Peer Review File

Article Information: https://dx.doi.org/10.21037/tlcr-22-52

Reviewer A

This is a very important and flawlessly written manuscript. It is a great contribution.
I have no major comments.

Comment 1

Minor comment:
1. It could be of interest to provide some additional information on KiSS1 protein-protein interactions (PPIs). This could be of great help to further highlight the value of the current study.

Reply 1

We thank the reviewer for the positive feedback and the suggestion on PPIs. The best studied protein interaction described for KiSS1 is that with its receptor. Bio-informatics approaches such as those available from the STRING interaction network (https://string-db.org) identify Tachykinin-3 as a functional interactor, suggesting a participation of KiSS1 in the Tachykinin receptor signaling pathway.

Changes in the text

The following statements have been added at the end of the Discussion: "A better understanding of the role of KiSS1 in cancer biology may also benefit from considering KiSS1 protein-protein interactions. In this regard, the best documented one is the interaction with its receptor (17). Besides, bioinformatics approaches such as those available from the STRING interaction network (https://string-db.org) identify Tachykinin-3 as a functional interactor, suggesting a participation of KiSS1 in the Tachykinin receptor signaling pathway".

Reviewer B

The study of Laura Gatti evaluates the levels of KISS1 derived peptides in NSCLC patients and performed some cell culture experiments with regard to cisplatin resistance and methylation.
Some aspects of the study are interesting and might deserve further analysis.
Unfortunately, the scope of the study is not clear, and the results do not give any substantial biological information either. Therefore, it would be important to improve the introduction substantially. The patient cohort is maybe also too small and heterogenous to identify any clinical associations. The biological experiments are too diverse and at the same time only use one cell line.
Abstract: please include patient numbers in the abstract and describe the results of the cell culture models.
Introduction: The text flow must be improved. The introduction is not updated.: Immunotherapy is now standard for advanced lung cancer. Drug resistance and epigenomics should be connected to KiSS otherwise it is not clear why the authors have evaluated it.
Methods: Patient characteristics should include histology.
Results: Figure 3: RNA levels after treatment with cisplatin and AZA should also be shown.
Figure 4: Why induce cisplatin more apoptosis in the resistant cell line. B) is the difference

between Cispl +/- KP54 significant? In that case, KP54 has no effect?
Figure 5 C: Differences significant, also between 0h and 48h? If not, it is difficult to interpret.

Comment 1

"…..the scope of the study is not clear"

Reply 1

We thank the Reviewer for all the comments that helped us to improve the manuscript. We apologize for not being clear enough in the original submission. We have rephrased the end of the introduction to clarify the scope of the study.

Changes in the text

Page 4, lines 12-18 of the Introduction: "Based on this background, since the role of KiSS1 in NSCLC is not well defined, the aim of the present study was to investigate the possibility of measuring KiSS1 levels in liquid biopsies from NSCLC patients in comparison to healthy donors and to explore the biological significance of KiSS1 in NSCLC experimental models. To this end, we designed a pilot study to analyse KiSS1 levels in liquid biopsies including three biological fluids (serum, plasma and urine) from healthy donors (controls) and NSCLC patients (cases) before and at follow up, thereby exploring potential applications of KiSS1 as a tool to monitor disease features in NSCLC patients."

Comment 2

"…..it would be important to improve the introduction……." and "Introduction: The text flow must be improved. The introduction is not updated: Immunotherapy is now standard for advanced lung cancer. Drug resistance and epigenomics should be connected to KiSS otherwise it is not clear why the authors have evaluated it."

Reply 2

We have improved the introduction mentioning the fact that immunotherapy is now the standard treatment for advanced lung cancer and it has been introduced also for early stages. We have added a few words to improve the text flow (In spite of, indeed). A recent reference has been added (reference 4). We have also detailed the connection between epigenomics and KiSS1, citing a comprehensive review (reference 17).

Changes in the text

Page 3, lines 6-8 of the Introduction: "Immunotherapy is now the standard treatment for advanced NSCLC and it is being introduced also at earlier disease stages (4). In spite of this, disease outcome remains unsatisfactory due to drug resistance and metastases (5). Indeed,…."

Page 4, lines 7-11 of the Introduction: "This study highlights a possible link between KiSS1 and epigenetic mechanisms active in drug-resistant cells. Indeed, the relevance of histone acetylation in KiSS1 regulation of drug-resistant cells emerged, together with additional evidence from the literature indicating KiSS1 regulation by DNA methylation or microRNAs (17)".

Comment 3

"The patient cohort is maybe also too small and heterogeneous to identify any clinical associations."

Reply 3

We agree with this comment. Please consider that no a priori knowledge was available on KiSS1 in NSCLC liquid biopsies. Accordingly, we implemented this pilot study with a prospective enrollment of NSCLC and healthy controls. This guarantees the

representativeness of all the entire spectrum of cases and complexity encountered in the study timeframe, during the routine clinical practice in our Institute. As concerns sample size, ≥55 patients/group can be considered an appropriate size for pilot experiments (Sim J, Lewis M. J Clin Epidemiol. 2012;65(3):301-308), by using a small to medium effect sizes.

Changes in the text

Discussion page 14, lines 4-9: "One of the strengths of our pilot study is the prospective recruitment of NSCLC cases and healthy subjects that guarantees the representativeness of the entire spectrum of cases encountered in the considered study timeframe, during the routine clinical practice in our Institute. As regards the study limitation, despite the pilot nature of this investigation, our results are encouraging and suggest a possible role of KiSS1 as NSCLC biomarker. Larger prospective studies are needed to better explore and characterize the association between KiSS1 and disease status."

Comment 4

"The biological experiments are too diverse and at the same time only use one cell line."

Reply 4

The "diverse" experiments are justified as follows and an explanation has been provided at page 4:

a) based on our previous work showing epigenetic regulation of KiSS1 upon treatment with HDAC inhibitors, we have carried out experiments with a standard epigenetic agent, i.e, azacitidine;
b) because KiSS1 is secreted, we have examined its levels by ELISA;
c) given that KiSS1 has been implicated in regulation of apoptotic response to cisplatin in head and neck cancer, here we used a gain of function approach carrying out combinations between KiSS1-derived peptides and cisplatin.

According to Reviewer's suggestion, we have now included results regarding apoptosis measured by the Annexin V binding assay in the H1975 cell line, a T790M-positive cell line that harbors the EGFR L858R/T790M double mutations conferring resistance to targeted therapy (i.e., gefitinib). Results regarding the parental cisplatin-sensitive H460 and cisplatin-resistant H460/Pt cell lines with the same assay have also been included.

Changes in the text

Page 4 of Introduction lines 18-24: "To explore the biological significance of KiSS1, based on our previous work showing epigenetic regulation of KiSS1 upon treatment with HDAC inhibitors (16), we first examined KiSS1 mRNA modulation by the epigenetic agent azacitidine. Then, because KiSS1 is secreted, we examined its levels by ELISA upon pharmacological modulation and, given that KiSS1 has been implicated in regulation of apoptotic response to cisplatin in head and neck cancer (14), we evaluated if KiSS1-derived peptides could increase cisplatin-induced apoptosis."

Page 6, line 10 Methods: the additional cell line used in the study, H1975, has been mentioned;

Page 7, lines 21-26; page 8, lines 1-3: the Annexin V binding assay used for quantitative analysis of apoptosis has been described.

Page 11, line 2-14: "Because KiSS1 may modulate cisplatin sensitivity in tumor cells (13), we examined the effect of KiSS1-derived peptide levels on cisplatin-induced apoptosis in NSCLC

cell lines by evaluating activation of caspases 3/7 (Figure 4). Using the KiSS1-derived peptide KP54, we observed activation of caspases by cisplatin in the H460/Pt cells but not in cells exposed for 48 h to the peptides *per se* (p-interaction <0.001). Of note, the combination of KP54 with cisplatin markedly increased apoptosis in cisplatin-resistant cells (contrasts p-value <0.001).
When the effect of the combination was examined using the Annexin V-binding assay (Figure 5), higher levels of apoptosis were observed comparing H460 or H460/Pt cells exposed to the combination of KP54 and cisplatin (p-interaction <0.01). The combination of KP54 and cisplatin induced a moderate (p-interaction = 0.14) increase in apoptosis levels also in the H1975 cell line, characterized by EGFR L858R/T790M double mutations conferring resistance to targeted therapy. Of note, higher values were observed both in cisplatin and KP54 factors resulting statistically significant".

Comment 5

Abstract: please include patient numbers in the abstract and describe the results of the cell culture models.

Reply 5

Patient number has been included in the Methods part of the abstract together with mention of the Annexin V binding assay

Changes in the text

Changes in red: **Methods** KiSS1-derived peptide levels in liquid biopsies from 60 NSCLC patients were assayed by ELISA. Preclinical experiments were carried out using qRT-PCR, ELISA, Annexin V binding and caspase activation assays.
Results: We compared KiSS1 release in 3 different matrices (serum, plasma and urine) and the highest levels were detectable in serum (range 0-4.5 ng/ml). We observed increased levels of seric KiSS1 in NSCLC patients as compared to healthy donors. KiSS1 serum concentrations, after surgical procedure and/or adjuvant therapy. We observed differences among disease stages in urine samples. In preclinical models, KiSS1 mRNA levels were increased by short term exposure to azacitidine, enhanced KiSS1 release was induced by the combination of azacitidine and cisplatin and KiSS1-derived peptides enhanced cisplatin-induced apoptosis. KiSS1 increase was observed upon exposure neurons enriched cultures to tumor cell conditioned medium.

Comment 6

"Methods: Patient characteristics should include histology".

Reply 6

Histology has been included

Changes in the text

Page 10 of Results lines 1-3: "As regards NSCLC patients, 79.66% of them had an adenocarcinoma, whereas the remaining ones were squamous cell carcinoma and sarcomatoid carcinoma (Table 1)."

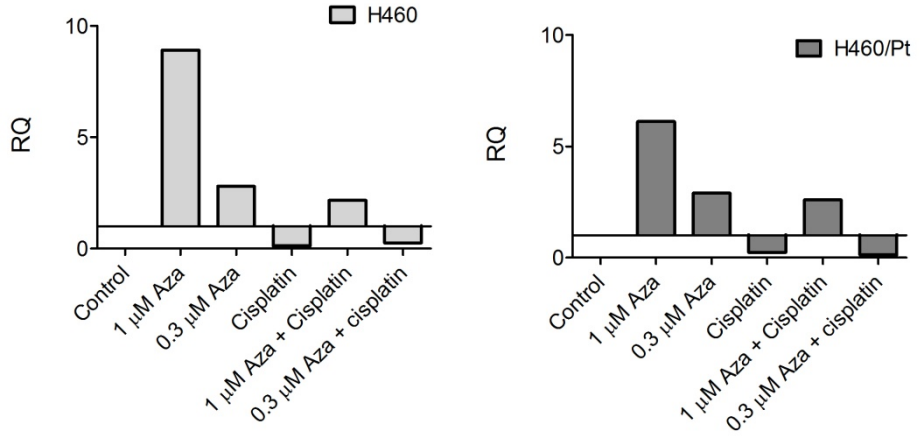
Comment 7

Results: Figure 3: RNA levels after treatment with cisplatin and AZA should also be shown.

Reply 7

The RNA levels after treatment with cisplatin and AZA in the H460/Pt cell line have been evaluated as requested by the Reviewer and are shown here. We have inserted a description in

the Results. However, we prefer not to include this analysis in the text because the mRNA levels are known to decrease in dying cells. In both cell lines the combination of AZA and cisplatin is synergistic with combination index values below 0.3 (not shown). Given that here an effective combination was tested the decrease of mRNA does not allow to better explain the biological relevance of KiSS1-derived peptides considering the mRNA level. However, released KiSS1 may cooperate to the efficacy of the combination.



Changes in the text

Results page 10, bottom 2 lines: the mRNA levels of KiSS1 in cells exposed to the combination were not increased (data not shown) likely reflecting the occurrence of cell death.

Comment 8

- Figure 4: Why induce cisplatin more apoptosis in the resistant cell line.
- B) is the difference between Cispl +/- KP54 significant? In that case, KP54 has no effect?

Reply 8

The degree of resistance of H460/Pt cells is around 2.6 (Peterson EJ, Menon VR, Gatti L, Kipping R, Dewasinghe D, Perego P, Povirk LF, Farrell NP. Mol Pharm. 2015 12(1):287-97. doi: 10.1021/mp5006867). The cisplatin concentrations used here correspond to the IC_{80} of the resistant (10 microM) and sensitive cell line (3 microM), respectively. Under our experimental conditions, 10 microM cisplatin can induce apoptosis that can be further enhanced by KP54. These data suggest that there might be a different kinetics in apoptotic response between sensitive and resistant cells, because-according to our experience - cisplatin-induced apoptosis may occur between 48 and 72 h following treatment start.

To reply to the issue, we implemented a two-way ANOVA model including the two main factors (cisplatin and KP54) and the first order interaction term. Significant interactions were decomposed by stratifying by cisplatin. As regards the H460 line, a significant effect of cisplatin was observed (i.e., higher values in cisplatin response with respect to control or KP54 alone). A difference between cisplatin and KP54 effect is reasonable, based on the pro-apoptotic effect of conventional cytotoxic agents like cisplatin. KP54 is not expected to be pro-apoptotic *per se* but to modulate drug-induced apoptosis as - in fact – it did. For H460/Pt cells we observed a significant interaction between cisplatin and KP54 (p-interaction

<0.001); by decomposing the interaction, a significant effect of the KP54 was observed in the cisplatin-treated H460/Pt cells (contrast p-value<0.001), supporting that the combination of KP54 with cisplatin markedly increased apoptosis in drug-resistant cells. We updated the Methods section accordingly.

Changes in the text

We updated the Statistical analysis section (page 9, lines 16-18) and the results section (page 11, lines 2-7)

Comment 9

Figure 5 C: Differences significant, also between 0h and 48h? If not, it is difficult to interpret.

Reply 9

Following the Reviewer comment, we investigated the association between conditioned medium of H460 cells at 48 and 0 h: although the mean value at 48h appeared to be higher that that measuread at 0 h, no significant changes were observed (one-sided Wilcoxon p-value = 0.19) [for review only].

Changes in the text

None