

Response to Reviewers for ESPS Manuscript NO: 21199

To the editor,

Please find our revised manuscript [ESPS Manuscript NO: 21199.R1], “**Association of *Fusobacterium nucleatum* with immunity and molecular alterations in colorectal cancer**”, which we would like to resubmit for consideration.

We have responded to each of the comments as indicated below. Reviewers’ comments are italicised, and in square brackets. Within the revised manuscript, changes to the text in response to the reviewers’ comments are underlined, using colored text.

We also have blotted out the name of Dr. Suzuki from the authorship at his own request. All of the authors agree with the new authorship order.

Thank you very much for this opportunity to respond to the reviewers’ comments. We believe that our manuscript has been much improved thanks to these constructive and valuable comments.

[Nosho K et al. presented a review article regarding the association between the gut microbiome, immunity and molecular alterations in colorectal cancer (CRC) patients for a better understanding of the relationship between microorganisms and immune cells in the tumor microenvironment. They showed that the frequency of F. nucleatum positivity in the Japanese colorectal cancer was 8.8% (44/511), which was lower than that in U.S. cohort studies (13%, 76/598). Similar to the U.S. studies, F. nucleatum positivity in Japanese colorectal cancers was significantly associated with microsatellite instability (MSI)-high status. This finding indicates that molecular features of CRC, especially MSI, influence T-cell-mediated adaptive immunity, and F. nucleatum possesses immunosuppressive activities by inhibiting human T-cell responses. MicroRNA-21 increases the levels of IL-10 and prostaglandin E2 (PGE2), which suppress antitumor T-cell-mediated adaptive immunity through the inhibition of the antigen-presenting capacities of dendritic cells and T-cell proliferation in CRC cells. The review seems informative and appealing; however, there are a lot of criticisms and have several issues that the authors need to address before the manuscript is suitable for publication.]

[Major Compulsory Revisions:]

We thank this reviewer for his/her positive and helpful comments.

[1. The major concern of the current study is the frequency of F. nucleatum positivity in the Japanese colorectal cancer was 8.8% (44/511), which was lower than that in U.S. cohort studies (13%, 76/598). If authors would say it on the more solid evidence, they should compare both cohorts by the statistical analysis.]

As requested, we have examined the frequencies of *F. nucleatum* positivities between the Japanese patients and the U.S. patients by the statistical analysis.

Our data showed that the rate of Japanese patients was significantly lower than that in the U.S. cohort study ($P = 0.027$) by the χ^2 test.

We have added the following sentences (page 8, paragraph 2):

Our current data demonstrated that *F. nucleatum* positivity was detected in 44 (8.6%) of 511 Japanese patients with colorectal cancer (**Table 1**). The rate of *F. nucleatum* positivity in the Japanese patients was significantly lower than that in the U.S. cohort study^[36].

[2. Authors mentioned in the text that microRNA-31 (miR-31) expression is significantly up-regulated in BRAF-mutated cancers compared with that in wild-type cancers using microRNA array analysis. In the current study, F. nucleatum positivity is also closely associated with BRAF-mutated cancers (Table 1). If any connection exists between miR-31 and F. nucleatum, and any relevant information is present regarding immunity and miR-31 in CRC tumorigenesis?]

As requested, we have examined the relation between *F. nucleatum* positivity and miR-31 expression in the Japanese colorectal cancers; however, no significant difference was found ($P = 0.37$). We also searched for papers which reporting the relation between

microRNA expressions and immunity in colorectal tumors as much as possible. Nevertheless, no previous study has reported the association of miR-31 expression with immune cells in colorectal tumorigenesis.

[3. In most studies, MSI in colon cancer has been associated with improved survival. We are curious about if MSI status in Japanese CRC patients would be related to survival.]

As requested, Kaplan–Meier method and log-rank test were performed to assess the association between MSI status and colorectal cancer mortality among Japanese patients. Our data showed that significantly lower mortality rates were observed in the MSI-high group ($P = 0.048$) compared with the MSS/MSI-low group.

We have added the following sentence (page 10, paragraph 2):

Similar to these reports from U.S. and Western countries^[27, 41, 50, 51], significantly lower mortality rate (log-rank test: $P = 0.048$) was observed in the MSI-high group compared with the MSS/MSI-low group by Kaplan–Meier method in our current Japanese population-based study (data not shown).

[Minor Essential Revisions:]

[1. Figure 1 should be a hypothesis of potential mechanism and this must be present in the text.]

As the reviewer request, we have revised the Figure legend and added the following sentences (page 14, paragraph 3 to page 15, paragraph 1):

Figure 1 Hypothesis of potential mechanism about the association of *F. nucleatum* in colorectal cancer with immune cells and molecular alterations

We have summarized the hypothesis of potential mechanism about the association of *F. nucleatum* in colorectal cancer with immune cells and molecular alterations in **Figure 1**. *F. nucleatum* increases the production of ROS and inflammatory cytokines in colorectal cancer. Inflammation and ROS can cause epigenetic silencing of the mismatch repair protein MLH1 leading to MSI. *F. nucleatum* possesses immunosuppressive activities by inhibiting human T-cell responses and modulates tumor-immune microenvironment suppressively. MicroRNA-21 increases the levels of IL-10 and PGE2, which suppress antitumor T-cell-mediated adaptive immunity in the tumor microenvironment.