Author's response to reviews

Title: Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression

Authors:

Mehmet Ender Avci (aender@bilkent.edu.tr)
Ozlen Konu (konu@fen.bilkent.edu.tr)
Tamer Yagci (tyagci@fen.bilkent.edu.tr)

Version: 3 Date: 11 November 2008

Author's response to reviews: see over
Dear Dr. Sabina Alam,

Thank you very much for your letter of October 23-2008, with regard to the review of our manuscript “MS: 1921301642211992” titled "Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression."

In light of the reviewer's critical comments, we restructured the whole manuscript and discussed in detail the aspects raised by the reviewers. Below are our point-to-point responses, in where each comment from the reviewers is shown in bold.

We sincerely appreciate the helpful comments of reviewers. We now hope that the revised version meets these requirements and will be found suitable for publication in BMC Cancer.

Sincerely yours,

Tamer Yagci

tyagci@fen.bilkent.edu.tr

Reviewer's report
Title: Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression
Version: 1 Date: 26 August 2008
Reviewer: Yutaka Midorikawa

Reviewer's report:
This manuscript describes the quantification of transcript of SLIT and ROBO genes about 14 hepatoma cell lines and 35 liver tumors, followed by two-way clustering analysis. The experiments were carefully done and are well described and discussed. However the study is still at a preliminary stage and therefore a careful tone should be used in the interpretation of the data. The manuscript would also benefit from a more focused presentation of the generated data.

Major comments

1) SLIT2-ROBO1 signaling is known to be involved in angiogenesis in cancer. However, SLIT2 and ROBO1 were clustered into different group in Figure 3. The author should discuss about this discrepancy.

In the tumor-normal tissue array, the expression profile of each SLIT-ROBO family gene was normalized to the average normal tissue expression thus scaling the values as log fold changes.
with respect to the mean control expression value. This type of scaling helps group genes and samples since it is relative for each gene reflecting its variability and represents increases or decreases in expression with respect to the normal tissue for each of the gene. Moreover, cluster analysis works in such a way that it groups genes that behave similar; yet this grouping is based on a distance/similarity matrix (Pearson) and the degree of similarity may range from low to high between genes. Although a two-group clustering for \textit{SLIT-ROBO} genes was observed, the degree of similarity between genes even within the group they belonged to was not greater than 0.7, except \textit{SLIT1-ROBO2} correlation in cell lines (0.76). This suggests that there is great variability in the expression of a gene, some of which might be correlated with the expression change in another group member. In this case SLIT2 and ROBO1 were in different clusters because there were other gene members to which they behaved closer than they did to each other. However, \textit{SLIT2} and \textit{ROBO1} expression were also upregulated in poorly characterized liver tumors suggesting that these two genes may also act synergistically. Indeed, knowing that SLIT2-ROBO1 ligand-receptor interaction was shown to be involved in angiogenesis suggests that the observed variability in \textit{SLIT2} gene expression might lead to a possibility that ligand-dependency of ROBO1 signaling may help understand the phenotypic variability among liver tumors.

We addressed this issue in the Discussion section of the revised manuscript as:

On page 12 we stated that "\textit{ROBO1, ROBO2, SLIT1,} and \textit{ROBO4, SLIT2, SLIT3} showed coordinate expression as two distinct modules, yet displaying high variability at gene level within each module."

On page 13 we added the following comments: "\textit{SLIT2} was present in most of the tumor tissues and HCC cell lines although at variable levels. Such variability might explain the clustering of \textit{SLIT2} in a different group than \textit{ROBO1} and \textit{ROBO2}, yet it is likely to be the main ligand for ROBO receptors. Nevertheless, this does not exclude interactions between
other SLIT and ROBO members, nor it does the possible ligand-independent activities of ROBO receptors in HCC. Additionally, SLIT2 and ROBO1 were both upregulated in HCCs with advanced stages and poor differentiation status (Figure 3 and Table 3). These findings also are in agreement with the expression of SLITs specifically in poorly differentiated HCCs [19]. Furthermore, in a tumor xenograft model, SLIT2-ROBO1 signaling was shown to have a role in angiogenesis, which supports tumor growth and metastasis [13].”

2) Finally, which SLIT-ROBO signaling pathway is activated in hepatocarcinogenesis?

SLIT-ROBO receptor-ligand family act in complex manners, regulating a plethora of processes, ranging from differentiation to angiogenesis. Interestingly, multiple ligands might interact with multiple of receptors, context dependently. Some of these are SLIT2-ROBO1-ROBO4 in angiogenesis. The antagonism between ROBO4 and SLIT2 has been shown in vascular disease progress (34).

We addressed this issue in the Discussion section of the revised manuscript as:

On Page 14 we added the following sentence: “In fact, recent findings indicated that SLIT2-ROBO4 interactions inhibited angiogenesis [34].”

And in the following paragraph, we concluded about the SLIT-ROBO signaling pathway contribution in HCC as:

“It is very likely that SLIT2-ROBO1-ROBO4 might contribute to some of the variability associated with the differentiation status of HCC while expression of ROBO1 and SLIT2 also helps explain the stage differences in this cancer. However, the expression variability is high among liver tumors suggesting that a combinatorial code with a possibility of ligand redundancy might be at work in hepatocellular carcinoma, which prompts further study using functional studies that include knock-down and overexpression.”

**Minor comments**
1) The results of Figure 1 obtained by RT-PCR should be left out, instead, the results by qRT-PCR in Table S2 should be involved.

Figure 1 was left out of the manuscript and transferred to Additional Files Section. Original Figure 3 (now Figure 2 in revised version) involves the results obtained by qRT-PCR results, which were represented in Table S2 (now Additional File 3 in revised version of the manuscript). Readers may infer the fold-change differences across cell lines or liver normal-tumor tissues using hierarchical clustering images, color coded as red and green, corresponding to fold-increase and fold-decrease, respectively.

Reviewer's report
Title: Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression
Version: 1 Date: 22 October 2008
Reviewer: James Woodgett
Reviewer's report:
This manuscript describes the RNA expression levels of the family of Robo/Slit genes in a variety of hepatocellular cell lines and liver tissue. The analysis is cleanly performed and the authors have clustered the expression levels into twocoordinated groups. While this study is of potential interest, it is too long in its present state to warrant the data being presented. Notably, no protein expression was determined, nor possible differential splice variants or insight into gene copy numbers in the cancer cell lines. Pure expression levels can often be calculated from the increasing data associated with microarray analysis of tissue and cell line atlases. Hence, this manuscript should be condensed by 30% or so unless the authors wish to add protein expression or copy number data.

We agree with the reviewer’s comments about cutting down the length of the original manuscript. Since the RT-data is partially redundant with the real-time RT data, and the latter is highly quantitative, we only reported the changes in expression as log-fold changes, reported them as mean +/- STD in the text, associated the mean differences with ANOVA results when needed, and finally represented the fold-change differences across cell lines or liver normal-tumor tissues using hierarchical clustering images, color coded as red and green, fold-increase and fold-decrease, respectively. The RT gel figure and its legend containing methodological and results information were moved to “Additional Files” section (Additional
We also removed a significantly lengthy part from the background information which pertained to the normal tissue expression in mice, since it was less relevant to the human cancers. Also, Figure 3 and 4 of the original manuscript were omitted, and mean expression values +/- STD and associated p values were represented in the text on page 10. Redundancy in references was corrected and the total number of references was decreased to 36. All together, these changes reduced the length of the original manuscript by about 30%.

Human Protein Atlas, a virtual immunohistochemistry tool online was searched to see whether ROBO-SLIT protein expression in liver tumors existed. Although there is some information at this site, since the tumors were not categorized into stages or differentiation states, we could not fully make use of it to validate our results. Moreover, this database also acknowledges the low validity of SLIT and ROBO antibodies. The possibility of splice variants in compartmentalization of the ROBO-SLIT expression profile is real and should be pursued to better clarify the active/inactive members of SLIT-ROBO signaling in a variety of liver tumors in different stages. Accordingly, on page 15, in Conclusion section we further elaborated on this point: “Elucidation of the mechanisms acting on the transcriptional regulation of SLIT-ROBO signaling pathway, such as alternative splicing, copy number variability and ligand/receptor redundancy in both HCC and other pathophysiological contexts will contribute to a better understanding of hepatocarcinogenesis.”

The examination of array cgh data in the OncoDB HCC (Oncogenomic Database of Hepatocellular Carcinoma: http://oncodb.hcc.ibms.sinica.edu.tw) suggested that ROBO or SLIT genes were not located in the chromosomal deletion/amplification domains frequently associated with human or rodent hepatocellular carcinoma. These perspectives were added in the Background section of the revised manuscript (page 4): “Moreover, karyotyping analyses
of HCC do not reveal any chromosomal gains or losses associated with SLIT-ROBO genes [23, 24].”