT and other HSPs on the cell surface send the signal to phagocytes to "eat me". Therefore, the data presented in Figure 1D consist of find-me signals only. It is equally important to analyze the cell surface expression of DAMPs such as ecto-CRT. Please see, Kepp O et al., Consensus guidelines for the detection of immunogenic cell death. Oncoimmunology. 2014; 3:e955691. [PMID: 25941621]

Answer
I would like to apologize for overlooking on this point. Exactly, CRT is one of DAMPs that play role in immune cell activation. According to time limited, we would like to discuss more about the effect of honokiol on CRT expression following your suggestion references that show in discussions.

3. The effect of honokiol on cancer cells have been quite well studied. Some have studied how honokiol modulates cancer immunogenicity, and the authors have missed referring to them. For example, (1). Liu SH. Et al., Honokiol confers immunogenicity by dictating calreticulin exposure, activating ER stress and inhibiting epithelial-to-mesenchymal transition. Mol Oncol. 2015; 9(4):834-49. (2). Lin CJ et al.,. Toxicol Appl Pharmacol. 2016; 304:59-69. (3). Li L, Han W, Gu Y, Qiu S, Lu Q, Jin J, Luo J, Hu X. Honokiol induces a necrotic cell death through the mitochondrial permeability transition pore. Cancer Res. 2007; 67:4894-903.
Answer I totally agree with your opinion. Therefore, the discussion in this point was inserted in the discussion section, in paragraph 4 as marked in yellow. Moreover, the references that talking about the mechanism of honokiol on cancer cell were added in first paragraph of discussion, as highlight in yellow.

4. The title. "...pulsed with DAMP components-derived tumor cell lysates". The phrase is a bit weird and I do not understand what the authors try to say.
Answer I would like to say that DCs were pulsed (or primed) with tumour cell lysates, which proved that contain with the DAMPs component.

5. Figure 4. The data were presented as mean +/- SEM. In fact, with so few data points, the right way to present the data is mean +/- SD.
Answer We have changed the data that presented as mean +/- SEM to mean +/- SD.

6. In all figure legends, it should be "p value", not some other symbol.
Answer We have followed reviewer's suggestion.

7. Page 13. "DCs pulsed with tumor cell lysates derived from honokiol-treated CCA cells induced T lymphocyte production of cytokines". From the subtitle, the authors implied that IFN-gamma and IL-12 were all produced from T lymphocytes in the co-

culture. This may not be true as DCs can produce these cytokines. Please modify the statement.
Answer I agree with your comment. Therefore, we modified that sentence to "DCs pulsed with tumor cell lysates derived from honokiol-treated CCA cells induced cytokines production"

(3) Reviewer no 02739495

In this paper, Jiraviriyakul A et al. found that DCs loaded with cell lysates derived from honokiol-treated tumor cells enhance efficacy against cholangiocarcinoma. DCs pulsed with honokiol-derived tumor cell lysates can induce T lymphocyte proliferation and enhance killing of cholangiocarcinoma cells compared to DCs and DCs pulsed only with tumor cell lysate cell lysates. Here are suggestions for this article:

1. This article validates the tumoricidal cell function of DCs plus Honokiol-derived cell lysates. These results indicate changes in cell phenotype, but no relevant mechanisms have been studied.
Answer Exactly, we only focus in the phenotype of anti-tumour activity of the dendritic cell loaded with tumor cell lysates. However, the result is enough for indicating that we can maximize the efficacy of dendritic cell based cancer vaccine by loaded with CCA cells pretreated with honokiol. For underlying mechanisms some of them we discussed in the discussion section at the ends of the paragraph 4 and we also ongoing investigates about that.

2. In the results section of the paper, the model in this paper was derived from mononuclear cells that were mature DCs, why immature DCs were selected instead of mature DCs for co-localization with tumor cell lysates?
Answer The main reason is the phenotype of immature DCs cells. Immature DCs highly express the phogocytosis receptor and also abundant with antigen processing machinery more than mature DCs. However, in coculture system we added the tumor necrosis factor-alpha and interferon-gamma for activation of DCs maturation, which highly express the costimulatory molecules that properly for activation of lymphocyte proliferation.

(4)Reviewer no 03291363

The authors have investigated the ability of honokiol primed dendritic cells to increase killing of an invitro cholangiocarcinoma line: a very interesting study My comments:

1. The results section has a lot of methods in it: they should be redirected to the methods and the results section should simply state the results without interpretation
Answer Thank you for your suggestion, unnecessary sentences about method and the interpretation were removed from result section as indicate with highlight.

2. Tables are required to define the data that were used to generate Figures 5 and 6
Answer We provided the data in term of Mean ± SD that were used to generate figures 5 and 6 in supplement table 1 and 2, respectively.

3. The significant effect of addition of honokiol to DC pulsing is derived from 3 experiments at 48/24: this is not enough, and the number need to be increased considerably
Answer Thank you for your suggestion. We would like to clarify that we did at least triplicate in every three independent experiment thus the number is at least 6.

4. Is the killing effect seen in Figure 6 simply due to free honokiol and not its presence in pulsed Dc? Please explain?
Answer The killing effect seen in Figure 6 is free from honokiol. According to the method that using effector cell, which harvested after coculture with DCs-loaded with tumour cell lysates. In cell harvesting process, we removed cell supernatant and resuspended in fresh complete medium. Therefore, during cultivation of effector T cell with KKU-213L5 (target cell) is free from honokiol.

(5)Reviewer no 03291363

Honokiol was shown to positively influence the immune system in different settings. Especially the action against Tumors (lung, breast, hepatocellular) makes this compound interesting. For the first time, the action of honokiol on cholangiocellular carcinoma was investigated. The authors could show with appropriate methods a positive influence of the drug also in this setting.
Answer Thank you very much. We appreciate your kind comments.