Author's response to reviews

Title: Modulation of RANTES expression by HCV core protein in liver derived cell lines

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Version: 4 Date: 16 February 2007

Author's response to reviews: see over
Dear Editor,

Thank you for reconsidering the manuscript “Modulation of RANTES expression by HCV core protein in liver derived cell lines” by A. Ruggieri, M. Franco, I. Gatto, A. Kumar and M. Rapicetta and for accepting the requested postponement of our re-submission.

We tried to further improve the manuscript according to the suggestions of reviewers. In particular, the results for parental CHL cell line and for Hep352 were added in Fig. 2 and 5; immunoblotting results of IRF-7 for the controls HepG2 and CHWT were also included in Fig. 6. Moreover, interpretation of the results regarding CH352 effect on RANTES expression were revised in Results section as well as in Discussion according to suggestions of the reviewer. The corroboration of semi-quantitative PCR results of RANTES mRNA expression in HepG2 cell lines was obtained by Real-Time quantitative PCR analysis using triplicate cDNA samples of each cell line on ABI Prism 7000 sequence detector system by TaqMan chemistry. We think that this analysis provides more clear-cut results than densitometry analysis of the gel bands.

Based on recommendations, the manuscript was read by a mother-tongue English reader, and rewording of the sentences in Conclusions section was done according to the reviewer indications. Wishing that the revised version of the enclosed manuscript will fulfil the standard required by BMC Gastroenterology journal the Authors thank you.

Best regards.

Dr. Maria Rapicetta
Reply to reviewer 1

Title: Modulation of RANTES expression by HCV core protein in liver derived cell lines

Version: 3 Date: 14 december 2006

Reviewer: Nischalke H.D.

1. It is a major shortcoming of this manuscript that control experiments (e.g. results in untransfected CHL cells and in HepG2 cells transfected with the HCV core-E1-E2-NS2-NS3 in figures 1, 2 and 5) are not reported consistently. For instance the authors showed that transfection of cell lines with HCV core alone leads to increased RANTES production in Chang Liver cells but to reduction of RANTES in HepG2 cells. In CHL cells transfected with HCV core-E1-E2-NS2-NS3 this effect was completely abolished. Thus, it is important to clarify if the longer construct also abolishes the opposite effect in HepG2 cells. Therefore transfection of HepG2 cells with core-E1-E2-NS2-NS3 had been suggested as a critical control in the previous review. However, this issue is not addressed at all by revised version of the manuscript.

   In Fig. 5 data relative to Hep352 and to parental CHL were included and showed as columns plot. The sentence :”Interestingly, in Hep352 cell line a 3.5 fold induction of RANTES promoter was detected in comparison to Hep39 cell line that suggested a counteracting effect of the structural E1-E2 proteins toward the core protein inhibition on RANTES promoter also in HepG2.” was added in Results section, page 12, line 230-233.

   In Fig2. A and B the data relative to parental CHL cell lines were also included.

2. Figure 3.: The authors state that transfection with their HCV core-containing vector (Hep39) results in down regulation of RANTES mRNA expression as compared to cells transfected with the empty vector. However, figure 3 seems to suggest that less β-actin mRNA was detected by the PCR. Thus, the presented data do not exclude the possibility that apparent changes in RANTES mRNA reflect differences in RT efficiency or effects of PCR inhibitors. Thus, we advised the authors to corroborate their data by providing densitometry data of the gel bands together with an appropriate statistical analysis. This improved evaluation of the data is missing in the current manuscript.

   In order to confirm the effect of HCV core protein expression on RANTES mRNA Fig.3 was updated with the results of Real-Time quantitative PCR analysis shown in Fig.3 (B). As described in Methods section (page 9, lines 161-171, subsection Real-Time Quantitative PCR analysis) the Real-Time quantitative PCR analysis was performed using RANTES primers on ABI Prism 7000 detector system using the TaqMan chemistry. Data normalized by the level of β2-microglobulin mRNA expression are reported as the relative expression unit of RANTES mRNA levels; values are means ± standard deviations of two independent experiments with three replicates /sample in each experiment. Accordingly, in Results section page 13, lines 230-233, the following modification of the text was done: “... that RANTES transcripts levels were decreased in Hep39 cell lines compared to the controls (Hepswx and HepG2) (Figure 3(A) upper panel). In order to corroborate these results, Real-Time quantitative PCR analysis was performed to determine relative RANTES mRNA levels in Hepswx and Hep39 cell lines. Results shown in Fig 3(B) indicated that in Hep39 cell lines RANTES mRNA level was 50% less than in Hepswx cell lines.”
Finally, legend of Fig.3 was also updated accordingly to the new data shown: “...(B) Real-Time quantitative PCR analysis using RANTES primers. Data were normalized by the level of β2-microglobulin mRNA expression in each sample and are shown as the relative expression unit. Shown are the means ± standard deviations of two independent experiments with three replicates /sample in each experiment.”

3. In figure 6, low panel, IRF-7 data for HepG2 parental cells and CHL cells transfected with control vector (CHWT) should be presented analogous to the presentation of the IRF-1 data in the upper panel.

   **Figure 6 : the low panel** was changed with the new picture of immunoblotting results including HepG2 parental cell line and CHWT.

4. In the results section the authors describe that in cells expressing HCV proteins C-E1-E2-NS2-NS3 (CH352) the fraction of RANTES positive cells was similar to the controls, suggesting a possible role of HCV non-structural proteins (NS2-NS3) in counteracting the effects of structural HCV proteins in RANTES induction. The authors did not consider the alternative possibility that E1-E2 counteracts the effects of core on RANTES in CH352 cell lines, since it has been recently reported by Nattermann et collaborators (J Viral Hepatitis 2004) that interaction of E2 protein with CD81 increased RANTES secretion by CD8+ lymphocytes from HCV patients. Nattermann et al. used recombinant E2 protein to stimulate T-cells extracellularly. This kind of stimulation is quite different from intracellular expression of multiple HCV proteins, because it is possible that E1 and E2 may counteract the HCV core mediated effects by direct binding to HCV core protein in the cytoplasm. Thus, the available data do not enable to dismiss this alternative interpretation of the results., which should be taken into account in the discussion of the results.

   **Page 11, line 216-218**: The suggested interpretation of the results has been considered and the sentence: “...However in CH352 cell lines the fraction of RANTES positive cells (7.5% ± 0.7%) was similar to the controls. This latter result suggests a possible role of HCV non-structural proteins (NS2, NS3) in counteracting the effect of the core protein in RANTES induction...” has been changed to: “...This latter result suggests a possible role of HCV structural E1 and E2 proteins in counteracting the effect of the core protein in RANTES induction ...”.

   **Page 17, line 325-330** : the following sentence was included: “Co-expression of the core protein with E1-E2 and non-structural NS2-NS3 proteins of HCV resulted in counteracted effect on RANTES expression with respect to the effect of HCV core singularly expressed in both cellular systems; this suggested that E1-E2 proteins, which have been reported to affect RANTES expression by CD8+ lymphocytes in HCV patients [20] may counteract the effect of HCV core protein on RANTES, consistently to the results by Soo et al. [18], possibly by direct interaction of E1 and E2 with the core protein in the cytoplasm.”

Minor Essential Revisions

1. Figure 2: The authors state that they omitted the misleading blue histogram in this figure which referred to CH827 cells expressing E1-E2. However, figure 2a submitted with the revised manuscript still show this blue histogram, contrary to the statement of the authors.

   **Figure 2 was updated** and the misleading histogram was omitted but data for parental CHL cell line were included as requested in point 1.
Reply to Reviewer 2: Alfredo Garzino Demo

Modulation of RANTES expression by HCV core protein in liver Title: derived cell lines

Version: 3 Date: 20 December 2006

Reviewer's report:
General
Ruggeri et al. have revised their manuscript following most of the feedback that they received on their original submission. I find their response, which include appropriate statistical analyses, and revision satisfactory and do not have major revisions to request, as I find that the revisions have significantly strengthened the manuscript.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Page 3, Abstract conclusion section: This sentence is not clear and should be reworded. My suggestion for a reworded conclusion reads: "HCV core protein have opposite effects on the expression of RANTES in different cell types in vitro, possibly reflecting a similar scenario in different microenvironments in vivo". However, this might not be exactly what the authors are inferring.

Page 3, Abstract: the sentence in conclusion section was corrected according to the suggestion.

Page 5, third paragraph: "NF90 belongs to a family [...] (evolutionary related to the flavivirus family [...]"evolutionary" should be changed to "phylogenetically"

Page 5: Done.

Page 10, last sentence of the Methods section: "The statistical analysis was performed by SPSS (Inc)"would read better "Statistical analyses were performed using SPSS software (SPSS Inc)"

Page 10: Done.

In the result section, some parts could be made clearer for example, on Page 11:
"Despite reduction in RANTES expression was detected also in Hepswx cells as compared to the parental cell line, suggesting a possible contribution of constitutive transfection on RANTES level, however the differences in percentage of cells positive for RANTES expression among Hep39 as compared to Hepswx cell lines was statistically significant (p<005). In contrast, flow cytometry quantitation of RANTES protein in CH39 cell lines showed (Figures 2A and 2B) a 2.5 fold increase in percentage (16.6% ± 2.9%) of cells positive for RANTES expression, as compared with the CHWT control cells (6.7% ± 1.2%). The differences observed between CH39 and control cell lines were significant according to the p value (<0.05) in a t Test’s."

my suggested rewording is:

"Reduction in RANTES expression was detected also in Hepswx cells as compared to the parental cell line, suggesting a possible contribution of constitutive transfection on RANTES level. However the differences in percentage of cells positive for RANTES expression IN Hep39 as compared to Hepswx cell lines was statistically significant (p<005). In contrast, flow cytometry quantitation of RANTES protein in CH39 cell lines showed (Figures 2A and 2B) a statistically significant 2.5 fold
increase in percentage of cells positive for RANTES expression, as compared with the CHWT control cells (16.6% ± 2.9% Vs. 6.7% ± 1.2%, p<0.05)

Page 11: Done

Page 18, Conclusions, first paragraph. Here too, the first sentence of the conclusion is not clear, see my note above regarding the abstract section.
In addition, the last sentence could be modified in "Our data suggest a role, that needs to be confirmed in vivo" for viral (HCV) proteins in contributing to immune evasion by tumor cells

Page 18, Conclusions, first paragraph ": the first sentence was changed to “HCV core protein have opposite effects on the expression of RANTES in different cell types in vitro, possibly reflecting a similar scenario in different microenvironments in vivo.”
The last sentence was rewarded according to the suggestion.

Discretionary Revisions (which the author can choose to ignore)
I recommend having the manuscript read by a mother-tongue english reader in case there are any grammar glitches that i might have missed.

What next?: Accept after minor essential revisions
Level of interest: An article whose findings are important to those with closely related research interests
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 interests