Author's response to reviews

Title: Quantitative evaluation of RASSF1A methylation in the non-lesional, regenerative and neoplastic liver.

Authors:

Sonia Di Gioia (sonia.digioia@unimi.it)
Paolo Bianchi (paolo.bianchi@humanitas.it)
Annarita Destro (annarita_destro@msn.com)
Fabio Grizzi (fabio.grizzi@humanitas.it)
Alberto Malesci (alberto.malesci@humanitas.it)
Luigi Laghi (luigi.laghi@humanitas.it)
Massimo Levrero (levmax@tiu.it)
Alberto Morabito (alberto.morabito@unimi.it)
Massimo Roncalli (massimo.roncalli@unimi.it)

Version: 6 Date: 22 February 2006

Author's response to reviews:

To Peter Newmark

Editor-in-Chief, BMC Cancer

Dear Prof Newmark,

The criticism raised by the referee Zhu and by the statistician have been, again, carefully evaluated and the paper amended accordingly.

We would like to underline that we have addressed the concerns of the statistician and that a novel statistical analysis, conducted following the indication of the statistician, fully confirmed the original results and their statistical value. As requested by the statistician, results shown in Figure 3 are now better detailed by including the median value of methylation index, 25 and 75% percentiles and the minimum and maximum values in the different settings of hepatitits and non-hepatitis liver. Given that results are not changed, the discussion was not modified.

As requested by the referee Zhu we have now enclosed a representative set of real graphs of our real time PCR results that, we think, would not improve the value and the clarity of the paper. As such they should eventually be enclosed as additional data.

I would like to emphasize that the second question raised this time by the referee Zhu sounds meaningless because he requested to perform an analysis (real time MSP PCR analysis of HCC) that we had already performed.

Referee Zhu:

1) It is necessary for author to provide further details on the real-time PCR analysis. For instance, a set of the real graphs from such an analyses are needed and the methylated state of which CpG is really looked at.

A set of the real graphs from the above analysis is now enclosed for the referee and, if necessary, it should be published as additional data. For sake of clarity we enclosed a representative set of real graphs from each group of lesions occurring in both hepatitis and non hepatitis liver (total number of graphs:12, total number of samples shown:60). In Materials and Methods, in the main text (page 6, lines 35-36), a sentence has been now added indicating that these results can be seen as additional data.

The fragments analyzed by MSP-real time analysis are exactly the same as those analyzed by conventional MSP (same set of primers). Therefore in our previous review we have already addressed the issue of the
methylation state at which CpG are really looked at (Figure 2).

2) It seems pretty natural for the authors to compare the MSP data with the data by the real-time experiments and discuss the implications to the methodological aspects of the methylation profiling. I wonder why the HCC samples have only been analyzed by MSP, excluding from the real-time PCR analyses.

From this question we retain that the referee did not carefully go through our paper. Indeed at page 6, line 3 we stated: "Real time methylation specific PCR was used for the quantification of the methylated and unmethylated RASSF1A promoters in all samples under study. That we conducted a real time MSP analysis on HCC samples is also detailed on Results (main text) and on Figure 3A.

3) "Human cell lines, LoVo and HeLa, previously shown to be respectively RASSF1A emimethylated and unmethylated by conventional MSP", I do not understand the word underlined in this sentence (emimethylated).

Emimethylated: methylation of one allele.

Statistician:

The authors have changed some of the statistical analyses to avoid the problem of multiple comparisons. Instead of 3 Mann-Witney tests, now they present 1 analysis of variance. However, still multiple samples from the same individual are considered independent to artificially increase the sample size and this is not correct from a rigorous statistical point of view. I already had advised to use linear mixed models which, if properly formulated, can account for the within subject correlation. These models usually show that the standard errors of correlated measures are greater than expected under the independence assumption and probably some of the results are no longer significant.

Linear mixed models (random effect model) accounting for the within subject correlation have been applied by a statistician (prof A. Morabito, now enclosed in the list of authors). All the results are now presented in Figure 3, showing standard errors of correlated measures. All the results have retained the original statistical significance. The adopted methodology is now detailed in the statistical analysis (page 7, lines 2-3).

Herewith enclosed the final version of our amended paper.

We think that the manuscript should hopefully be accepted for publication; we hope to hearing from you very soon.

Sincerely,

Massimo Roncalli MD PhD