Author's response to reviews

Title: c-Met in esophageal squamous cell carcinoma: an independent prognostic factor and potential therapeutic target

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Author's response to reviews: see over
Dear Drs. Dafne Solera and Jochen Lennerz (the reviewer of our manuscript):

We appreciate your thoughtful comments on our manuscript entitled “c-Met in esophageal squamous cell carcinoma: an independent prognostic factor and potential therapeutic target.” We have revised the manuscript as per your suggestions. Please find our detailed responses to your comments enclosed herewith. Our responses are presented in bold, and the changes within the manuscript text are in red.

We also attached certificate of English editing.

We hope that the revised manuscript is acceptable for publication in *BMC Cancer*. If you have any questions and/or comments regarding the revised manuscript, please do not hesitate to contact me.

I look forward to hearing from you soon.

Yours truly,

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Major points

1. How did the authors determine that the observed efficacy of PFE-2341066 is truly related to interference with MET? PFE-2341066 interferes with a variety of other molecules that converge (in part) on the same downstream signaling molecules.

Authors' response: Thank you for your thoughtful comment. PF-2341066 has other inhibitory effects such as in the gene rearrangement of anaplastic lymphoma kinase (ALK) and ROS1 tyrosine kinase. However, to the best of our knowledge, these rearrangements have not been reported in ESCC. In addition, our results demonstrated that the inhibition of cell function by PF-2341066 depended on the presence of HGF, a c-Met specific ligand. Therefore, we considered that the inhibitory effects by PF-2341066 detected in our present study were due to c-Met inhibition. We have included this rationale in the “Discussion” of the revised manuscript as follows:

Lines 326–330: PF-2341066 is also known to inhibit the gene rearrangement of anaplastic lymphoma kinase and ROS1 tyrosine kinase [42, 43] in addition to c-Met inhibition. However, to the best of our knowledge, such rearrangements have not been reported in ESCC. In addition, our results demonstrated that the inhibition of cell function by PF-2341066 depended on the presence of HGF, a c-Met specific ligand. Thus, PF-2341066 functioned as an efficient c-Met inhibitor in the context of our study.

2. Efficacy in one (of three cell lines) with a reduction of “proliferation” of ~15% is a weak basis for some of the rather drastic statements regarding 'generalized' efficacy of PFE-2341066 in ESCC. Reduction of proliferation by 15% means that 85% of the tumor cells continue to proliferate. Did the authors consider using other read-outs e.g. apoptosis?

Authors’ response: Thank you for your comment. We agreed with your assertion that the change in proliferation was weak. Therefore, we performed an additional experiment using all three ESCC cell lines (KYSE150, 170, and 180) to evaluate the inhibitory effects of PF-2341066 on cell proliferation as well as the effect of HGF (Figure 5). We added a pre-treatment by incubation in FBS-free medium for 24 hours prior to the cell proliferation assay. This step was also performed in the invasion assay and immunoblotting analysis in our study. We still detected a slight suppression of cell proliferation by PF-2341066 (as shown in Figure 5), indicating that the inhibitory effects of PF-2341066 on ESCC cell proliferation was rather weak compared to that on
cell invasion, as you mentioned. Therefore, we have revised the “Methods,” “Results,” and “Discussion” sections of the manuscript to reflect our methodology and findings.

3. The authors used three ESCC cell lines for some of their experiments; however, a direct comparison of efficacy between cell lines is not provided. Why do the authors focus on KYSE170?

Authors’ response: Thank you very much for your comment. KYSE170 cells showed the most abundant expression of c-Met mRNA among the three cell lines tested. Therefore, they were used for all functional experiments in our original study. However, we completely agree with you that a direct comparison of efficacy among the cell lines is certainly required. Therefore, we have performed additional cell proliferation and invasion experiments using all three cell lines (Figures 4 and 5). The results of the more recent assays indicated that in the presence of rHGF, PF-2341066 significantly inhibits cell invasion in KYSE170 cells, but not KYSE150 and KYSE180 cells, which was consistent with our original results. In addition, cell proliferation was suppressed by PF-2341066 and promoted by HGF but only slightly so. These results suggested that PF-2341066 inhibited ESCC cell invasion and proliferation, especially in the cell line with rather abundant c-Met mRNA expression (KYSE170), under HGF stimulation. The inhibition was also more profound on invasive than proliferative properties. Therefore, we have revised the “Methods,” “Results,” and “Discussion” sections of the manuscript to reflect our methodology and findings.

4. The definitions of ‘cut-offs’ for high vs. low MET and HGF should be revised. Did the authors test other cutoffs (i.e. high as only 3+ etc..)? A clear definition is paramount because HGF clearly had an effect in the cell-line experiments and the authors report differences in the clinical phenotype of patients; however, there was no effect on survival. These findings are in contrast to other studies (Ref 34, 35) and the difference might be related to these cutoffs.

Authors’ response: Thank you for your comment and we apologize for the unclear definition of the cut-off values. However, the cut-off levels for evaluating immunoreactivity of c-Met and HGF are unknown, at least in ESCC. Other studies (Ref 34, 35) evaluated serum or tissue HGF concentrations instead of immunohistochemistry results. Therefore, in this study, we calculated the “H-score,” incorporating both the percentage of immunopositive cells and the immunointensity, and reevaluated the
optimal cut-off values by using the receiver operating characteristic (ROC) curve. The optimal cut-offs were as follows:

<table>
<thead>
<tr>
<th></th>
<th>Tumor depth</th>
<th>Lymph node metastasis</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met</td>
<td>42.8</td>
<td>55.0</td>
<td>40.2</td>
</tr>
<tr>
<td>HGF</td>
<td>40.2</td>
<td>36.2</td>
<td>40.8</td>
</tr>
</tbody>
</table>

These results indicated that “40” was the optimal cut-off for survival outcome when analyzing c-Met and HGF immunohistochemistry results. It was also close to the optimal cut-offs for tumor depth, lymph node metastasis, and patients’ clinical outcome. Therefore, we redefined the cut-off value of high vs. low c-Met and HGF expression as “an H-score of 40.” Such a cut-off value led to slightly different results in our statistical analysis, but by no means changed the conclusions of our study.

We have added this information to the “Methods” as follows:

Lines 111–119: Five different high-power fields were analyzed per slide, with each field containing more than 100 carcinoma cells. The H-score was determined using the percentage of immunopositive cells and their immunointensity. Immunointensity was evaluated and scored according to the following criteria: 0, completely negative; 1+, weakly positive; 2+, moderately positive, and 3+, markedly positive. The H-score was then calculated by multiplying the percentage of immunopositive cells to the immunointensity score (H-scored ranging from 0 to 300). We also determined the optimal cut-off values for patient survival using the receiver operating characteristic (ROC) curve method. Cases with an H-score below the cut-off value was tentatively categorized as “low expression,” whereas those with an H-score greater than the cut-off value were considered as “high expression.”

Minor points
1. Abstract: The statement that MET as a poor prognostic factor in ESCC is unexplored should be revised. Similarly the statement “a molecular targeted therapy has not been fully developed” should be reworded.

Authors’ response: Thank you for your suggestion. We have revised the “Abstract” as follows:

Lines 31–35: Previous studies have suggested the involvement of c-Met and/or its ligand, hepatocyte growth factor (HGF), in esophageal squamous cell carcinoma (ESCC), but the correlation between c-Met status and clinical outcome remains unclear.
Furthermore, the identification of a novel molecular therapeutic target might potentially help improve the clinical outcome of ESCC patients.


Authors’ response: Thank you for your suggestion. We have revised the “Background” as follows:

Lines 79–81: However, the correlation between c-Met status and survival of ESCC patients is virtually unexplored despite the reported correlation of c-Met and/or HGF status to various clinicopathological features of ESCC [29, 30].

3.1 Methods Line 109-113: “Relative immunointensity was evaluated and scored according to the following criteria: 0, completely negative; 1+, weak; 2+, moderate, and 3+, marked immunoreactivity. High c-Met group was defined as immunopositive cells with greater than 40% and a relative immunointensity of above 1+ as previously reported [30]. High HGF group was defined as immunopositive cells with greater than the median value and relative immunointensity of above in accordance with our previous report [31]. “What do the authors mean with “Relative immunointensity”? relative to what normal epithelium (which is known to express MET; or relative to non-tumor/stroma/fibroblasts)?

Authors’ response: We apologize for the confusion and have revised the “Methods” as follows:

Lines 111–116: Five different high-power fields were analyzed per slide, with each field containing more than 100 carcinoma cells. The H-score was determined using the percentage of immunopositive cells and their immunointensity. Immunointensity was evaluated and scored according to the following criteria: 0, completely negative; 1+, weakly positive; 2+, moderately positive, and 3+, markedly positive. The H-score was
then calculated by multiplying the percentage of immunopositive cells to the immunointensity score (H-scored ranging from 0 to 300).

3.2 The cut-off between low and high was apparently set as: 0/1+ vs. 2+/3+. How is the statement “immunopositive cells with greater than 40%” related to that? 40% of cells, 40% of one category? Similarly, High HGF was defined as “greater than the median value and relative immunointensity of above...”. I am not sure what that means? Given that these definitions will drive application by other scientists – and the findings related to that are central to the current manuscript determination of these cutoffs have to be more clear (see major point 2).

Authors’ response: We apologize for the confusion and have revised the “Materials and Methods” to address this ambiguity as mentioned in our response to major point #4 above.

4. Statistics: quantitation = quantification?

Authors' response: Thank you for your comment. We have changed the term from “quantitation” to “quantification” in “Materials and Methods” section.

5. Statistics: which features/characteristics did the authors include in the multivariate model?

Authors’ response: The multivariate model in our study included all clinicopathological features with a P-value of <0.05 in the univariate analysis (sex, lymphatic invasion, TNM-T, TNM-N, TNM stage), c-Met, and HGF status. We have added this information to the “Methods” section as follows:

Lines 182–184: The multivariate analysis performed in this study included clinicopathological features with a P-value of <0.05 in the antecedent univariate analysis, c-Met expression, and HGF expression.

6.1 Results. Line 182-183 “c-Met status of carcinoma cells was significantly correlated with pathological stage” Does this mean that higher stages had higher “intensities” of MET immunoreactivity? If the authors truly tested correlation: what statistical method did they use?
Authors’ response: Thank you for your comment. We used the Pearson’s chi-square test to assess the correlation of c-Met and HGF status with various clinicopathological factors. We have revised our manuscript as follows:

Lines 199–204: c-Met status was significantly correlated with tumor depth \((P = 0.013)\) and pathological stage \((P = 0.010)\). On the other hand, HGF status was significantly correlated with tumor differentiation \((P = 0.024)\), tumor depth \((P = 0.028)\), lymph node metastasis \((P = 0.014)\), and pathological stage \((P = 0.022)\). However, no statistically significant correlation was detected between c-Met and HGF status of the patients. Results of the correlation analysis of ESCC patients’ clinicopathological variables and c-Met/HGF immunoreactivity in carcinoma cells are summarized in Table 1.

6.2 Several other “correlations” are mentioned – the authors should specify/clarify this.

Authors’ response: Thank you for your comment. Please refer to our response to minor point #6.1.

7 Discussion. The authors mention cancer-associated fibroblast; however, do not provided data on expression of HGF in this cellular compartment?

Authors’ response: Thank you for your comment. HGF immunoreactivity in CAFs was heterogeneous and difficult to evaluate in our study. We have included this information in the “Results” and revised the “Discussion” to reflect this as follows:

Lines 284: Nonetheless, HGF immunoreactivity in CAFs was heterogeneous and difficult to evaluate.

8 Statement “owing to the high frequency of alcohol consumption and smoking history” – these are not provided in Table 1.

Authors’ response: Thank you for your suggestion. We have added these features in Table 1 of the revised manuscript. However, the data were not available for a few patients.

9 The authors may want to discuss the efficacy of PF-2341066 in ESCC in comparison to...
other tumors because reduction of proliferation by ~15% (in one cell line!) is not compatible with findings in other tumors. Especially because inhibition of cell proliferation by PF-2341066 was not detected without HGF pre-stimulation.

Authors’ response: Thank you for your comment. The suppression of cell proliferation by PF-2341066 and promotion by HGF were detected in all three cell lines examined but only slightly (11–16%). Some of the carcinoma cell lines were reported to demonstrate more than 50% of anti-proliferative effects (Tu WH, et al. Efficacy of c-Met inhibitor for advanced prostate cancer. BMC cancer. 2010;10:556), but results in other carcinoma cell lines were similar to ours (Zillhardt M, et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, reduces tumor burden and metastasis in a preclinical model of ovarian cancer metastasis. Neoplasia. 2010;12(1):1-10.). However, the experimental methodology or conditions (e.g. reaction time) in the above-mentioned studies were somewhat different from those in ours. Therefore, we considered the anti-proliferative effects in our study to be consistent with the results in other tumors. However, further investigation is certainly needed for clarification.

10 Figures Supplement: In “Additional file 1”, specifically the figure, the 5-year survival is displayed over time. Is this truly what is displayed or is it overall survival over a 5 year period?

Authors’ response: The figure in Additional file 1 demonstrates the “5-year overall survival” and “5-year cause-specific survival” from the day of surgery. We have also revised the axis label for clarification.

12 What do the authors mean by cause-specific survival?

Authors’ response: Cause-specific survival indicates the survival rate from cancer-specific death (ESCC in our case). In our present study, the statistical difference in cause-specific survival between c-Met high and low cases was larger than that in 5-year overall survival. This result indicated that c-Met high expression could serve as a prognostic factor in ESCC patients. We have added this information to the “Discussion” sections as follows:

Lines 270–272: Furthermore, the statistical difference of CSS between the two groups was more prominent than that of 5-year overall survival. This result suggested that
c-Met high expression could be a prognostic factor in ESCC patients.

13 Additional file 2 – same as with Additional file 1 (minor point 10)

Authors' response: Please refer to our response to minor point #10.

14 Additional file 3 – the authors likely "normalized" the starting amount of proliferation to 100; however, that is rarely the case. Please change axis label, normalize to 1, or provide absolute numbers.

Authors’ response: Thank you for your suggestion. We have deleted Additional figure 3 owing to the changes in the cell proliferation assay methodology. The comparison between HGF treated and untreated cells was integrated in the new proliferation assay (Figure 5). The axis label was also revised.

15 Additional file 4: The axis labels in the figure are not self-explanatory. The legend of this figure mentions “correlation”. Now showing this figure is not absolutely necessary – however, if there was no correlation or rather statistically significant difference, the author should mention the test applied and the P-value. Showing the datapoints/distribution is optional.

Authors’ response: We have deleted Additional figure 4 owing to the absence of statistical correlation by Student's t-test. However, serum HGF concentration increases in inflammatory diseases, and therefore, an evaluation of serum biochemical test and respiratory function test in ESCC could be important in our study. Thus, we have revised the “Methods” and “Results” sections as follows:

Lines 120–126: Preoperative serum biochemical test and respiratory function test

In order to evaluate the possible influence of inflammatory processes upon c-Met and HGF expression, the preoperative levels of percent vital capacity (% VC), forced expiratory volume in one second percentage (FEV 1.0%), C-reactive protein (CRP), the retention rate of indocyanine green 15 min after administration (ICG-R15), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were evaluated because of the high frequency of alcohol consumption and smoking history in ESCC patients.

Lines 219–222: The association between c-Met/HGF expression and preoperative serum
**biochemical test and respiratory function test**

There were no significant correlations between c-Met/HGF expression and preoperative AST, ALT, ICG-R15, % VC and FEV 1.0% in ESCC patients (data not shown).

16 Table3. Tumor size cutoff is ay 48.7 mm – which is a strange value. How was this determined and what if size follows established criteria (AJCC/TNM)? How is this different from pT3/4 vs. pT1/2. Continuous testing? How was the cutoff determined?

Authors’ response: Thank you for your comment. “Tumor size” indicates the macroscopic maximum diameter of the resected specimens. The value 48.7 mm represented the mean value of tumor size of all patients’ specimens in our study. However, tumor depth (pT) indicates the microscopic tumor depth in the esophageal wall. Therefore, these parameters are considered different concepts. We have evaluated the tumor size according to the Japanese Classification of Esophageal Cancer, 10th edition (Japan Esophageal Society 2009).

17 Figure 1. The panel is ok; however for ease of the reader the authors should consider indicating their cut-off of c-MET (high) vs. c-MET (low) in the panel.

Authors’ response: Thank you for your suggestions. We have prepared a new Figure 1.

18 Figure 2. Please explain 5-year survival plotted against time after surgery? Is this overall survival? Please change label? What is cause-specific survival?

Authors’ response: Thank you for your comment. The 5-year overall survival was calculated from the date of surgery. We have revised the axis label as per your suggestion. Cause-specific survival indicates the survival rate from cancer-specific death (ESCC in our case).

19 Figure 4. Axis labels (i.e., “percentage”) should be revised. The figure consists of 12 panel and the numbering is A-F. The authors may want to consider relabeling (for easier referral to the specific panel) or separate the figure.

Authors’ response: Thank you for your suggestion. We have separated the original Figure 4 according to each experimental assay and revised the axis labels for clarity.
20 Figure 4 data. The maximum effect of PF-2341066 on “proliferation” is a reduction to ~85% meaning 15% of cells are affected. The efficacy is mild (see major point 1).

Authors' response: Please refer to our responses to major point #2 and minor point #9 above.

21 The comparisons in the three “grey-bared” graphs on the right (D, E, F) show highly significant differences (i.e. ***) and presumably the comparison was made to the PF-2341066/+HGF bar; however, this is not clear.

Authors' response: Thank you for your suggestions. We have prepared new Figures 6 and 7 and revised the figure legends for clarity.

22 Figure 4 legend. The authors should mention the cell line(s) used for these assays?

Comparison of efficacy between three cell lines.

Authors' response: We used KYSE 170 cells for these assays in the original manuscript. In the revised version, KYSE150, 170, and 180 cells were used to compare the results among three different cell lines in invasion and cell proliferation assays.

23 Grammar: e.g. “motility of ESCC cells were regulated”; or “considered pivotal in the ESCC patients.”

Authors' response: We apologize for the grammatical errors. We have revised and corrected those accordingly.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, and I have assessed the statistics in my report.
Declaration of competing interests: I declare that I have no competing interest.