May 8, 2015

Dear Reviewer,

Please find enclosed the edited manuscript in Word format (file name: hur17394.docx.).

Title: HuR mediated post-transcriptional regulation as a new potential adjuvant therapeutic target in chemotherapy for pancreatic cancer

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First of all we would like to thank the reviewer for such an in-depth analysis and valuable comments. Please, find below a point by point reply (italic font) to the reviewer's comments (normal font) and information regarding the revisions of the presented manuscript.

Major points

1. The authors showed that expression levels of HuR mRNA and protein were decreased in PDA tissues compared to normal tissues. However, expressions of COX-2 and HO-1, well-known targets of HuR, were increased in PDA. These results strongly suggested that HuR would not be main cause of COX-2 and HO-1 overexpression observed in pancreatic cancer tissues. In other word, HuR could modulate these expressions only under stress condition, because GEM-induced HuR modulated COX-2 and HO-1 expression in pancreatic cancer cells as the author shown in this manuscript.

Answer/action:
We fully agree with the above statement of the reviewer and present similar idea in the conclusion section (see pg. 14): „Our in vitro study confirms that GEM treatment strongly induces HuR expression in the pancreatic cancer cells lines. The stabilization and increased translation of HuR target mRNAs results in overexpression of cytoprotective and anti-apoptotic proteins COX-2 and HO-1, which translates in the increased resistance to GEM treatment. The HuR silencing significantly decreases COX-2 and HO-1 expression and sensitizes pancreatic cancer cells to GEM treatment."

To clarify that HuR could modulate COX-2 and HO-1 expression only after GEM treatments, they need to show that treatment with HuR siRNA alone do not affect on these expressions in Fig 6. Same approaches should be taken in Fig 7, 8 and 9.

Answer/action:
We demonstrate by Western blot analysis, that anti-HuR siRNA results in the decreased HuR protein expression in pancreatic cancer cells 72 hours after transfection (fig. 5). The Western blot analysis and quantification also clearly demonstrated that treatment with GEM induces expression of HuR, COX-2 and HO-1, while transfection with HuR siRNA prior to the GEM treatment effectively reduces the expression of these molecules. Based on the reviewer's suggestion we included the data from MTT experiments demonstrating that HuR silencing alone has no effect on pancreatic cancer cell viability (fig. 7). We believe there was sufficient data to show that transfection with control siRNA and/or HuR siRNA has no effect on the cell viability, therefore we didn't carry out the analysis of these subgroups in the later stages of the research that are presented in figures 8-9.

Moreover, they had better to investigate the expression of HuR, COX-2 and HO-1 in PDA tissues treated or untreated with GEM by immunohistochemistry. These approaches will be useful to prove their hypothesis.

Answer/action:
This is indeed a very interesting point for further research and discussion, however, it's not a routine practice to give neoadjuvant chemotherapy with Gemcitabine (indeed with any other chemotherapy drug too) prior to pancreatoduodenal resection in most of the medical centres (and it's not part of the medical practice in our hospital), therefore it's not possible to get cancer tissue samples after GEM treatment to investigate the expression of HuR, COX-2 and HO-1 in our institution. However, we agree that this issue is very important and we decided to include the section called "study limitations" on page 15 addressing this problem: "It would be useful to investigate and compare the expression of HuR, COX-2 and HO-1 in PDA tissue obtained both from GEM treated and non-treated patients in order to fully understand the underlying mechanism and the role of HuR mediated post-transcriptional regulation for the exceptional resistance of pancreatic cancer to the conventional treatment. However, it's not a routine practice to give neoadjuvant chemotherapy with Gemcitabine (or any other chemotherapeutic drug) prior to pancreatoduodenal resection in our hospital, therefore, it's not possible to obtain pancreatic cancer tissue samples after GEM treatment for research purposes in our institution. This issue demonstrates certain limitations of this study, however, we believe the overall results are sufficient to demonstrate that HuR mediated plays an important role in the chemoresistance of pancreatic cancer cells"

2. It is hard to compare the intensity of immunoblot across the different membranes. In Fig 2, the authors need to apply protein samples from normal pancreas, pancreatic cancer, and colon cancer on same membrane. Same approach should be used for the Fig. 6.

Answer/action:

Protein samples from normal pancreas, pancreatic cancer, and colon cancer were blotted on same membrane during the experiments; however, a representative figure (fig. 2) was developed after removal of some artefacts (see attached figures HuR_tissue1 and HuR_tissue2). Same approach was used for the samples represented in Fig. 6. Protein extracted from GEM treated or HuR siRNA transfected and GEM treated cells was blotted on the same membrane as protein from the control non-treated cells, however, the representative and easily understandable figures were developed (see attached original blots in figures HuR_cells_control_GEM and HuR_cells_siRNA_GEM). It was not possible to blot all three groups of samples represented in Fig. 6 on one membrane because the running gel only had 10 slots/wells for the loading.

3. In this study, usage of beta-actin for loading control is inappropriate, because it has been reported that HuR binds to beta-actin mRNA and regulates its expression (Dormoy-Raclet et al. Mol Cell Biol. 2007). The authors need to use other internal control, such as GAPDH, and re-evaluate their results.

Answer/action:

With all the due respect we believe that re-evaluation of the results and their validation using the other internal controls should not be required. Despite the above mentioned publication by Dormoy-Raclet et al. beta-actin remains a widely used internal control in studies on Hur and there is little evidence that the results could be significantly biased because of this. Journals with relatively high citation indexes continue to publish the papers on the research of HuR with the use of beta-actin as internal control, e.g. Kakuguchi W et al. HuR knockdown changes the oncogenic potential of oral cancer cells. Mol Cancer Res. 2010 Apr;8(4):520-8. PubMed PMID: 20332213. Nevertheless, we were aware of the publication by Dormoy-Raclet et al. and have used GABDH (as shown in fig. 5) as a loading control during the crucial stage of the research when we testing the effects of HuR siRNA on pancreatic cancer and successfully demonstrated that transfection significantly reduces the expression of HuR protein. We continued to use beta-actin as internal control for other experiments as we evaluated mainly the expression of downstream molecules (HO-1 and COX-2), furthermore, the effects seen in the functional experiments (MTT and CV) during HuR silencing were comparable to those seen in the experiments when direct silencing of HO-1 was employed: Dambrauskas Z et al. Inhibition of heme oxygenase-1 increases responsiveness

of pancreatic cancer cells to anticancer treatment. Clin Cancer Res. 2005 May 15;11(10):3790-8. PubMed PMID: 15897578.

4. While the effects of HuR-knockdown in sensitivity to GEM were much in MiaPaca2 and SU.86.86 compared to Capan-1 and -2 cells, the activation levels of caspases 3 and 7 after HuR siRNA plus GEM-treatments looks quite similar in all cells. The results of fluorescence microscopy analysis, as presented in Fig 10, are hard to evaluate. Quantitation of caspase activation using luminescent assay is required. The authors also need to examine the effects of HuR-overexpression on chemoresistance in pancreatic cancer cells, in addition to HuR knockdown experiments.

Answer/action:
The reviewer rightly points that the mechanisms of the apoptosis and their effects need to be further investigated after combined treatment with HuR siRNA and GEM. This is a very interesting area of the research, however, in this study we did not aim to look at more precise mechanisms of apoptosis and/or its intensity. We only attempted to demonstrate that different cancer cell lines react very similarly to the GEM treatment and that silencing of HuR makes them more susceptible to the chemotherapy. It was also quite surprising and interesting that treatment with GEM alone did not significantly induce the cleavage of caspases 3 and 7, while the combined treatment induced apoptosis in all the cell lines. We believe that the presented data is sufficient to prove this point, while more detailed investigations would be a valuable part of further studies in the future.

Minor points

1. In Fig 3, the authors need to show the HuR expression of each tissue sample.

Answer/action:
Tissue samples from 20 patients were used in this study for QRT-PCR and/or Western. We believe that in this case Western blot is only a semi-quantitative study which only supports the data obtained during QRT-PCR analysis and shows that expression at mRNA and protein levels coincide, thus few representative protein samples are sufficient to demonstrate the tendencies. It's quite unusual to blot and quantify all the tissue samples used in the study.

2. In Fig 9, it is hard to distinguish the morphological changes of cancer cells. The authors should show the photomicrographs with appropriate brightness and contrast. Same approaches should be taken in Fig 10.

Answer/action:
Much to our regret the photos with a higher resolution could not be readily obtained in our lab within a short period of time. Nevertheless, we believe that fig. 8-9 and fig. 10 are sufficient that the cell proliferation significantly differs between different treatment groups and the changes in the shape and/or sizes as well as fragmentation are the first signs of apoptosis, which is also demonstrated by the high expression of activated caspases. We strongly believe that presented data is sufficient to prove the above mentioned notion.

3. In Fig 11, it is difficult to distinguish translocation of HuR from nuclear to cytoplasm. The authors need to show photomicrographs with high magnification. Furthermore, they need to show the images merged with DAPI stain and HuR expression.

Answer/action:
Much to our regret the photos with a higher resolution could not be readily obtained in our lab within a short period of time. As required by the reviewer the merged images with DAPI stain and HuR expression were included in the fig. 11.

4. I do not understand the explanation ("Study showed that HuR silencing sensitized pancreatic cancer cells to GEM, but didn't have an effect on cell viability.") since HuR knockdown further decreased cell viability in GEM-treated cells (Fig 7).

Answer/action:
Figure was edited to include the effects of HuR siRNA transfection and description of the figure was revised accordingly: "Figure 7. Cell viability analysis (MTT). Viability was analysed in control cells, cells treated with GEM IC50, siHUR alone and treated with GEM IC50 after HuR siRNA. Transfection with HuR siRNA had little or no effect on cell viability, while HuR silencing dramatically increased response of pancreatic cancer cells to the GEM treatment."

Also the manuscript has been improved according to the suggestions of editors:

1. The final language editing of the manuscript was done by the members of the Lithuanian Centre for Evidence-based Medicine who are graduates of the Oxford University and lecturers at the Centre for Evidence-based Medicine of the Oxford University. Thus, perhaps, only a minor language polishing could be required for the final version of the manuscript. The last author of the manuscript also has achieved a pass in the University Entrance in English for Speakers of Other Languages and demonstrated proficiency in language (Level B, see attached copy of the certificate).

2. The running title and ethics approval was included in the manuscript's title page.

4. All the reference numbers was reformatted (superscript with square brackets). Normal line space was used.

5. Comments list was included.

Sincerely yours,

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