Author's response to reviews

Title: Copy Number Amplification of the PIK3CA Gene Is Associated with Poor Prognosis in Non-lymph node metastatic Head and Neck Squamous Cell Carcinoma

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Author's response to reviews: see over
Thank you for your response to our manuscript, “Copy Number Amplification of the PIK3CA Gene Is Associated with Poor Prognosis in Head and Neck Squamous Cell Carcinoma Prospective Cohort”, which we submitted to BMC Cancer.

We provide point-by-point response to each of the reviewers’ comments.

Reviewer #1: A minor comment on the arrangement of the manuscript
1. Why are there two discussion sections (p5 and p7)?
Response; We corrected Page 8 “Result and Discussion ⇒ Result”.

2. Why does the text start with Table 3 (p5) instead of Table 1?
Response; We corrected Table number.

3. The tables and graphs should be self-explained. A few notes may be needed to explain the tables/graphs.
Response; We added each explains in Legends.

Reviewer #1: Other major comments:
1. Results and discussion section:
   a. Did the authors collect the drinking information?
Response; In a Japanese, there is various kinds of alcohol (sake, beer, whiskey, shochu), and an accurate evaluation is difficult. Therefore we did not evaluate alcohol.

   b. In terms of smoking status, do the authors mean the smoking status at diagnosis? Lifetime smoking status? Did the authors collect the smoking and drinking information after diagnosis, which are also important parameters that influence the HNSC prognosis?
   c. Page 6, line 2, “in terms of smoking status (pack-years), one patient had a ‘high score’, and the other had ‘low score’.” How did the authors define the “scores”?
Response; The smoking status is Lifetime smoking status. And we defined smoking status high score as pack-years>30. We added reference No 19.

   d. Page 7, line 1, “… status (with or without amplification) in PIK3CA and the overall survival and disease-free survival... (Figure 1).” Is the Figure 1
completed;
there is only “disease-free survival” in Figure 1...?
Response; We added Figure 1-B (Over-All-Survival with PIK3CA copy number status).

2. Method section:
a. Page 11, 2nd line from the bottom, “… 115 tumors were obtained from HNSCC patients...” It sounds like some patients may have two or more samples. Please clarify that the 115 tumor samples were from 115 different individuals.
Response; We added a sentence Page 5, 5th line from bottom “115 tumors were obtained from 115 HNSCC patients......”.

b. Was any quality control strategy conducted during the lab measurements?
   Response; We analyzed each normal tissues for controls. This strategy based on reference No 17.

c. Statistical analyses:
i. How did the author define the “disease-free” (outcome)?
ii. How were the patients followed?
iii. How were the outcomes identified?
Response; We added some sentences in Statistical analyses (Page 7) about how to follow up and outcomes.

3. Table 6, Figure 1 and Figure 2:
a. How many patients with lymph node metastases is PIK3CA amplification? It is a bit confusing when looking at the numbers in Table 6 and the Figures.
Response; We correct Table 6, Figure 1 and 2. And we revised sentence Page 10 Line 1.

Reviewer#2
Reviewer's report:
The paper “Copy Number Amplification of the PIK3CA Gene Is Associated with Poor Prognosis in Head and Neck Squamous Cell Carcinoma: Prospective
Cohort Study” deals with the frequency and the clinical implications of mutations and amplification of KRAS, BRAF and PIK3CA in HNSCC. The topic is quite interesting but I believe that the paper cannot be suitable for publication unless very important and deep major revisions. Furthermore, in my opinion, the title of the article does not seem to be appropriate because of the constraints of the survival analysis and the disagreement with the objective the Authors wanted to pursue.

Response; We changed the title to “Copy Number Amplification of the PIK3CA Gene Is Associated with Poor Prognosis in non-lymph node metastatic Head and Neck Squamous Cell Carcinoma”.

Major Compulsory Revisions

Abstract
1. I would like to ask the Authors to check the sentence “Kaplan-Meier survival analysis revealed that copy-number amplification of PIK3CA was markedly associated with cancer relapse in patients without lymph node metastasis (Log-rank test, p=0.447 and p=0.2626)” and “Copy number amplification of the PIK3CA gene is associated with poor prognosis in HNSCC patients without lymph node metastasis” because, according to the statistical tests which were reported, there is no evidence of significant association.
Response; We corrected the text in reference to Fig2.

Introduction
2. I would suggest the Authors to better define the objective of the study with respect to type of mutations which were under investigation
Response; We added a sentence about determination of the type of mutations in this study and referenceNo9, 10. (Page 4 Line 12)

Methods
3. Were cases consecutively enrolled among patients admitted to the surgical department or was there a source of selection bias?
Response; All patients received surgical treatment. There is no patient who received chemotherapy or radiotherapy for first treatment in this study. We only discuss the progression factor after surgical treatment.

4. The description of follow up of patients as well as of outcomes (in particular
survival) and covariates is completely missing.
Response; We added sentences about how to follow up and outcome in Statistical analyses (Page 7,11th Line).

5. The description of statistical analysis is poor. The outcome of the study should be to analyze the prevalence of mutations and amplifications and to describe if there are dependent on patients’ characteristics. Furthermore, mutations as well as amplifications and other covariates should be put in relation with survival. In this case, I would suggest a multivariable approach too (at least for PIK3CA because of the higher number of patients with amplification).
Response; We added the multivariable analysis using the coxs hazard model to table7 and Results.

Results
6. In table 3 I would add relative frequencies for all qualitative variables.
Response; We added each relative frequencies in table 1.

7. The percentage of patients with mutation in KRAS exon 1 is 5.2% and not 6%. Response; We corrected it (6%⇒5.2%).

8. The Authors stated to investigate the correlations between the presence of mutations and the clinical data: this was not described in the methods section and was not dealt with thoroughly in results. The only data which were provided were those about cancer site and the smoking status (which, furthermore, was not reported in the Table 3).
Response; Significant association was absent about the association between mutation and clinical data (localization, sex, stage etc). We added sentence in Result-Mutation analysis of KRAS, BRAF and PIK3CA. (Page 9, Bottom Line)

9. The numbers of patients with amplifications are not the same of the abstract!! Response; We corrected this point in abstract.(Page 2, 10th Line)

10. Table 6 could be implemented adding relative frequencies. Furthermore, the Authors did not speak about the application of the Mann Whitney test: they should specify why they chose it. According to me if the variable “number of lymph nodes sites” is categorized, the Chi square test could be applied.
Furthermore, I suppose that in some cases the Fisher exact should have been necessary because of the presence of cells with an expected count less than 5.

Response; We conducted similar examination about lymph node metastases in our previous article (Reference No3), it was acceptable on Mann Whitney test. Therefore we conducted same investigation in this study.

11. The Authors did not specify anything about the follow up study in the methods and in results. They speak about overall survival (never cited before) and disease free survival without any previous clarification.

Response; We added sentences about how to follow up and outcome in Statistical analyses (Page 7, 11th Line).

12. Why the Kaplan-Meier curves and the log rank test were used only for investigating the role of the status of PIK3CA? I would suggest studying also KRAS even though only 10 (or 12 as in the abstract??) showed the amplification.

Response; We added a sentence that conducted similar examination in KRAS copy number status, and the significant difference was not found in copy number status and prognosis in Result. (Page 9, Bottom Line)

13. Figure 1 does report only disease free survival. The title is moreover misleading.

Response; We added Figure1-B(Over-All-Survival with PIK3CA copy number status).

14. The sentence “Of those with PIK3CA copy number alteration, 31% were disease-free at 2.0 years, whereas 90% of the patients without PIK3CA copy number gain survived without recurrence during the study period” is misleading because Authors did not address censoring. Did someone die being an informative censoring? Furthermore, I would suggest reporting median time to progression.

Response; We added a sentence about information of median follow-up period etc in Result; Page 9, 11th line.

Discussion

15. The limits of the study are not discussed at all.
Response; We added a sentence about the limits of the study in Discussion. (page11, 12st Line)

Conclusion
16. Conclusion must be checked because mostly in contrast with the content of paper (see also the comments on the abstract).
Response; We checked abstract and conclusions and corrected it.

Minor Compulsory Revisions
Abstract
17. Please revise the percentages in the abstract: 32.1% should be rounded to 32.2% and 19.3% is indeed 19.1
Response; We corrected this point. Please check Page2, 11st Line.

Methods
18. Please provide the reference for to the “6th UICC TNM classification and stage groupings”.
Response; We added reference No14.

Results
19. The results of mutation in KRAS exons 2 are not reported in results even though it was said (in methods) that they were searched.
Response; We added a sentence about exon2. (page 8,12st Line)

20. Please check the 32.1% percentage because it should be rounded to 32.2%
Response; We corrected this point. Please check Page9, 9thLine.

Discussion
21. I would suggest the Authors to report the right percentages: 2.6% showed mutations in KRAS gene, and 2.6% in PIK3CA.
Response; We corrected it according to your indication.