Reviewer A

General remark:
Mutational signature is the main topic of the article and is not a routine diagnostic tool.

Comment 1:

So mutational signatures should be more extensively described in the abstract: the interest of the data provided by mutational signatures should be highlighted in the "background" section. The "methods" section should be more specific about mutational signatures.

Reply 1:

We have highlighted the interest of the data provided by mutational signatures in the Abstract background as advised and modified the Methods section to include specific text on mutational signatures (page 3, line 2 to line 16). We also added precise explanations of each signature in the Methods section of the main text (page 9, line 8 to line 11).

Changes in the text:

In the abstract, background was changed from "Programmed death-ligand 1 (PD-L1) as well as tumor mutational burden (TMB) have been reported as predictors for the response to anti-programmed cell death (PD)-1 antibody therapy in non-small cell lung cancer. TMB has been shown to correlate with smoking history in lung adenocarcinoma. Our previous study could not find an association between TMB and clinicopathological background factors, such as smoking history, in squamous cell carcinoma of the lung. Here, we analyzed the mutational spectrum in lung squamous cell carcinoma to identify the significance of mutational signatures." (page 3, line 2) to

"Tumor mutational burden (TMB) has been identified as one of the predictors for the response to anti-programmed cell death (PD)-1 antibody therapy and reported to correlate with smoking history in lung adenocarcinoma. However, in squamous cell carcinoma of the lung, the association between TMB and clinicopathological background factors, such as smoking history, has not been reported, including in our previous study. The mutational signature is a tool to identify the mutagens that are

contributing to the mutational spectrum of a tumor by investigating the pattern of DNA changes. Here, we analyzed the mutational signature in lung squamous cell carcinoma to identify mutagens affecting the TMB." (page 3, line 2 to line 9)

The text in the Methods section was changed from

"Mutational signatures were analyzed in Japanese patients with lung squamous cell carcinoma (n=67) using next-generation sequencing consisting of a 415-gene panel. The relationships between signatures and clinico-pathological data including TMB and PD-L1 expression were analyzed."

to

"Seven representative mutational signatures including signatures 7 (ultraviolet-related), 4 (smoking), 6/15 (mismatch repair), 2/13 (APOBEC), and 5 (clock-like) were analyzed in Japanese patients with lung squamous cell carcinoma (n=67) using data generated by next-generation sequencing consisting of a 415-gene panel. The relationships between signatures and clinico-pathological data including TMB and PD-L1 expression were analyzed." (page 3, line 10 to line 15)

We also added a precise explanation of each signature in the Methods section of the main text as follows: "These signatures are related to exogenous mutagens, with SI7 related to ultraviolet (UV), SI4 to smoking, SI6 and S15 to mismatch repair (MMR) defect, SI2 and S13 related to apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC), and SI5 to aging or "clock-like" (7)."(page 9, line 8 to line 11)

Comment 2:

Mutational signature should also be better described in the introduction, for example with references to some relevant previously published results. For readers who are not aware of the different types of mutational signatures, a brief reminder of the current catalogue could have been welcome.

Reply 2:

We have added a brief introduction on mutational signatures with references(page 6 line 18). We also inserted a short summary to the Discussion section (page 14, line 6).

Changes in the text:

We added a paragraph and a reference in the Introduction section as follows: "The

mutational signature is a tool to identify the mutagens that contribute to the mutational spectrum of a tumor by investigating the pattern of DNA changes. Thirty distinct signatures have been identified, and signature (SI) 4 is related to tobacco smoking with transcriptional strand bias for C>A (and also T>A mutations). SI4 is very similar to the mutational signature induced by exposing cells to benzo[a]pyrene in vitro (7)." (Page 6 line 18 to Page 7 line 4)

We also added a sentence to the Discussion section as follows: "In this study, we referred to mutational signatures version 2, which revealed 30 distinct signatures based on an analysis of 10,952 exomes and 1,048 whole genomes across 40 distinct types of human cancer (19)."(Page 14 line 6 to line 8)

Specific remarks:

Comment 3:
The total number of cases was 67. However there is no precise number in the results. It would be interesting to know how many cases (or what proportion of cases) showed smoking-related signature 4? Signatures 2 and/or 13? Signatures 6 and/or 15? Did any cases displayed several different mutational signatures and if so, how many?

Reply 3:
We have added the numbers of the patients with specific signatures in the revised text (page 12, line 5 to line 8). However, direct comparison between signatures cannot be performed because the data were drawn from a limited panel sequencing, as we discussed in the text (page 14, line 12).

Changes in the text:
We added sentences to the Results section as follows: "Fifty-six patients (83.6%) had more than four signatures, with SI6 as the most frequently observed (64 cases, 95.5%). Other signatures were observed as follows: SI2 in 26 cases (38.8%), SI4 in 45 (67.2%), SI5 in 59 (88.1%), SI7 in 40 (59.7%), SI13 in 44 (65.7%), and SI15 in 21 (31.3%)."(Page 12 line 5 to line 8)

Comment 4:
Abstract page 3 line 14. The sentence "the distributions of signatures were comparable among samples." Is not clear. "several tissue samples from each case" or similar, would

be more explicit.

Reply 4:

We have added the precise number of patients with each signature to clarify this information (page 4, line 1 to line 2).

Changes in the text:

We have changed the text from "the distributions of signatures were comparable among samples. The smoking-related signature 4 was related with smoking history." to "the distributions of signatures were comparable among samples, with 56 cases containing more than four signatures. The smoking-related signature 4 was found in 45 cases and was significantly related with pack-year index (p=0.026)." (Page 3 line 17 to Page 4 line 1)

Comment 5:

Abstract page 3 lines 5-6: "Our previous study could not find an association between TMB and clinicopathological background factors, such as smoking history" This is in contradiction with the last line of the abstract "Tobacco smoking is the mutagen most related with TMB." I have not found any interpretation of this discrepancy in the article.

Reply 5:

We have added a paragraph to explain the discrepancy (page 15, line 4).

Changes in the text:

We have added a paragraph in the Discussion section to explain the question as follows:" Most patients had more than four signatures within each tumor. Our results indicated that SI4 was most related with total mutation count, but other signatures also contributed to total mutation count. Tobacco mutagen is reported to induce mutations in TP53, which results in many other genomic alterations, causing cancer initiation and progression (15). Yoshino et al reported no survival difference in patients with lung squamous cell carcinoma divided according to PYI (0, 1–20, 20–50 and 50+), whereas a significant difference in survival was observed in patients with lung adenocarcinoma divided according to PYI (21). When we compared estimated mutation counts belonging to SI4, patients with PYI more than 50 had more SI4 counts than patients with PYI 50 or below, suggesting that tobacco mutagen may continue to influence total mutation count even after a tumor has been established." (Page 15, line 4-13)

Comment 6:

Page 12 line 16. High PYI (≤50PY) should be replaced by ≥50PY. Beyond this typographical error, the authors should explain why they have chosen this threshold, which has no clinical relevance. Has a difference between heavy and very heavy smokers in terms of mutations been previously reported?

Reply 6:

We have corrected the typographical error (page 13, line 1). We chose PYI of 50 as a cut-off because it was a median value; therefore, we added the range and median of PYI in the patient background (page 12, line 1) and added a reference where a cut-off value for PYI of 50 was used (page 15, line 10).

Changes in the text:

We have corrected the typographical error from "(≤median PYI, 50 PYI)" to "(≥median PYI, 50 PYI)" (Page 13, line 1)

We have added the following text: "all patients were smokers with median pack-year index (PYI) of 50 (range 7–150)" (Page 12, line 1)

We have added the following sentence: "Yoshino et al reported no survival difference in patients with lung squamous cell carcinoma divided according to PYI (0, 1–20, 20–50 and 50+), whereas a significant difference in survival was observed in patients with lung adenocarcinoma divided according to PYI (21)."(Page 15, line 7 to line 10)

Comment 7:

Abstract conclusion page 4 line4: "Mutational signatures can predict the cause of lung squamous cell carcinoma." Was it necessary to perform such complex analyses to discover that lung squamous cell carcinoma was associated with smocking? Beyond smocking, this study did not predict the cause of squamous cell carcinoma. Has neon exposure already reported as a cause of a lung carcinoma?

Reply 7:

We discussed the importance of the smoking-related signature in terms of a predictive marker for anti-PD-1/PD-L1 therapy in the Discussion section (page 16, line 5). We have also added the importance and future availability of this analysis in the Discussion session (page 15, line 1). We have revised the discussion on neon exposure as advised

(page 15, line 17).

Changes in the text:
We have added the following sentence: "As the techniques and cost of next-generation sequencing are rapidly improving, this issue will be resolved when whole exome (or whole genome) sequencing becomes more available for clinical use."(Page 15, line 1 to line 3)

We have modified the Discussion as follows; "Occupational exposure to UV is a risk for skin malignancy, but not for lung malignancy; there could have been an occult skin malignancy in that patient." (Page 15. Line 17 to 18)

Comment 8:
Is it possible to imagine that mutational signature could help to differentiate tobacco-related primary squamous lung carcinoma from HPV-related metastatic carcinomas? This and possibly other applications could be discussed.

Reply 8:
We have added sentences in the Discussion section as advised (page 16, line 1).

Changes in the text:
We have added the following sentences: "Similarly, APOBEC mutagenesis has been related to human papillomavirus- associated carcinomas. However, it is difficult to distinguish whether the lung tumor is primary or metastatic, because previous studies did not distinguish between HPV-positive and –negative tumors (22). "(page 16, line 1 to line 4)

Reviewer B

Comment 1:
 They should reveal clinical characteristics of patients in this study.

Reply 1:
 We have added a citation to our previous study because this study is a follow-up report. (Page 11, line 18) We have also added the data on smoking status to the text, because the smoking status is particularly important to this study (page 12, line 1).

Changes in the text:
 We have modified the text as follows; "The characteristics of the 67 patients were previously reported (3), for example: 60 (89.6%) patients were male, all patients were smokers with median pack-year index (PYI) of 50 (range 7–150), and the median age of all patients was 70 (range 47–84 years) (3)" (Page 11 line 18 to page 12, line 2)

Comment 2:
 Did they examine the relationship between signatures and clinical factors?

Reply 2:
 We have examined the relationship as described in the revised text (page 12, line 16). Other factors did not statistically differ according to estimated mutation counts from each signature.

Changes in the text:
 We added the following sentence as advised (see Page 13, line 3) "No other significant correlations between clinical characteristics and signature were observed."

Comment 3:
 In Materials and Methods section, they should reveal IRB number.

Reply 3:
 We have added the IRB numbers as instructed (page 8, line 11).

Changes in the text:
 Page 8, line 9: We modified the sentence as follows: " This study was approved by the

Institutional Review Boards of both the Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University Hospital (IRB number: 674-01) and the Division of Thoracic and Cardiovascular Surgery, Niigata University Hospital (IRB number: 774). Written informed consent was obtained from all participants." (Page 8 line 9 to line 13)

Comment 4:

On figure 3, A/B/C and D/E/F were separated into two portions. Those should be submitted as one figure.

Reply 4:

We have modified Figure 3 as advised (see Figure 3).

Changes in the text:

We have modified figure 3 as advised.