Author’s response to reviews

Title: Gene Expression Profiling of Laterally Spreading Tumors

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Author's response to reviews: see over
To reviewers

Thank your comment. We revised our manuscript.

Reviewer's report (2055524232162580_comment)

1. Some of the conclusions the authors make are only observational so there are some statements that there should be downsized.

   Thank you for your comment. We revised conclusion.

   (Abstract, p5, line 9)

   LSTs have an unusual profile of gene expression compared to other tumors and BCL2L1 might be concerned in the organization of LSTs.

2. The inverse correlation between expression of BCL2L1 and apoptosis is highly speculative unless some functional assays are performed. There is no biological rationality to claim that the inverse relation between expression of BCL2L1 and apoptosis happens specifically in LSTs.

   Thank you for your comment. As the reviewer pointed out, we did not clarify the relation between BCL2L1 and apoptosis directly. Previous studies showed the role of BCL2L1 on apoptosis. Although Suzuki et al. reported that the apoptotic index and the Bcl-2 expression changed significantly in non polypoid type with a significantly lower apoptosis, we did not show that the inverse correlation between BCL2L1 and apoptosis is happened only in LST. We added the sentences in Discussion.

   (Discussion, p 19, line 6)

   …Our analysis for apoptosis and gene expression has some limitations. We did not clarify the relation between BCL2L1 and apoptosis directly in LST, and did not perform other methods except TUNNEL assay. And we could not show the question which the inverse correlation between BCL2L1 and apoptosis in LST is occurred also in other types of tumor. Further analysis is needed.

3. If possible, the correlation between mRNA and the protein should be performed in the same set of samples.

   Thank you for your comment. Unfortunately, we did not perform the expression analysis of mRNA and protein in the same samples. Sorry.

4. The methodological description of the immunohistochemistry should include
each dilution factor utilized for each antibody.
Thank you for your comment. We showed the dilution factor.
(Material and Methods, p11, line 3)
…We used the following antibodies: TNFRSF25, AKT1, BCL2L1, MTA2 and
ERBB2 (1:200, Abcam plc., Cambridge, UK).

5. In Figure 1, some L-Can cluster within the groups of adenomas, both LTS and
non-LTS. Given the fact that cancers should actually be the most well
separated entities in the hierarchical clustering, the authors should attempt to
find an explanation about their results.
Thank you for your comment. As the reviewer pointed out, cancer cases were not
separated from adenoma ones perfectly. We commented as follows.
(Discussion, p19, line 10)
…In the hierarchical clustering, cancer cases were not separated from adenoma ones
perfectly. We suggested that a small number of genes analyzed by PCR array might
cause this contingent result.

6. Is the overexpression of BCL2L1 a marker of high-grade dysplasia in LTS only
or it is also true for other tumor subtypes?
Thank you for your comment. We analyzed BCL2L1 expression in high grade
adenoma again.
(Results, p15, line 2)
…In high grade adenoma, 14 out of 20 LST cases (70%) showed high expression of
BCL2L1 by IHC, on the contrary, only 7 out of 17 Ip adenoma cases (41%) showed
high expression of it (p=0.078, chi-squared test).

7. The results of the qRT-PCR would be much easier to follow by the reader if
they are presented in a plot (e.g. box plot).
Thank you for your comment. We added Fig 2.
(Figure Legends, p28, line 9)
Fig. 2: The mRNA Expression Level in Colon Adenoma. The ratios of mRNA
expression level in adenomas compared to normal mucosa corrected by
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were shown. White bars
indicated LST-adenoma and dotted bars indicated Ip-adenoma. *p<0.01, unpaired
t-test. AKT1, v-akt murine thymoma viral oncogene homolog 1; BCL2L1, B cell
leukemia/lymphoma 2-like1; ERBB2, v-erb-b2 avian erythroblastic leukemia viral
oncogene homolog 2; MTA2, metastasis associated 1 family, member 2; TNFRSF25, tumor necrosis factor receptor superfamily, member 25.

8. Although it is mentioned by the authors in the Discussion, it would be informative to know whether BCL2L1 upregulated cells are Ki-67 positive. This could give some insights in the mechanism of action.

Thank you for your comment. Ki-67 is one of the reliable markers for cell cycle in adenoma or cancer cells. Unfortunately, our analysis did not clarified the important role of the genes associated with cell cycle. We added the sentences.

(Discussion, p17, line 1 from the bottom)

…Ki-67 is one of the reliable markers of cell cycle in adenoma or cancer cells. On the contrary, apoptosis also involved in clinical or morphological characteristics of tumor. In fact, in a previous study, the expression levels of Ki-67 and Bcl-2 antigen in adenomatous colorectal polyps showed a good correlation (29). Together with our results and other markers, it might lead to further understanding the pathophysiology of LST.

9. The rationale as to why the authors dropped off the follow-up of TNFRSF25 is not clear.

Thank you with your nice comment. As the reviewer pointed, the TNFRSF25 protein levels were highly up-regulated in LST-adenoma, compared to Ip-adenoma (p≺0.001), however, in 10 cases (45%) of Ip-adenoma cases, the protein levels of TNFRSF25 were also up-regulated. We suggested that TNFRSF25 was up-regulated not only in LST-adenomas but also Ip-adenomas and the importance of TNFRSF25 expression was less than that of BCL2L1. TNFRSF25 is a member of the TNF-receptor superfamily and is involved in carcinogenesis. Further study for TNFRSF25 is needed.

(Discussion, p19, line 4 from the bottom)

…According to our IHC analysis, TNFRSF25 protein was also up-regulated in LST-adenoma, compared to Ip-adenoma (p<0.001). However, of the Ip-adenomas, 10 (45%) were TNFRSF25 positive. Up-regulation of TNFRSF25 is not specific in LST-adenomas. Therefore, we speculated that the importance of TNFRSF25 expression was less than that of BCL2L1.
Minor revisions:

1. The name of the genes must be in italics.  
   We corrected all.

2. Last paragraph in the abstract needs to be rewritten. 
   We corrected it.

3. In Line 251 "claim" should "replace" declare. 
   We corrected it.

4. In Line 258 "to" should replace "with". 
   We corrected it.
Minemura et al. have analyzed the gene expression profiling of 41 colorectal tumors including 17 laterally spreading tumor-type (LST-type) adenomas, 12 LST-carcinomas, and 12 Ip adenomas, and have found that the five genes, AKT1, BCL2L1, ERBB2, MTA2, and TNFRSF25, were significantly up-regulated in LST adenomas compared to Ip adenomas. They have also confirmed that BCL2L1 proteins were significantly up-regulated in another small cohort of 38 patients with LST adenoma. The study may provide additional data that could help to clarify molecular mechanisms underlying the oncogenesis of this specific tumor type, LST. However, I feel that the paper shows only preliminary results for the expressions of some genes and proteins using a quite small number of patients, and lacks mechanistic experiments to support their hypothesis.

Thank you for your comment. We recognized the numbers of samples and analyzed genes are small. In fact, in clustering analysis, cancer cases were not separated from adenoma ones perfectly. We suggested the small number of genes might influence it. In this study, there some limitations, but in previous studies to analyze the gene expression of LST, a limited number of genes were analyzed. We think that our study is the important report to clarify the gene expression profile by the array method which could clarify the expression of many genes all at once.

(Discussion, p19, line 10)

…In the hierarchical clustering, cancer cases were not separated from adenoma ones perfectly. We suggested that a small number of genes analyzed by PCR array might cause this contingent result.

(Discussion, p 19, line 6)

…Our analysis for apoptosis and gene expression has some limitations. We did not clarify the relation between BCL2L1 and apoptosis directly in LST, and did not perform other methods except TUNNEL assay. And we could not show the question which the inverse correlation between BCL2L1 and apoptosis in LST is occurred also in other types of tumor. Further analysis is needed.

(Discussion, p16, line 3 from the bottom)
…Most of these studies have targeted genetic and/or epigenetic changes, or analyzed limited sets of genes. Our study is the first to clarify the gene expression profile by array analysis which could clarify the expression of many genes all at once.