Author’s response to reviews

Title: The effects of the location of cancer stem cell marker CD133 on the prognosis of hepatocellular carcinoma patients

Authors:

Yao-Li Chen (31560@cch.org.tw)

Ping-Yi Lin (69221@cch.org.tw)

Ming Ying-Zi (myz_china@aliyun.com)

Wei-Chieh Huang (Wei-Chieh@nhri.org.tw)

Rong-Fu Chen (den2.th1@gmail.com)

Po-Ming Chen (050611@nhri.edu.tw)

Pei-Yi Chu (chu.peiyi@msa.hinet.net)

Version: 1 Date: 11 Nov 2016

Author’s response to reviews:

Reviewer reports:

Reviewer #1: Review

Q1. The authors heavily cited the property of CD133 as a cancer stem cell (CSC) marker to build study rationale. However, according to the CSC theory, CSCs are believed to represent only a minority number of the tumor mass. This is directly contradictory to the data shown in Fig. 1, in which a ubiquitous staining pattern, regardless cytoplasmic or nuclear, was shown for CD133. It should be noted that it is the AC133 epitope (glycosylated N-terminal of CD133) but not the CD133 protein in general, accurately serves as marker of stemness (see Int J Biochem Cell Biol. 2005 Apr;37(4):715-9. Cancer Res. 2010 Jan 15;70(2):719-29. and many other studies). Apparently, the polyclonal CD133 antibody used in this study is not a suitable tool to interrogate stemness. Other biological roles associated with CD133 are thus suggested, which needs to be thoroughly discussed in the revised manuscript.

Answer: We agree the comment of the reviewer. According to the CSC theory, CSCs are believed to represent only a minority number of the tumor mass. CD133 has been applied as a marker for CSCs in several cancers (24-27). Actually, to identify CSCs are dependent on glycosylated CD133 protein, not native CD133 protein (28). Recent studies show highly CD133 protein expression has poor prognosis in various cancer patients (14-16, 29). CD133
overexpression induces epithelial-mesenchymal transition (EMT) (30) and increased in vitro invasion and resists to chemotherapy (31). Interestingly, Y828 phosphorylation level of CD133 can bind to P85 for activation of PI3K/AKT pathway to promote tumorigenic capacity. Additionally, CD133 transcription is upregulated by SP1 and Myc, and inhibition of CD133 transcription is require for P53 tumor-suppressive activity and methylated CpG islands of its promoter.

Notablely, another study has firstly showed that CD133 protein expression levels in both the cytoplasm and nucleus were significantly higher in non-small cell lung cancer (NSCLC) compared with corresponding peritumoral tissue that agreed with our study, and high CD133 expression in both the cytoplasm and nucleus is associated with unfavorable outcomes in NSCLC (33). Anomalous localization in the nucleus has been reported for several other cell-surface and secreted molecules in various cancer, and some molecules can move to nucleus to be the transcriptional factors, such as epidermal growth factor receptor (EGFR), Cyr61-Ctgf-Nov (CCN), epidermal growth factor EGF, and fibroblast growth factor (FGF) (34, 35).

The above description has been added in conclusion section in the revised manuscript (line 14, page 13).

Q2. Considering this is the first time reporting the nuclear localization of CD133 in HCC, it is imperative for the authors to thoroughly validate the specificity of the CD133 antibody used in this study (preferably in a HCC cell line). Both immunoblot (specificity test) and immunofluorescence (or other type of immunostaining, to suggest staining pattern) are needed. Further, these tests should be also accompanied by a validated siRNA (or shRNA) to suggest the signal from the testing antibody can be abrogated by CD133 knock-down.

Answer: We agree the comment of the reviewer.

(1) The liver cancer cell lines HepG2 and PLC-5 were obtained from the National Health Research Institutes (Taiwan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 0.1 mM sodium pyruvate, 10% FBS, 2 mM l-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Briefly, 5 × 105 cells were respectively transfected with 10 µg of the lentiviral vector pLKO (control) or pLKO/shCD133 (target sequence
which were purchased from the National RNAi Core Facility at Academic Sinica, Taiwan. After 48 h, CD133 expression was confirmed by CD133 antibody (orb18124) for Western blot as Supplementary figure 1:

(2) 2.5×10⁴ PLC-5/PLKO and PLC-5/shCD133 cells were respectively seeded on cover slips for 150 mins in complete medium and then fixed with 4% formaldehyde for 5 minutes at room temperature prior to immunofluorescence assay. Cells were washed with phosphate-buffered saline three times, treated with 0.1% Triton for 10 min, and blocked with 5% goat serum for 1 hour, cells were then incubated with CD133 antibody (orb18124) at 200X dilution at 4°C overnight followed by binding with Alexa Flour 488 goat anti-Rabbit for green fluorescence by Leica DM2500 Upright Fluorescence Microscope as Supplementary figure 2:

Q3. The authors also need to exclude the possibility (although less likely) that the nuclear staining is a kind of antibody-specific artifact. This can be done by repeating the staining with the same specimen showing nuclear CD133 with another CD133 antibody.

Answer: CD133 is known to show both cytoplasmic and membranous staining. Please have a look at http://www.proteinatlas.org/ENSG00000007062-PROM1/cancer/tissue/liver+cancer/#img?utm_source=custserv&utm_medium=email&utm_campaign=CSE of a hepatocellular carcinoma sample, and the CD133 antibody (orb18124) is used to recognize an epitope corresponding to residues NHQVRTRIKRSRLDSNFKD (Supplementary figure 3). The figure shows that highly nuclear CD133 was observed using immunohistochemistry.

Q4. In fact, for a study exclusively focusing on IHC study, "no primary antibody" as negative control is not enough. Instead, normal rabbit IgG (in this case) is needed. For the six cases shown in Fig.1A, please supply the corresponding isotype control.

Answer: We agree the comment of the reviewer. We have supplied the corresponding isotype control of CD133 antibody by using normal rabbit IgG in Figure 1A that showed as Figure 1:
Reviewer #2:

Q1. It's not enough to draw such a conclusion "cytoplasmic CD133 expression was significant for poor prognosis, while contrarily nuclear CD133 expression was significantly correlated with favorable prognosis". You should better compare the groups with their other clinicopathological parameters like age, gender, differentiation, tumor stage, hepatitis B surface antigen, hepatitis C virus to see whether the two groups are comparable or not before draw this conclusion.

Answer: We agree the comment of the reviewer. The results showed that no statistically significant correlation between the C-/N-, C+/N-, C-/N+ and C+/N+ groups (C: cytoplasmic CD133; N: nuclear CD133) and age, gender, differentiation, tumor stage, hepatitis B surface antigen, and hepatitis C virus. The results has been added in Supplementary table 1.

Q2. "The median followup time after surgery was 982 days and the median overall survival of all patients was 1092 days." How do you explain the median OS is longer than that of the followup time?

Answer: Primary tumor tissues were obtained from 119 HCC patients receiving surgical resection. The survival time was defined to be the period of time from the date of primary surgery to the date of death. The median follow up time after surgery was 982 days in 119 HCC patients. During this survey, 39 patients died, and the median overall survival of 80 patients was 1092 days.