Author's response to reviews

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

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Dear dr. Newmark

The issues raised by both referees have been carefully considered and the paper amended accordingly.

Specifically

Referee Zhu J
1) The frequency of changes detected by MSP technique in methylation of both the genes under studies are reported in the text, page 4-5 for hepatitic liver (RASSF1A HCC, HN and cirrhosis) and lines 22-23 for non hepatic liver (RASSSF1A, liver adenoma, focal nodular hyperplasia, non lesional liver). For NORE1A the results are reported on page 7, lines 2-3. The methylation index of RASSF1A in the various settings is reported in the Figure 3 (grouping previous Figures 3&4), upper panel (A) for the hepatitic liver (HCC, HN, cirrhosis) and bottom panel (B) for non-hepatitic liver (HA, FNH and non lesional liver).
2) The referee requested to discuss the frequency of methylation in the cirrhotic and HCC settings according to results obtained from different geographic regions. A sentence now discusses the role of RASSF1A methylation in the cirrhosis-HCC sequence on the basis of studies from different regions (Discussion, page 9, lines 10-16):
"Interestingly, the RASSF1A methylation rate in cirrhosis (81-82%) and corresponding HCC (98-100%), as detected by MSP (qualitative assay), occurred with a surprisingly overlapping frequency in 3 different geographic regions: Italy (Mediterranean area, present work), North Europe (Lehmann et al, 2005) [32] and China (Yu et al 2002) [33]. Given the different etiology of liver cirrhosis in these regions the above results might suggest that the very high rate of RASSF1A methylation in the cirrhosis-HCC sequence is a peculiar early molecular feature of hepatocarcinogenesis in the cirrhotic setting, unrelated to a specific etiology". All the above papers were quoted in the original version of the manuscript.
3) The methylation status of CpG in clinical sample is now reported in the context of the sequence in Figure 2 which now incorporates both the original Fig 1 and 2.

Referee Fruhwald MC
1) All the samples under study were run by real-time MSP and each experiments were repeated three times. This is now reported in Material and Methods page 5, lines 31-33. As requested by the referee the calibration curve is now shown in a new figure (Figure 1 and corresponding Legend 1). The impact of calibration curve on the methylation index is now reported in Material and Methods, page 6, lines 17-20 and lines 24-25.
2) As requested by the referee a detailed section of how and when the amounts of DNA were measured is now reported in Material and Methods, page 4, line 19-20 and page 5 line 6.
3) The original Figure 1 is now Figure 2. For sake of clarity this figure has been implemented according to Referee Zhu with the sequence analysis. What is now shown in Figure 2 is just an example of MSP qualitative analysis in cirrhosis and HCC, the main sources of material of the present work. We do not think
that the addition of analogous figures for focal nodular hyperplasia and hepatocellular adenoma would increase the clarity of the paper.

4) We agree with the referee that methylation is the correct word to be used consistently throughout and that silencing/repression should never be used in this context. Accordingly we have now substituted silencing/repression with methylation.

5) We agree with referee that an increased number of samples including material from fetuses and children would be helpful to strongly support our results. This is now acknowledged in a sentence (Discussion, page 10, lines 22-24):

"Because our results were obtained in a limited number of cases, the gradual increase of RASSF1A methylation index by age should be confirmed in larger series including younger subjects such as children and fetuses".

Unfortunately we do not have access to material from children and fetuses in our hospital (www.humanitas.it) and so we are unable to perform these analysis. An additional issue to be taken into account when working with normal liver from children and fetuses is that it is only collectable from autopsies and we do not think post-mortem material (not less than 24 h from death for the Italian law) optimal for this type of analysis.

6) As requested by the referee Figure 3 and 4 are now incorporated in a single one (Figure 3 A&B).

7-8) We agree with the referee that the full conversion of unmethylated cytosines by bisulfite is very critical for MSP and real-time MSP analysis evaluation. To be confident on the reliability of our results we originally addressed this issue by carefully checking the presence of additional peaks in the sequences (both raw data and sequences analyzed by base caller). As reported also in Figure 2 we never documented additional peaks in our samples and control cell lines.

We acknowledge that the cloning of bisulfite modified DNA is the gold standard for the sequencing but it is currently rather infrequently used, because extremely laborious, expensive and time-consuming. Actually, we have to say that most of papers accepted for publication in the current literature, as those quoted in our study, do not even perform any kind of sequencing to confirm the methylation status and that a consistent number of published papers documented their methylation status by direct sequencing of MSP-PCR products (see Wen Chang H et al, Clin Cancer Res 2003; Reddy AN et al, Cancer Res, 2003; Poone LM, Clin Chem 2002, Tamura G et al, JNCI 2002, Kuroki T et al, Clin Cancer Res, 2003).