Author’s response to reviews

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

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Reviewer #1: Review

Q: It is highly recommended that the authors incorporate all of their responses to the revised manuscript (e.g. the validation of the antibody as well as all other supplementary materials).

Answer: We agree the comment of the reviewer. All of our responses have been incorporated to the Materials and Methods, Results and Figure legends sections of the revised manuscript as follows (line 17, page 7; line 1, page 13; line 17, page 34):

Line 17, Page 7

Immunohistochemistry and scoring

Immunohistochemistry (IHC) was used to detect CD133 protein expression. The CD133 antibody (orb18124) was purchased from Biorbyt (USA). Paraffin-embedded HCC tissue sections (4-μm) on poly-1-lysine-coated slides were deparaffinized and rinsed with 10mM Tris-HCl (pH 7.4) and 150mM sodium chloride. Peroxidase was quenched with methanol and 3%
hydrogen peroxide. Slides were then placed in 10mM citrate buffer (pH 6.0) at 100 °C for 20 min in a pressurized heating chamber. After incubation with 1 : 200 dilution of CD133 antibody (orb18124) for 1 h at room temperature, slides were thoroughly washed three times with phosphate-buffered saline (PBS). Bound antibodies were detected using the EnVision Detection Systems Peroxidase/DAB, Rabbit/Mouse kit (Dako, Glostrup, Denmark). The slides were then counterstained with hematoxylin. At last, the slides were photographed with the microscope (BX50, OLYMPUS, Japan). Negative controls were obtained by performing all of the IHC steps, but leaving out the primary antibody. The immunohistochemical staining scores were defined as described previously (21) and the intensities of signals were evaluated by a board certified pathologist. The immunostaining scores criteria was defined as the cell staining intensity (0= nil; 1=weak; 2=moderate; and 3=strong) multiplied by the percentage of stained cells (0–100%), resulting in scores from 0 to 300. A score higher than mean score were defined as ‘high’ immunostaining, while a score equal to or lower than mean score was categorized as ‘low’ in tumor. Although CD133 is known to show both cytoplasmic and membranous staining, our results revealed that highly nuclear CD133 was observed using immunohistochemistry. Please also have a look at


Of a hepatocellular carcinoma sample, and the CD133 antibody (orb18124, Biorbyt) is used to recognize an epitope corresponding to residues NHQVRTRIKRSLADSNFKD (Supplementary figure 1).

Cell lines

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 GCGTCTTCTATTCAGGATAT) 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 blotting and.
Western blotting

After whole cell protein extracts were prepared in ice-cold RIPA lysis buffer and quantified by BCA (bicinchoninic acid) protein assay, equivalent amounts of cell lysates were separated by 8–12 % SDS polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was then blocked in 5 % non-fat milk in PBST (1X Phosphate Buffered Saline Tween-20) and probed overnight at 4°C with the primary antibodies against human CD133 antibody (1: 1000, orb18124, Biorbyt) and β-actin (Sigma-Aldrich Corp., St. Louis, MO, USA). Anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase was used as the secondary antibody for detection using an enhanced chemiluminescence (ECL) western blot detection system (Millipore, Bedford, MA, USA), and band intensities were quantified by densitometry (Digital Protein DNA Imagineware, Huntington Station, NY).

Immunofluorescence

2.5×10^4 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, Biorbyt) 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.

The validation of the CD133 antibody (orb18124)

We used the lentiviral vector pLKO (control) or pLKO/shCD133 (target sequence GCGTCTTCTATTCCAGGATAT) which were transfected into HepG2 and PLC-5 cells. Western blotting showed that CD133 protein expression level was decreased in HepG2 and PLC-5 cells were transfected with pLKO/shCD133 compared with HepG2 and PLC-5 cells were transfected with pLKO using the specific CD133 antibody (orb18124) (Figure 2A). We further examined CD133 protein location in PLC-5/pLKO and PLC-5/pLKO/shCD133 by labeling CD133 antibody (orb18124, Biorbyt) with Alexa Flour 488 goat anti-Rabbit for green fluorescence by Leica DM2500 Upright Fluorescence Microscope. Fluorescence images revealed that cytoplasmic and nuclear CD133 protein expression was higher in PLC-5/pLKO than in PLC-5/pLKO/shCD133 cells.
Different effects of OS and RFS on CD133 location of HCC

We further investigate the association of clinico-pathological parameters and CD133 with patients’ survival was statistically verified by using univariate analysis. Results showed that several characteristics including age, gender, differentiation, tumor stage, hepatitis B surface antigen, hepatitis C virus, cytoplasmic CD133 and nuclear CD133 on OS and RFS of HCC. (OS: p=0.330 for age, p=0.761 for gender, p=0.134 for differentiation, p=0.003 for stage, p=0.552 for hepatitis B surface, p=0.152 for hepatitis C virus, p=0.022 for cytoplasmic CD133 and p=0.025 for nuclear CD133; RFS: p=0.851 for age, p=0.881 for gender, p=0.104 for differentiation, p=0.001 for stage, p=0.861 for hepatitis B surface, p=0.189 for hepatitis C virus, p=0.022 for cytoplasmic CD133 and p=0.013 for nuclear CD133; Table 2). Kaplan-Meier analysis showed that patients with high level of cytoplasmic CD133 expression (C) had shorter OS and RFS periods when compared to patients with low level of cytoplasmic CD133 (C) expression (Figure 2A and 2D). Unexpectedly, we found high nuclear CD133 expression (N) of HCC patients had longer OS and RFS periods when compared to those patients with low level of nuclear CD133 expression (N) (Figure 2B and 2E). We further stratified CD133 expression into C-/N-, C+/N-, C-/N+ and C+/N+ groups to estimate the OS and RFS of HCC. The results showed C+/N- group had the shortest OS and RFS periods (Figure 2C and 2F). However, No statistically significant correlation was found 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.

Figure 2. CD133 expression is decreased using the lentiviral vector pLKO/shCD133, and the CD133 antibody (orb18124, Biorbyt) was to validate CD133 protein expression level and location in liver cancer cells. (A) CD133 expression was depleted upon transfection of HepG2 and PLC-5 cells with pLKO/shCD133 respectively. The levels of the CD133 protein expression was evaluated by western blotting. β-actin was used as a loading control. (B) CD133 antibody (orb18124, Biorbyt) was used to probed CD133 location in PLC-5 cells with pLKO and pLKO/shCD133 at 4°C overnight followed by binding with Alexa Flour 488 goat anti-Rabbit for green fluorescence by Leica DM2500 Upright Fluorescence Microscope. Nuclei were stained with 4’,6’-diamidino-2-phenylindole (DAPI).