Author's response to reviews

Title: Implication of metastasis suppressor gene, Kiss-1 and its receptor Kiss-1R in colorectal cancer

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Author's response to reviews: see over
RESPONSE TO REVIEWER'S COMMENTS

Response to Reviewer-1

We are grateful for the constructive comments from the reviewer. The following are our detailed modifications.

Background.

We are very grateful for the comments. We have removed the word 'first' to avoid confusion. We have also cited the suggested reference by Dr Sánchez-Carbayo's group. This is now seen as Reference-14 in the new version and has also been commented on page 15 (lines 309-313).

The sections now read:

1. In this study, we examined the expression of Kiss-1 and Kiss-1R in human colorectal cancer.

2. Finally, a recent report has shown that the reduction/loss of Kiss-1 in colorectal cancer may be the result of hypermethylation of Kiss-1, providing a plausible explanation for the reduction of Kiss-1 observed in the current study [14]. It will be interesting explore the mechanisms by which Kiss-1R is reduced/lost in this cancer type in the future.

Materials and Methods.

We are grateful for the comments and have updated all the materials and methods as can be seen by the extensively modified methodology section on pages 5-8, lines 102-202. All the methods are fully described in sufficient details. In addition, the sequences for all the primers and oligos are now included in a new table, namely Table-1 and information on Kiss-1 and Kiss-1R messenger RNA secondary structures as part of revised figure-2 (Figure-2A).

These sections now read:
Antibodies to Kiss-1 (SC-101246) and Kiss-1R (SC-48220) were purchased from Santa Cruz Biotechnologies Inc., (Santa Cruz, CA, USA).

2.4 RNA isolation, cDNA synthesis, RT-PCR, Q-PCR and Immunohistochemical staining
RNA extraction Kits and reverse transcription Kits and RT-PCR Mix were purchased from Promega (WI, USA) and Bio-Rad (CA, USA). Conventional PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen (Paisley, Scotland, UK). Follow the manufacturer’s protocol, total RNA had been isolated using a standard guanidine isothiocyanate. The concentration of RNA was detected by a UV spectrophotometer at 260 and 280nm. cDNA samples were synthesized in the total 20ul volume of reaction mixtures. Primers used for RT-PCR are given in table-1. We have used GAPDH as the house keeping control.

Real-time quantitative PCR, based on the Amplifluor™ technology, was used to quantify the level of mRNA expression of Kiss-1 and Kiss-1R from the cDNA samples of the colorectal tissues and cells, mentioned above. All colorectal cDNA samples were synchronously examined for Kiss-1 and Kiss-1R along with a set of internal control. Q-PCR primers (Table-1) were designed using Beacon Design software (PREMIER Biosoft, Palo Alto, CA). Real-time PCR was carried out using IcyclerIQ™ (Bio-Rad, Hemel Hempstead, UK) following the cycling conditions: 94˚C for 5 min, 80-90 cycles of: 94˚C for 10 sec, 55˚C for 35 sec and 72˚C for 20 sec.

The frozen sections of colorectal tumours and adjacent background tissues were sectioned at a thickness of 6µm using a cryostat [15].The samples were mounted onto Super Frost Plus microscope slides. After air-drying, the samples were fixed in a mixture of 50% acetone and 50% methanol and then aeration-dried once again. After rehydration and blocking with 0.6% horse serum solution, the sections were probed with the primary antibody and subsequently the secondary antibody. Following the instructions, the avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) was applied before staining with diaminobenzidine chromogen. Nuclei was counterstained in Gill's haematoxylin. Sections from fresh frozen human planceta were used as positive controls.

2.5 Generation of Kiss-1 and Kiss-1R ribozyme transgenes and stable transfectants
Hammerhead ribozymes targeting Kiss-1 and Kiss-1R were designed using Zuker’s mRNA Fold programme (Zuker, 2003) based on the secondary structure of Kiss-1 and Kiss-1R mRNA (Figure-3A and 3B), respectively. The ribozymes were....

2.6. In vitro cell function assays

In vitro cell growth assay
Cells were seeded into 96-well plate at 2,500 cells/well, which were cultured using normal media (1% fetal cattle serum, 0.1% antibiotics). The cells were cultured in triplicate for 1.3 and 5 days. After incubation the cells were fixed in 4% formalin and stained by 0.5% crystal violet (w/v). The stained crystal violet was then extracted using 10% (v/v) acetic acid, and the absorbance was determined using a spectrophotometer (Bio-Tek, ELx800) at a wavelength of 540nm.

In vitro cell adhesion assay
A 96-well plate was precoated with 5µg of Matrigel (CollaborativeResearch Products, Bedford, Massachusetts, USA) and allowed to air dry. Following rehydration using serum free media, 40,000 cells were seeded into each well. After 40 minutes of
incubation, non-adherent cells were washed off using BSS. The adherent cells were then fixed with 4% formalin and stained using 0.5% crystal violet. The number of adherent cells was counted under a microscope.

**In vitro invasion assay**

Transwell inserts (with 8µm pore) were precoated with 50µg of Matrigel and air dried. Following rehydration, 40,000 cells were seeded into each insert. After incubation for three days, cells which had invaded through the matrix and adhered to the other side of the insert were fixed in 4% formalin, and stained with 0.5% (weight/volume) crystal violet. The number of invaded cells was then counted under a microscope.

**In vitro Wounding assay for cellular migration**

Cells were seeded into a 24-well plate at a density of 200,000 per well and allowed to form a monolayer of cells. The monolayer of cells was then scraped to create a wound. Migration of the cells at wounding edges was monitored over a period up to 18 hours. Optimas 6.0 motion analysis (Meyer Instruments, Houston, Texas) was used to tracked the leading edge of the cells to measure the distance of the migration.

Survival analysis and multivariate analysis were carried out using the SPSS 20 software package.

New and modified display materials include New Table-1, modified Figure-2 as follow:

**Table 1. Primers and oligos used in the current study**

<table>
<thead>
<tr>
<th>ssGenes</th>
<th>Sense (5'-'3)</th>
<th>Anti-Kiss1 ribozyme1</th>
<th>Anti-Kiss-1 ribozyme2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kiss-1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional PCR</td>
<td>TGAACCTCAGTTTCTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>CATTAGAAAAGGTGGCTCT</td>
<td>ACTGAACCTGAAGC</td>
<td></td>
</tr>
<tr>
<td>Anti-Kiss1 ribozyme1</td>
<td>CTGCAGCTCTCGGGGGCGAGCG</td>
<td>ACTAGTGCAGCTCTCTCGGAGGA</td>
<td></td>
</tr>
<tr>
<td>Anti-Kiss-1 ribozyme2</td>
<td>CTGCAGCAGCAGCGCGCTGGG</td>
<td>ACTAGTGGTCGCTCTCAGGGACT</td>
<td></td>
</tr>
<tr>
<td><strong>Kiss-1R</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional PCR</td>
<td>CTTCATGTGCAAGTTCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantitative PCR</td>
<td>GCTTCATCATCTACGTCATCT</td>
<td>ACTGAACCTGACCTTC</td>
<td></td>
</tr>
<tr>
<td>Anti-Kiss-1R ribozyme1</td>
<td>CTGCAGTTCCGATCGTGCTCTGGG</td>
<td>ACTAGTGTCGCTCTCGGACT</td>
<td></td>
</tr>
<tr>
<td>Anti-Kiss-1R ribozyme2</td>
<td>CTGCAGAGCTCCAGAGTACCCAG</td>
<td>ACTAGTGCCCAGACTCT</td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional PCR</td>
<td>GGCTGCTAAAAACTCTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QuantitativePCR</td>
<td>CTGAGTACGTCGTGGAGTC</td>
<td>ACTGAACCTGCAGTTTG</td>
<td></td>
</tr>
</tbody>
</table>

'New Figure-2:
We wish thank the reviewer for the question. To start a cut-off point is hard. We decided to start from the level of Dukes-B as the starting point and have done a great deal to test the correlation, based on this level, by moving upward and downward, until a satisfactory conclusion as the one indicated in this manuscript was achieved. Thus, a large number of values have been tested to achieve the current conclusion.

4 The authors need to perform multivariate analysis for survival in their study.
We are very grateful for this highly valuable point. Multivariate analyses have been carried out with the data now provided in a new table, namely Table-4. This has been commented on page 10 (lines 200-201), page 11 (lines 224-231), in Table-4 and table-4 legend in the new version.

This section now reads

'In contrast to Kiss-1, the expression pattern of Kiss-1R has revealed that high levels of the Kiss-1R transcript are associated with both a poor overall survival (Figure 1.c, p=0.0011) and poor disease free survival (Figure 1.d, p=0.0033). Multivariate analyses have further demonstrated that T-stage and Kiss-1R are independent prognostic factors (p=0.025 and p=0.003, respectively) for colorectal related death. Furthermore, TNM staging and Kiss-1R (p=0.03 and p=0.012, respectively) are independent prognostic factors for colorectal cancer related incidence (death, recurrence and metastasis) (Table-4).'

'New table-4 is as following:

**Table 4. Multivariat analysis for prognostic factors for colorectal related death and incidence.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>With colorectal related death*</th>
<th>With colorectal related incidence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes stage</td>
<td>0.507</td>
<td>0.249</td>
</tr>
<tr>
<td>T-stage</td>
<td>0.025</td>
<td>0.092</td>
</tr>
<tr>
<td>TNM stage</td>
<td>0.146</td>
<td>0.030</td>
</tr>
<tr>
<td>Node status</td>
<td>0.230</td>
<td>0.057</td>
</tr>
<tr>
<td>Differentiation</td>
<td>0.601</td>
<td>0.326</td>
</tr>
<tr>
<td>Kiss-1</td>
<td>0.435</td>
<td>0.566</td>
</tr>
<tr>
<td>Kiss-1R</td>
<td>0.003</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* p values

5 The authors demonstrated the immunochemical staining of tumor tissues in figure 1a. Then the authors need to evaluate more detail of the expression of Kiss-1 and Kiss-1R protein expression in colon cancer tissues by IHC analysis with clinical outcome.

The immunohistochemical staining was carried out on a portion of the fresh frozen tissues that were available to this study, as the majority of available tissues of the study cohort were small and were processed for extracting genetic materials. The current IHC study meant to provide a preliminary information on the staining pattern in normal and tumour tissues of the colon. Thus, the information on the full clinical outcome of the entire cohort was not available. We hope to be able to address this in a future study. This is indicated on page 11 (lines 233-235) in the new version.

This section now reads:

'Immunohistochemical staining was carried out on a portion of paired normal and tumour tissues (n=23 pairs). The staining showed intensive staining of Kiss-1 and Kiss-1R was primarily..'
The author need to use the positive control such as placenta for the expression of Kiss-1 and Kiss-1R.

We are grateful for pointing this out. The positive control by using placenta for IHC is now included in figure-1A and its figure legend. The positive control by using placenta for PCR is now provided in figure-3A and its figure legend in the new version.

'which lost the expression of Kiss-1 and Kiss-1R were used in the subsequent experiments. During these experiments, cDNA generated from human placenta were used as positive control.'

The new results are presented in the revised figure-1 and figure-3:

![Image](image-url)
The authors state that, “in summary, it is suggested that Kiss-1 inhibits ERK activation and consequently reduces the enzymatic activity of MMP-9 caused by the degradation of NF-κB, which contributes to the suppression of tumor metastasis.” The reviewer strongly recommend to show the degradation of NF-κB in their original work.

We are very grateful for this comment. The statement in the previous version was based on the recent findings in the literature with regard to CCMP-9 and NF-kappa B, including that by Yan et al. Presently, we are unable to carry out this analysis due to a methodology challenge. However, Our institute is about to recruit scientist(s) to establish this line of method and research work. We hope to be able to address this important issues in our future studies. We have indicated this matter on page 16 (lines 329-330) in the new version.

This section reads

‘It will be indeed very interesting to examine the degradation of NF-Kappa B in response to Kiss-1 signalling in future studies.'
Response to Reviewer-2

We are grateful for the constructive comments from the reviewer. The following are our detailed modifications.

In this paper the authors for the first time shed light in the intracellular pathway of the Kiss-1/Kiss-1R system (ERK and MMP9) in colon cancer and they verified that the kiss-1 gene is presents anti-metastatic actions in colorectal cancer and its expression is associated with better prognosis in colon cancer patients.

Line 232: Suggest that the presence of Kiss-1R is significantly associated with disease free survival, and at line 234 the authors say that high expression of kiss-1R transcript is associated with poor prognosis. Can the authors please clarify this part?

We have clarified the confusion in the new version by providing clear statement which reads: 'In contrast to Kiss-1, the expression pattern of Kiss-1R has revealed that high levels of the Kiss-1R transcript are associated with both a poor overall survival (Figure 1.c, p=0.0011) and poor disease free survival (Figure 1.d, p=0.0033). Multivariate analyses have further demonstrated that T-stage and Kiss-1R are independent prognostic factors (p=0.025 and p=0.003, respectively) for colorectal related death. Furthermore, TNM staging and Kiss-1R (p=0.03 and p=0.012, respectively) are independent prognostic factors for colorectal cancer related incidence (death, recurrence and metastasis) (Table-4).’ This can be seen on page 9 (lines 224-231).

Figure 4a: It is not clear what is what. It seems that the kiss-1R KD responds to exogenous administration of kisspeptin 10 similarly to the kiss-1 KD. The kiss-1 expression in the cell lines used is quite low (fig 1 c western blots) maybe a cell line with a higher expression would represent a better model for the kiss-1 KD study.

We have revised figure-4 by providing colour line graphs to aid the reading. The new figure-4 is as following:
We are very grateful for this comment. A positive control using human placenta for IHC and PCR has now been provided in Figure-1A (IHC) and Figure-3A (PCR) and has been indicated in the respective figure legends.

The new results are presented in the revised figure-1 and figure-3:
The results obtained by the authors could be verified by an overexpression experiment in these cell lines used in this study whereas the exogenous administration of a blocking anti-Kiss-1 antibody could verify the autocrine / paracrine effect of kiss-1.

This is indeed a very valid comment. Both cell lines, naley HT115 and HRT18 had an adequate level of expression of Kiss-1. We had search for a more suitable human colon cancer cell line(s) which is negative for Kiss-1, which would allow a distinction between the wild type negative and over-expression. This was unfortunately without success, as a number of human colon cancer cell lines we tested including RKO, HT29, HT55 and CaCo2 were all had the same profile to HT115 and HRT18. We chose HT115 and HRT18 for the suitability in the cell based assays. We have exhausted with available human colorectal cancer cells available to us and are currently looking at other non-colon cancer cells which are negative for Kiss-1 and hope to carry out further studies of autorine/paracrine regulation by Kiss-1.

What was the amount of exogenous Kiss-1 used?
This information has now been provided on page-9 (lines 185-192) and reads as following:

"HT115 Cells were prepared for six repeats per group and seeded at 40,000 cells per well in 200µl of DMEM medium alone or medium supplemented with 200nM ERK small inhibitor, 300nM Kisspeptin-10 and 300nM Kisspeptin-234.

2.8 Gelatin zymography assay
1x10⁶ cells were counted and seeded to a tissue culture flask. After an overnight incubation and 4 hours treatment (300nM Kisspeptin10, 300nM Kisspeptin234 or 200nM ERK inhibitor), the medium was collected. The concrete use method refers to the section of Materials and Methods of Sun et al. [16]."