Reviewer's report

Title: SIP1 is Downregulated in Hepatocellular Carcinoma by Promoter Hypermethylation

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Reviewer: Janet JM Mertz

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The authors investigated the RNA and protein levels of SIP1 in primary HCCs, normal liver tissue, and cell lines derived from HCCs. They claim that SIP1 expression was completely lost or significantly reduced in most of the primary tumors. They show that incubation of SIP1-negative cells with the combination of a DNA methyl transferase and an HDAC inhibitor can lead to increased expression of SIP1. Finally, they look at promoter methylation to try to correlate hypermethylation of the SIP1 promoters with expression of this gene in HCCs.

SIP1 likely plays a significant role in many cancers. Thus, it is important to understand its regulation and expression in cancers. These authors attempt to do so with HCCs. The experiments they performed are appropriate ones, and the data they present is of considerable interest to the field. Unfortunately, some key controls are missing (e.g., proof of the specificity of their SIP1 antibody) and some additional experiments, outlined below, are needed to strengthen the interpretation of their findings. Without these additional experiments, it is not clear whether the primary conclusions stated in the title are truly valid, i.e., whether SIP1 is really downregulated rather than alternatively expressed, and whether this downregulation is occurring primarily at the level of promoter hypermethylation rather than indirectly via miRNAs, the SIP1 NAT RNA, alternative splicing of the primary transcript, or decreased efficiency of translation of the SIP1 mRNA.

Major compulsory revisions:

1. Figure 1A – Please explain from where the slightly less than 100-bp band is originating. It almost looks like it, too, could be a SIP1 band, e.g., a variant from alternatively spliced processing of the transcript. If true, one could be looking at alternatively spliced SIP1 RNA rather than lower levels of SIP1 RNA.

2. Figure 2 – How sure are you that your antibody is staining only for SIP1 protein? Figure 4A of Oztas et al. (Exp. Mol. Path. 89:182, 2010) shows an immunoblot probed with your other SIP1 mab in which numerous bands are visible. Can you please show an immunoblot probed with the mab you used here in IHC to document that it truly is highly specific for endogenous SIP1, i.e., it does not significantly cross-reacting with other cellular proteins? Such a control is crucial to the interpretation of IHC results.

3. Given P2 is likely the primary promoter from which SIP1 transcripts are synthesized, your finding that it is rarely hypermethylated in the tumors may be
the more important finding than that the P1 and P3 promoters are usually hypermethylated in the primary HCCs. If you believe that your finding of hypermethylation of P3 may be physiologically important, you need to assay your samples as well for the anti-sense NAT RNA whose synthesis may be regulated by the P3 promoter. It would also be nice to assay them for at least one of the 200 family of miRNAs to determine whether SIP1 expression correlates with these RNAs better than it does with hypermethylation of some of the putative promoters. If so, maybe SIP1 RNA levels are being primarily regulated at a post-transcriptional level.

Minor essential revisions:
1. Figure 1B - Please clearly describe what the y-axis represents.
2. Figure 2 – What do you think the physiological meaning might be of SIP1 being present primarily in the cytoplasm in some cases?
3. Figure 3A – Again, what is the origin of the band slightly less than 100-bp? Why is it abundant in this HepG2 lane, but not in the HepG2 lane in Fig. 1A?
4. Figure 3B – Please indicate clearly what is the sample in the rightmost lane?

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.