

## Original Article

# Effect of hepatitis B virus infection on trophoblast cell line (HTR-8/SVneo) and choriocarcinoma cell line (JEG3) is linked to CD133-2 (AC141) expression

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**Abstract:** Mother-to-infant transmission of hepatitis B virus (HBV) plays an important role in the chronic carrier state in China. In our studies, the response of trophoblast cell and choriocarcinoma cell to HBV infection regarding the expression of CD133-2 (AC141) was evaluated. Western blot and RT-PCR showed that a high level of CD133-2 protein and mRNA in HTR-8/SVneo cells, but a low level in JEG-3 cells. Lower proliferation and mobility, and higher apoptosis were observed in HTR-8/SVneo cells and JEG-3-CD133-2<sup>+</sup> cells after HBV infection than those in HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells. Our main finding is that CD133-negative cells (HTR-8-CD133-2<sup>-</sup> and JEG-3) are prone to HBV infection. In the last, our data indicated that the activation of Smad signaling pathway and the induction of epithelial-mesenchymal transition (EMT) in CD133-negative cells after HBV infection. In summary, our study demonstrated that CD133 is a key factor that mediated HBV infection to trophoblast cell and choriocarcinoma cell.

**Keywords:** HBV infection, trophoblast, choriocarcinoma, mobility, Smad pathway

## Introduction

China is a high incidence of hepatitis B virus (HBV) infection country [1]. Mother-to-infant transmission of HBV plays an important role in the chronic carrier state in China [2]. Intrauterine route is the most common route of HBV transmission, however, only 5-15% of infants of HBV-positive women are infected by intrauterine transmission [3]. Primary placental trophoblasts are more resistant to virus infection than non-trophoblastic cells [4]. One reason is that human trophoblasts could produce high levels of inflammatory cytokines that comprises a subsequent immunological barrier restricting microbes from accessing the fetus [5]. Therefore, the mechanisms by which placental trophoblast combats virus, including HBV, need elucidating.

In the current investigation, it was hypothesized that CD133 on trophoblastic cells is involved in the prevention of intrauterine transmission of HBV. CD133, a glycoprotein also known in humans and rodents as Prominin 1 (PROM1),

localized in membrane protrusions or microvilli [6]. CD133 gene codes two splice variants named PROM1.s1 and PROM1.s2, and the protein product is recognized by three different antibodies (CD133-1: clone AC133 or CD133-2: clone 293C3 and clone AC141) directed toward different epitopes [6, 7]. Pötgens et al. [8, 9] found that CD133-2 (AC141) is a positive marker for the characterization of trophoblast cell line.

In this study, the response of trophoblast cell and choriocarcinoma cell to HBV infection regarding the expression of CD133-2 (AC141) was evaluated. We confirmed that CD133-2 expression in trophoblast cell and choriocarcinoma cell was negatively associated with the presence of HBSAg.

## Materials and methods

### Cell lines and culture conditions

The trophoblast cell line HTR-8/SVneo (kindly provided by Dr. Charles H. Graham, Queen'

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University, Kingston, ON, Canada) and human choriocarcinoma cell line JEG-3 (ATCC; Manassas, VA, USA) were maintained in RPMI-1640 medium (Hyclone; Logan, UT, USA) and Dulbecco's Modified Eagles Medium (DMEM, Hyclone) which was supplemented with 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO<sub>2</sub>.

### *CD133-2/pcDNA3.1 plasmid construction*

The human *CD133-2* gene was obtained by PCR from a cDNA library of placenta. Total RNA was isolated from placenta tissues using an RNeasy Mini Kit (Biomed, Beijing, China). cDNA was reverse transcribed with 1 µg of total RNA using a TaKaRa Reverse Transcription Kit (TaKaRa, Dalian, China) and was amplified using the following primers. *CD133-2* primers were 5'-TATAAAGCTTACCATGGCCCTCGTACTCGG-3' (sense) and 5'-TATAGGATCCTCAATGTGTGATGGGCTTGTC-3' (antisense) (Shanghai GenePharma, Shanghai, China). The amplified *CD133-2* cDNA was digested and inserted into the pcDNA3.1 vector (TaKaRa) between BamH I and Hind III (TaKaRa).

### *Transfection*

Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, cells were plated in medium with 10% FBS and cultured until they achieved 70-80% confluence. Culture medium was then replaced with low-serum media (containing 0.5% FBS). HTR-8/SVneo cells were transfected with *CD133*-specific siRNA fragment (sense: 5'-GGGCUAUCAUCCCUUAAUTT-3', antisense: 5'-AUUAAGGGAUUGAUAGCCCTT-3', Shanghai GenePharma, Shanghai, China). A nonspecific siRNA sequence (sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3') was designed and synthesized as negative control (data were not shown). JEG-3 cells were transfected with *CD133-2/pcDNA3.1* plasmid.

### *Retro-transcription PCR (RT-PCR)*

Total RNA was isolated from cells using an RNeasy Mini Kit (Biomed). cDNA was reverse transcribed with 1 µg of total RNA using a TaKaRa Reverse Transcription Kit (TaKaRa) and

was amplified using the following primers. *CD133-2* primers were described in *CD133-2/pcDNA3.1* plasmid constructs. *HBsAg* primers were 5'-ATGGAGAACATCACATCAGGATTC-3' (sense) and 5'-TGCCCAAAGACAAAAGAAAATTGG-3' (antisense). *GAPDH* primers were 5'-AG-AAGGCTGGGGCTCATTG-3' (sense) and 5'-AGGGGCCATCCACAGTCTTC-3' (antisense). The PCR products were electrophoresed on a 1.5% agarose gel, and visualized by ethidium bromide staining under a UV imaging system (UVP, LLC, Upland, CA, USA).

### *HBV infection*

The serum samples with a high level of HBV DNA (> 1.0 × 10<sup>8</sup> copies/ml) were collected from HBV carriers. A 0.22 µm filtration device (Millipore, Shanghai, China) was used to filter away the bacteria. 20% HBV positive serum (complement inactivated) was added into culture medium of HTR-8/SVneo cells and its *CD133-2* negative clone, and JEG-3 cells and its *CD133-2* positive clone.

### *Colony formation assay*

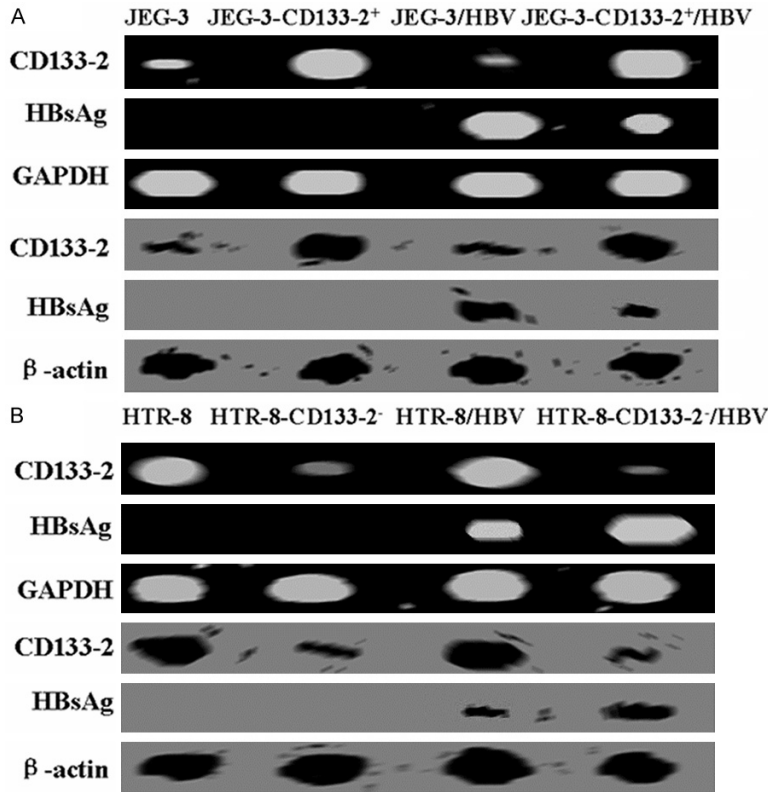
Cells were seeded at 300 cells/well in twelve-well tissue culture plates. The plates were incubated in a humidified incubator at 37°C. Colonies were stained with 0.05% crystal violet containing 50% methanol, and counted. The colonies were counted in five random fields. Photographic images were taken using an Olympus CX21 microscope (Olympus, Tokyo, Japan).

### *Detection of apoptotic cells*

*In situ* TUNEL assay was employed to detect the apoptotic cells. Cells were grown on slides and fixed with 4% buffered formaldehyde. TUNEL assay was performed using the *in situ* Cell Death Detection kit (KeyGEN BioTECH, Nanjing, China) according to the manufacturer's instructions. Coverslips were mounted onto glass slides using an Olympus CX71 microscope (Olympus, Tokyo, Japan).

### *Wound healing assay*

For scratch wound healing assay, cells were cultured in serum-free medium for 24 h and wounded with pipette tips. Fresh medium was replaced. The wound closing procedure was observed for 48 h.



**Figure 1.** Detection of HBV infection in HTR-8/SVneo cell and its CD133-2 negative clone (HTR-8-CD133-2), and JEG-3 cell and its CD133-2 positive clone (JEG-3-CD133-2<sup>+</sup>). A. & B. The protein and mRNA levels of CD133-2 and HBsAg were determined by using western blot and RT-PCR.

#### Gelatin zymography

Cell culture supernatants were loaded onto a 10% SDS-PAGE containing 1 mg/ml gelatin (Biomed). After electrophoresis and washing with a buffer containing 20 mm Tris-HCl (pH = 7.5) and 2% Triton X-100 for 1 h, the gel was incubated in MMP reaction buffer containing 20 mm Tris-HCl (pH = 7.5) and 10 mm CaCl<sub>2</sub> at 37°C for 16 h. Gelatinolytic activity was detected by staining with Coomassie Brilliant Blue G-250 staining solution (Biomed). Gelatinolytic activity appeared as a clear band on a blue background.

#### Western blot

Then equal protein (30 μg) from cells was subjected to SDS-PAGE and then blotted onto NC membrane (GE Healthcare Corp., Piscataway, NJ, USA). CD133-2 (AC141) was purchased from Miltenyi Biotec Technology & Trading (Shanghai, China). HBsAg (sc-53300), p-Smad3 (sc-130218), Smad3 (sc-101154), p-Smad2

(sc-135644), Smad2 (sc-6200), E-cadherin (sc-7870), vimentin (sc-6260), N-cadherin (sc-7939), and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, Shanghai, China). The secondary antibody was anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (Amersham Biosciences, Needham, MA, USA). Then the results detected by enhanced chemiluminescence (KeyGEN BioTECH).

#### Statistical analysis

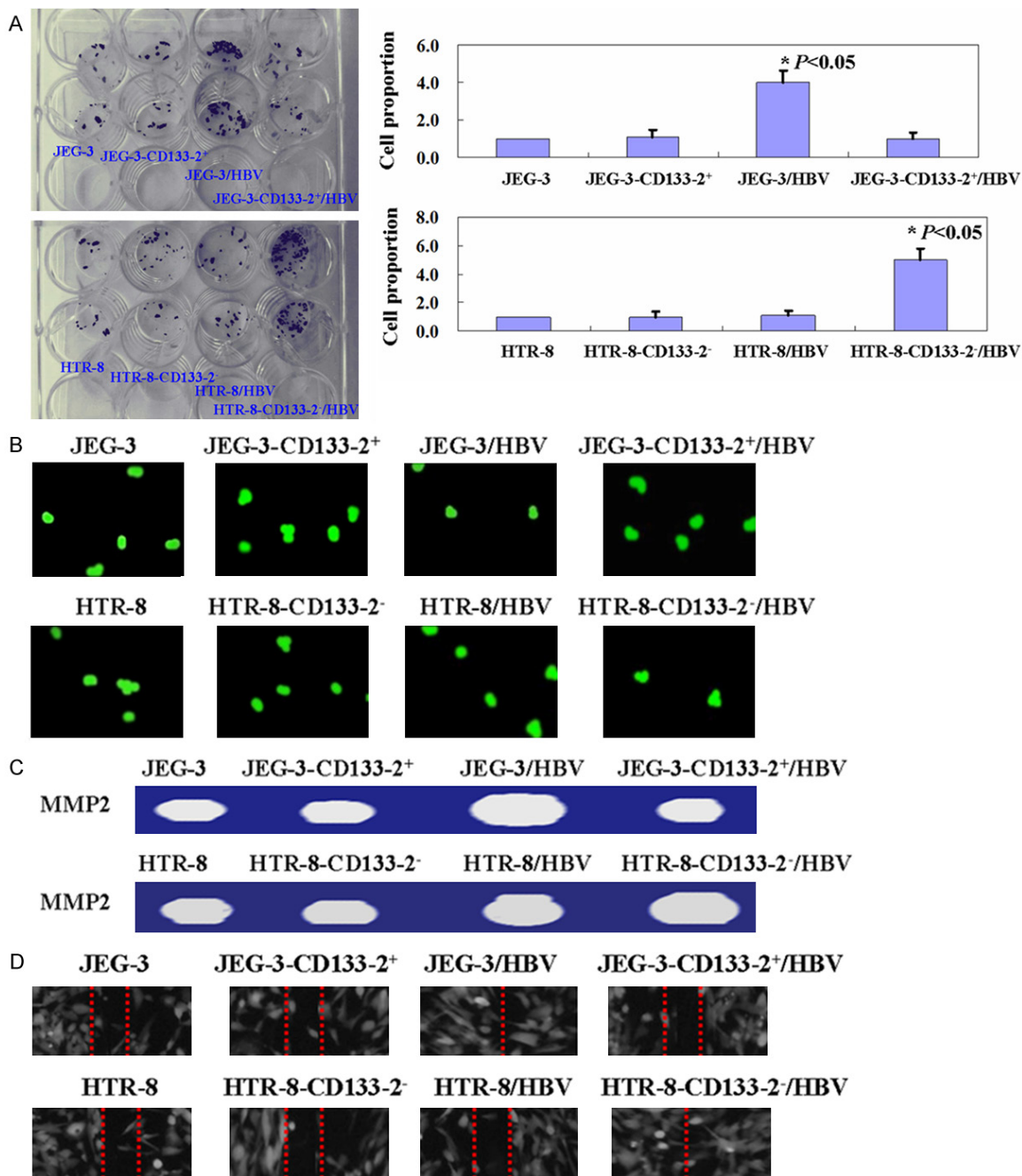
Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data comparisons in relation to control were performed by one-way ANOVA. Data were expressed as mean ± standard deviation of three independent experiments performed in triplicate. Differences were considered

statistically significant if a *P* value < 0.05 was achieved.

#### Results

##### The mRNA and protein level of CD133-2 and HBsAg in HTR-8/SVneo and JEG-3 cell lines

The results of western blot and RT-PCR showed that a high level of CD133-2 protein and mRNA in HTR-8/SVneo cells, but a low level in JEG-3 cells (**Figure 1**). HTR-8/SVneo cells transfected with CD133-specific siRNA and JEG-3 cells with CD133-2/pcDNA3.1 plasmid showed the levels of CD133-2 were reversed in these cells (**Figure 1**). These results showed that the transfection was successful. Then, HTR-8/SVneo cell and its CD133-2 negative clone (HTR-8-CD133-2), and JEG-3 cell and its CD133-2 positive clone (JEG-3-CD133-2<sup>+</sup>) were infected with HBV. We found that the levels of HBsAg mRNA and protein were increased significantly in infected HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells (**Figure 1**). However, slightly increased HBsAg mRNA and protein levels were observed in HTR-8/



**Figure 2.** The effects of HBV infection on HTR-8/SVneo, HTR-8-CD133-2-, JEG-3 and JEG-3-CD133-2<sup>+</sup> cells. A. The proliferation ratio of cells was determined by colony formation assay. B. Apoptotic ratio of cells was analyzed by using TUNEL assay. C. Gelatin zymography was used to detect the activity of MMP2. D. Wound healing assay was performed to detect the mobility of cells.

SVneo cells and JEG-3-CD133-2<sup>+</sup> cells (Figure 1).

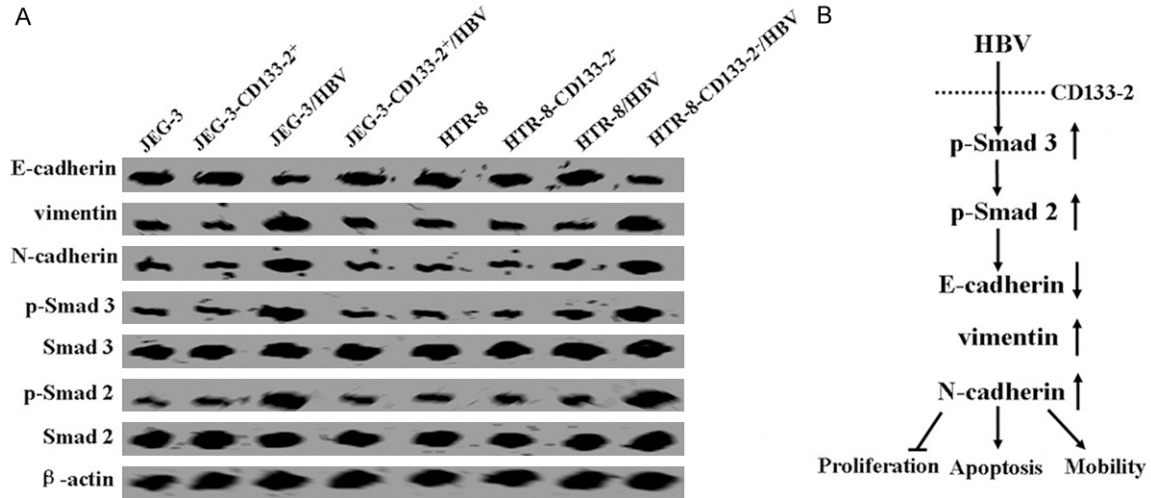
The effects of HBV on HTR-8/SVneo and HTR-8-CD133-2-, and JEG-3 and JEG-3-CD133-2<sup>+</sup> cells

Cell viability was monitored by using colony formation assay, and Figure 2A showed the prolif-

eration rates of HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells were induced by HBV infection ( $P < 0.05$ ). Then, the apoptotic cells were detected by using TUNEL staining. The apoptotic number of HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells after infected with HBV was decreased significantly (Figure 2B). And we found the activity of MMP-2 was induced by HBV in HTR-8-CD133-2<sup>-</sup> cells



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**Figure 3.** Western blot analysis of the Smad signaling pathway. A. Smad2, Smad3 p-Smad2, p-Smad3, E-cadherin, N-cadherin and vimentin were detected by using western blot. B. A proposed model for signaling pathways of CD133 mediated HBV infection.

and JEG-3 cells (**Figure 2C**). Motility was significantly increased in HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells with HBV infection by using wound-healing assay (**Figure 2D**). However, no significant changes of proliferation, apoptosis and motility were found in HTR-8/SVneo cells and JEG-3-CD133-2<sup>+</sup> cells (**Figure 2**).

*HBV activated the Smad signaling pathway and induced epithelial-mesenchymal transitions (EMT) in HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells*

Furthermore, we found that a lower level of E-cadherin, while higher levels of vimentin and N-cadherin in HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells after HBV infection (**Figure 3A**). Compared with uninfected cells and HBV infected HTR-8/SVneo cells or JEG-3-CD133-2<sup>+</sup> cells, HBV infected HTR-8-CD133-2<sup>-</sup> cells and JEG-3 cells showed higher levels of phospho-Smad3 and phospho-Smad2, while the total of Smad3 and Smad2 were no changes (**Figure 3A**). No significant changes of these proteins were found in HTR-8/SVneo cells and JEG-3-CD133-2<sup>+</sup> cells with HBV infection (**Figure 3A**).

### Discussion

The maternal-fetal transmission of HBV is still one of the important causes of chronic HBV infection in China [1]. The question of viral entry is crucial, since the trophoblasts are not the target cell for HBV [10]. In this study, the mech-

anisms by which placental trophoblast combats HBV were elucidated. Consistent with the results of Pötgens et al. [8], we found that AC133-2, an intracellular protein, expressed in trophoblast cell line (HTR-8/SVneo), but not in choriocarcinoma cell line (JEG3). Interestingly, in this study, our main finding is that CD133-negative cells (HTR-8-CD133-2<sup>-</sup> and JEG-3) are prone to HBV infection. Based upon the literature reports [11-13], the apoptotic ratio of both HTR-8/SVneo and JEG-3 cells that infected with HBV was decreased. We also found that the proliferation and motility were higher in CD133-negative cells with HBV infection than those of CD133-positive ones with HBV infection. Yeh et al. [14] found that CD133 expression in hepatocellular carcinoma (HCC) was negatively associated with the presence of HBsAg. Both of these data indicated that CD133-expressing cells could resist HBV infection. However, no other reports showed the roles of CD133 in HBV infection.

Moreover, we detected the molecular mechanisms of CD133 mediated reactions to HBV infection. Unfortunately, we did not find the deeper mechanism. Our findings in this study were no more than previous studies [14, 15]. Examination of the Smad signaling pathway showed that both Smad2 and Smad3 were phosphorylated after HBV infection. We also confirmed that HBV infection increased the motility of cells is involved in Epithelial-Me-

senchymal Transition (EMT) which is important in the progression of human carcinomas [16].

In summary, our study demonstrated that CD133 is a key factor that mediated HBV infection. The up-regulation of CD133 could decrease HBV infection. Moreover, our data indicated that the activation of Smad signaling pathway and the induction of EMT in both trophoblast cell and choriocarcinoma cell with HBV infection (**Figure 3B**).

**Disclosure of conflict of interest**

None.

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