Author's response to reviews

Title: mRNA expression of the DNA replication-initiation proteins in epithelial dysplasia and squamous cell carcinoma of the tongue

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Author's response to reviews: see over
Dear Dr. Edmunds,

Thank you and the reviewers of our manuscript for your considerations and constructed comments. I am now submitting the revised manuscript which contains revisions that address and answer all of the concerns and questions raised by the reviewers, as detailed below.

**The review 1, Dr. Takuji Tanaka, asked for three Major Compulsory Revisions:**

1. Describe how to remove stromal cells in the lesions (cancer and dysplasia) for the analysis.

Response:

The surrounding normal tissues including stromal cells were largely removed from the surgical samples based on the gross morphology before formalin-fixation and paraffin-embedding. Serial section slices were then obtained, and some of the slices were used for pathological diagnosis after hematoxylin and eosin staining, while some other slices were used for QRT-PCR. When we analyzed samples by QRT-PCR with adequate specimens with multiple replicas followed by statistical analysis, we could reduce the error of incorrect sampling.

The first two sentences above have been incorporated into the Methods section of the revised manuscript on page 5.

2. Page 4, line 3-6, in the Introduction section: The description is incorrect.
   Rewrite two sentences.

Response:

We agree with the reviewer that the original sentences here were inaccurate. So we modified them in the revised manuscript on page 4, lines 2-7, as follows:

Many oral carcinomas had pre-cancerous lesions including dysplasia. Although the oral cavity is easily accessible for clinical examination and biopsies, precancerous lesions are often missed, and oral cancers are usually diagnosed at late stages. Therefore, many patients still face poor prognosis. Currently only the gold standard of histological examination of a scalpel biopsy or surgical specimen can provide a definitive diagnosis.

3. Is the accuracy of the analysis superior to pathological determination? If so, state and discuss.

Response:
The quantitative real-time PCR and pathological diagnosis are complementary in diagnosing oral dysplasia and SCC. We agree with the reviewer that it is better to briefly summarize the virtues of our analysis in comparison to pathological diagnosis. Therefore, we added the following to the last paragraph of the Discussion section on page 16 of the revised manuscript:

The QRT-PCR assay described in this paper demonstrates high diagnostic sensitivity and accuracy. This assay is relatively rapid, technically amendable, and requires only a small amount of total RNA extracted from formalin-fixed and paraffin-embedded specimens. Furthermore, QRT-PCR test has the potential for automation and standardization. Therefore, the assay has potential clinical utility as a complementary technology to the current pathological diagnosis methods in distinguishing malignant SCC from benign dysplasia.

The reviewer 2, Dr. Masatoshi Fujita, raised three Major Points:

1. This study was carried out using patients’ samples. Therefore, I think it should be clearly stated in the manuscript that the study was approved by the Institute (or the samples were obtained from patients with consent).

Response:

We indeed obtained ethical approval for our study and sorry for not including the statement in the original manuscript. Now we have added the following to the Methods section on page 5:

This study was approved by the Medical Ethical Committee of the First Affiliated Hospital, Sun Yat-sen University.

2. Page 13, Discussion. The authors state “We found that CDC6, Cdt1, MCM2 and CDC45 mRNA expressions were significantly higher in malignant SCC than precancerous epithelial dysplasia”. However, Figure 2 shows that expression levels of Cdt1, MCM2 and CDC45 mRNAs are higher in severe dysplasia than SCC. I think this description may represent the result obtained with comparison between overall dysplasia and overall SCC. If so, please provide the data.

Response:

The review’s assessment is correct, as we did not make it clear that our statement meant to represent comparison between overall SCC and overall dysplasia. In fact, it is more accurate to state that “In this study, we found that CDC6, CDT1, MCM2 and CDC45 mRNA expression analyzed by quantitative real-time PCR are significantly higher in malignant SCC than mild precancerous epithelial dysplasia, and the expression levels in general increase with increasing grade of dysplasia.” This has been modified in the revised manuscript, across pages 13-14.

Accordingly, we changed the first sentence of the Abstract to:
We found that the expression levels are significantly higher in malignant SCC than mild precancerous epithelial dysplasia, and the expression levels in general increase with increasing grade of precancerous lesions from mild, moderate to severe epithelial dysplasia.

3. Table 3. The title of the table is “Relationship between histological grade and CDC6, Cdt1, MCM2 and CDC45 mRNA expression” and in the text, the authors say “Significant correlations between the target gene expression and the clinicopathological parameters were observed” (page 12, lines 4-7). However, in other parts, the authors state that there was no statistically significant overall correlation between histological grade and CDC6 (page 9, lines 15-18), Cdt1 (page 10, lines 9-11), MCM2 (page 11, line 4-7), or CDC45 (page 11, line 15-17). What are the clinicopathological parameters in Table 3?

Response:

The reviewer’s comment is correct, and the question was raised because we incorrectly used the term “histological grade”, instead of “clinicopathological parameters”.

The clinicopathological parameters in Table 3 include precancerous epithelial dysplasia and overall OSCC. The analysis is about correlation between overall precancerous dysplasia and overall OSCC. In its entirety, significant correlations between the target gene expression and the clinicopathological parameters were observed. In summary, the clinicopathological parameters in Table 3 are overall precancerous epithelial dysplasia and overall OSCC. Therefore, we changed the term in Table 3.

We also did as suggested for the Minor Point: Page 9, line 13: I suggest adding “between” before “moderate epithelial dysplasia and SCC”. Thanks.

I hope that we have answered all questions and addressed all concerns from the reviewers. Please let me know if anything else needs to be done.

I look forward to, and thank you in advance for your favorable decision on our revised manuscript.

Sincerely yours,

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