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Knockdown of CD44 inhibits proliferation, migration, and invasiveness in hepatocellular carcinoma cells by modulating CXCR4/Wnt/β-Catenin Axis

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Hepatocellular carcinoma (HCC) has high mortality and incidence worldwide. The molecular mechanism associated with HCC is largely unexplored. Objective: To investigate the impact of CD44 knock-down on the proliferation, migration, and invasiveness in HCC cells. Methods: Colony formation and MTT assay were used to observe cellular proliferation and viability. In addition, cellular invasion and migration were studied by Transwell and wound healing assays respectively. Finally, western blotting was utilized to check the protein expression levels. Results: The cellular proliferation, invasion and metastasis in Huh7 cells were inhibited after the silencing of CD44. Furthermore, expression levels of MMP-2, MMP-9, CXCR4, GSK-3β and β-catenin was significantly decreased. However, opposite results were demonstrated when CD44 was overexpressed. Conclusions: Interference with the expression of CD44 significantly inhibits the invasion and metastasis in the HCC cell line, Huh7. Furthermore, CD44 was found to regulate the expression of MMP-2, MMP-9, CXCL12, CXCR4 and Wnt/β-catenin signal pathway.

Key words: CD44, Hepatocellular carcinoma, invasion, metastasis, Wnt/β-catenin

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Abbreviations: CSCs, Cancer stem cells; DMSO, Dimethyl sulfoxide; ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; EpCAM, epithelial adhesion molecule; HCC, hepatocellular carcinoma; MMPs, matrix metalloproteinases; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide); PVDF, polyvinylidene fluoride

INTRODUCTION

In the primary liver cancer class, HCC is the first one in frequency and accounts for 80–90% of all malignant tumors (Davis *et al*., 2008; Ghouri *et al*., 2017). Worldwide, HCC has emerged as a major health issue increasing continuously due to its association with viruses like hepatitis B and C (El-Serag, 2012). The world trends are unevenly distributed, finding the highest incidence in eastern Asia (McGlynn *et al*., 2015). Incidence ratio of HCC varies among sex, and the reasons for such is still unknown (Wilson and Buetow, 2020). Concerning age distribution, it also varies depending on the geographic situation (Sung *et al*., 2021). HCC is mostly found in the late stage when radiotherapy, chemotherapy and other treatments are ineffective. However, surgery in the early stage is currently the most effective treatment. Consequently, it has become one of the research focuses on exploring the detailed mechanism of metastasis and malignancy to explore novel treatment options for HCC.

The CD44 antigen, as an important epigenomic regulator, is involved in tumor development (Luo & Tan, 2016; Asai *et al*., 2019). Numerous studies have confirmed that CD44 can be used as a molecular marker for different cancer (Malhotra *et al*., 2010; Moldovan *et al*., 2017). Zhang and colleagues demonstrated that CD44 could promote HCC progression by up-regulating YAP (Zhang *et al*., 2021). Shah and colleagues found that interfering with CD44 could lead to the death of ovarian cancer cells (Shah *et al*., 2013). It has been found that up-regulation of CD44 can promote metastasis and poor prognosis of Hepatocellular carcinoma, however, the mechanism by which CD44 regulates HCC is unclear (Asai *et al*., 2019). Epithelial to mesenchymal transition (EMT) in cancer cells results in the acquisition of stem cell-like characteristics and increased CD44 expression (Mani *et al*., 2008). Because of the clinicopathological effects that CD44 and its isoforms have on carcinogenesis, CD44 may one day serve as a molecular target for cancer treatment (Li *et al*., 2014). Additionally, the demonstrated function of CD44 in preserving stemness and the ability of cancer stem cells to regenerate tumors after treatment raises the possibility that CD44 may play a significant prognostic marker. Clinical research on treatment plans that concentrate on CD44 or lessen CD44 expression is ongoing (Matzke-Ogi *et al*., 2016; Todaro *et al*., 2014). These methods include ectodomain mimics, aptamers, tumor-delivery shRNAs, and CD44 neutralizing antibodies (Orian-Rousseau and Ponta, 2015; Iida *et al*., 2014). Consequently, it is crucial to further clarify the functional roles of CD44 as a focus of research.

Growing data indicates that cancer stem cells (CSCs) are responsible for the recurrence and metastasis of many malignancies (Vlashi *et al*., 2011; Gao *et al*., 2013). CSCs are essential for starting and maintaining tumour phenotypes because they can self-renewal and differentiation, which other cancer cells (non-CSCs) lack (Ayob & Ramasamy, 2018). The presence of CSCs in numerous malignancies, including those of the brain, breast, lung, colon, and liver, has been demonstrated utilizing particular CSC markers (Yang *et al*., 2020). Epithelial adhesion molecule (EpCAM), CD13, CD44, and/or CD133 are among the markers that liver CSCs display and studies

have shown that the expression of these molecules on HCC cells is associated with a poor prognosis (Yamashita *et al*., 2009; Zhu *et al*., 2010). Although traditional therapies could eradicate non-CSCs, it is claimed that surviving CSCs eventually induce tumour recurrence and metastasis because they exhibit the characteristics of tumorigenicity and resistance to conventional chemotherapy and radiotherapy (Cross & Laidler, 1990; Gao *et al*., 2013). Therefore, eliminating CSCs is crucial for fully curing cancer.

Tumor invasion and metastasis is a complex biological process (Nguyen *et al*., 2009a). Gene regulation is crucial in different processes, like unlimited growth potential, epithelial-mesenchymal transition (EMT), and apoptosis avoidance (Perlikos *et al*., 2013). Wnt signaling is an essential pathway affecting tumor cells' cellular migration and invasion ability. The Wnt/β-catenin signaling system is a conserved signalling axis involved various physiological processes, including tissue homeostasis, migration, invasion, differentiation, proliferation, and apoptosis (Salik *et al*., 2020). There is mounting evidence that certain solid tumors and hematological malignancies were aided in their development and progression by deregulation of the Wnt/ß-catenin cascade (Gajos-Michniewicz & Czyz, 2020). Early events in carcinogenesis are brought on by aberrant regulation of the transcription factor ß-catenin, a crucial part of the Wnt signaling pathway, in the Wnt/ß-catenin pathway (Zhang *et al*., 2020). GSK3ß and CK1a, two enzymes in the degradation complex, facilitate the phosphorylation of ß-catenin, boosting its ubiquitination and subsequent proteasomal destruction (Wiese *et al*., 2018). When β-catenin is accumulated at a certain amount, it gets translocated to nucleus, and binds to the target transcription factor to form a transcriptional complex. This complex subsequently activates its downstream target genes matrix metalloproteinases (MMPs), p21, and C-myc (Wiese *et al*., 2018; Tai *et al*., 2015). MMPs are a group of proteolytic enzymes which are highly homologous and zinc-dependent. The extracellular matrix (ECM) holds cells together and is essential for cell survival, motility, differentiation, and proliferation. The ECM components that serve as the physical impediments to cell migration must be locally broken down for a tumour cell to spread from the main tumour to other organs. Matrix metalloproteinases (MMPs) are the primary enzymes responsible for the breakdown of the ECM (Conlon & Murray, 2019).

Chemokine (CXCL12) and its receptor (CXCR4) have emerged as key factors in the development of tumors and their metastasis. CXCL12 has been reported to induce signaling *via* AKT and ERK pathways and thereby induce cancerous growth (Scotton *et al*., 2002). In breast cancer, CXCL12 expression has been linked with pathological features and clinical outcomes (Kang *et al*., 2005). The expression levels of CXCL12 have been reported on the higher side in different human cancers, including HCC (Sakai *et al*., 2012) (Ghanem *et al*., 2014; Teng *et al*., 2016). The essential role of CXCL12 is yet to be fully explored in most cancers. The involvement of the CXCL12/CXCR4 axis in tumor progression, survival, metastasis and angiogenesis is well known. The current investigation aims to study the effect of CD44 on proliferation, migration, and invasiveness in HCC cells for CXCL12/CXCR4/Wnt/β-Catenin Axis.

MATERIALS AND METHODS

Cell culture and cell transfection

The human hepatocellular carcinoma (HCC) cell line, Huh7 was purchased from ATCC. Huh7 were grown in DMEM containing 10% FBS (Sigma). The medium was put in a saturated humidity incubator at 37°C with 5% CO_2. SiRNA (Si-CD44) was obtained from Ruibo Biotechnology Co., Ltd (Guangzhou, China). Over-expressing plasmid pcDNA3.1-CD44 (CD44) along with control vector (Vector) was purchased from General Biol (Anhui, China). Huh7 cells were grown in 6-well plates and divided into six groups, namely: blank group (Blank), Si-CD44 group, Si-NC, CD44 and Vector. Lipofectamine was used for the transfection of different vectors into the cells.

MTT assay

Cell viability in each group was observed MTT assay (Gibco, USA). Huh7 cells were grown into 96-well plates (6×10^3 cells/well) for 48 h. It was followed by transfection studies using siRNAs (Si-CD44 and si-NC) and vectors (empty vector and vector-CD44) in the Huh7 cells, using lipofectamine and in accordance with the manufacturer's protocol. The efficacy of transfection was checked by western blotting. After incubation, the medium was removed from the wells, followed by the addition of 20 μl MTT reagent (5 mg/ml; Gibco, USA) to each well. At the end of the experiment, MTT (Sigma) stock solution of 5 mg/mL concentration and volume 100 μL was supplemented to cells with 4 h of incubation. The formazan crystals then produced are dissolved with DMSO, and thereafter, absorbance was measured at 540nm using a microplate reader. Each experiment for individual drug concentrations and controls was performed thrice.

Colony formation assay

Cell viability was observed via colony formation assay. Cells were rinsed twice with PBS. Afterward, individual cell in each group was obtained with 0.25% trypsin and then inoculated into culture dishes for one hour. Serially dilute the samples to obtain 100 cells in a 10 mL culture medium. At last, the cells were inoculated into other culture dishes for 10–14 days, followed by an observation of the cell colony formation under a microscope.

Transwell assay

The anti-invasive and anti-migratory effects of each group were monitored *via* transwell chambers assay. The upper chambers of the transwell were loaded with 600 μL of DMEM medium and 3×10^4 Huh7 cells (transfected or un-transfected) each well. In the transwell's lower chambers, only cultural medium of 800 μL with FBS 10% was filled. Cells in each group were cultured in upper chambers for 24 h at 37°C. Then clean off the non-migrated cells and the migrated cells were processed routinely by 10 minutes of fixation with formalin 4%. Afterward staining was accomplished with crystal violet (0.1%) for 12 minutes, followed by photographing the randomly selected 5 fields using microscopy with 100× magnification. Finally, the invasion was determined, except transwell chambers were coated with Matrigel.

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
CD44	5′-ACTTGGAGGCCTTGGCTAAC-3′	5′-GACAGACAGACTGCGACCTG-3′
GAPDH	5′-TGTGTCCGTCGTGGATCTGA-3′	5′-TTGCTGTTGAAGTCGCAGGAG-3′

Wound healing assay

Huh7 cells were cultured in 12-well plate overnight for wound healing assay purposes. With the help of a 10μl tip, a scratch was created on the monolayer cells. After scratching, cells were washed with culture media to remove floating cells. Cells were kept untreated or either transfected with different vectors. The transfection of siRNAs (Si-CD44 and si-NC) and vectors (empty vector and vectore-CD44) was performed in the Huh7 cells, using lipofectamine and according to the manufacturer's protocol. The efficacy of transfection was checked by western blotting. Images of fresh scratch were captured immediately with the help of a digital camera. After treatment completion, cells were washed thrice with culture media and followed by capturing pictures of the scratch. The scratch area was determined using Image-Pro software. Cell migration was determined by calculating the scratch closure.

RT-qPCR assay

RNA from Huh7 cells was obtained using TRIzol method and then reversed to cDNA with RT Kit. Quantitative PCR was carried out using SYBRGreen (Takara) with appropriate primers designed by Primer 5.0 (Table 1) according to the conditions, including an initial step of 10 minute in 95°C, and then 40 cycles of amplification, which includes 10 s in 95°C, 20 s in 58°C and 25 s in 72°C. Quantification was determined by $2^{-\Delta\Delta CT}$ (26). The internal control used was GAPDH.

Western Blot

Total cellular protein from each group was extracted, followed by protein concentration determination using a BCA protein quantification kit (Pierce, 23225). From each sample, 45 μg of proteins were loaded and run on SDS-PAGE gels, which were processed for blotting to PVDF membranes. Blocking PVDF membrane with 5% non-fat dry milk was done at room temperature for 2 h. It was followed by overnight incubation with primary antibodies like anti-MMP-9 (abcam, ab58803, 1:1000) anti-MMP-2 (Santa Cruz, sc-13594, 1:800), anti-β-catenin (Sigma Aldrich, C7207. 1:1000), anti-GSK-3β (Santa Cruz, sc-81462, 1:1000), anti-CXCR4 (abcam, ab124824, 1:800) and anti-GAPDH (Cell Signaling Technology, 5174, 1:1000). Next day, after washing with PBS thrice, membranes were incubated with HRP-linked secondary antibodies (Cell Signaling Technology, 7074 and 7076, 1:3000) for 90 minutes at room temperature. Finally, ECL chromogenic substrate was added for color reaction.

Statistical analysis

The experimental data were expressed as mean ± standard deviation (S.D.), and SPSS 21.0 software was utilized for statistical analysis. In addition, *t*-test and ANOVA were used for comparison between groups. Each experiment was repeated thrice. $P<0.05$ was considered that the results were statistically significant.

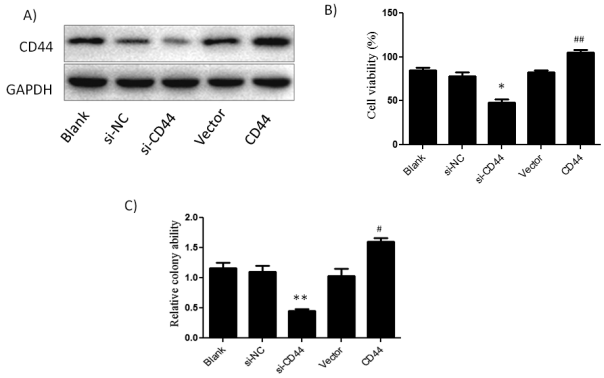

Figure 1. Transfection efficacy (A) and Effect of CD44 expressions on proliferation and cell viability of Huh7 cells (B, C).
Cell viability of Huh7 cells was detected by MTT while as Cell viability was detected by colony formation assay. **P*<0.05 and ***P*<0.01 *vs.* si-NC group; #*P*<0.05 and ##*P*<0.01: *vs.* Vector group.

RESULTS

Inhibition of CD44 inhibits proliferation and cell viability

Knockdown of CD44 can inhibit its expression in Huh7 cells, while overexpression can reverse the result (Fig. 1A). The results of immunoblotting showed that CD44 is expressed in Huh7 cells. The expression level of CD44 significantly decreased in the Si-CD44 group compared to the Si-NC group. However, the expression level of CD44 increased significantly in the CD44 group compared to the vector group.

MTT assay showed that the proliferation ability of Huh7 cells were significantly reduced in the si-CD44 group compared to the si-NC group (Fig. 1B). On the contrary, the proliferation ability of Huh7 cells increased significantly in the CD44 group compared to the vector group. Furthermore, knockout of CD44 significantly in-

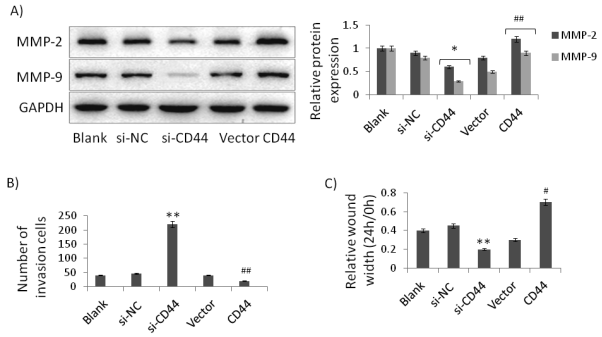

Figure 2. Effect of CD44 expression on invasion and metastasis of hepatocellular carcinoma cells.
(**A**) The expression of MMP-2 and MMP-9 in Huh7 cells detected by western blot. (**B**) Effect of CD44 expression on invasive ability of Huh7 cells detected by Transwell assay. (**C**) Effect of CD44 expression on migration ability of Huh7 cells detected by wound healing assay. **P*<0.05 and ***P*<0.01 *vs.* si-NC group; #*P*<0.05 and ##*P*<0.01: *vs.* Vector group.

hibited cell viability of Huh7 cells, while overexpression of CD44 significantly motivates it (Fig. 1C). The results showed that CD44 was involved in the proliferation and cell viability of hepatocellular carcinoma cells.

Inhibition of CD44 can inhibit invasion and metastasis

Further, we studied the role of CD44 on cellular invasion and metastasis in Huh7 cells. Transwell and wound healing assay were used to evaluate the invasive and migration ability of Huh7 cells. The results (Fig. 2A–B) suggested that the invasion and metastasis of Huh7 cells decreased significantly after silencing CD44 compared to si-NC group. Instead, cellular invasion and metastasis were significantly increased after overexpressing CD44 compared to the vector group ($P<0.05$). To investigate the possible mechanism through which CD44 regulates functional changes in Huh7 cells, MMP-2 and MMP-9 expressions were examined (Fig. 2C). The expression level of MMP-2 and MMP-9 was significantly increased after CD44 silencing and a reverse trend was observed after overexpressing CD44.

Inhibition of CD44 can inhibit the expression levels of CXCR4/CXCL12 proteins

The effect of CD44 expression on CXCR4 proteins associated with induction of cancer growth was detected. Western blot indicated (Fig. 3) that the expression levels of CXCR4 were significantly decreased in the si-CD44 group, and opposite results were observed in the CD44 group compared to the control group. These results confirmed that the activation of CXCR4 signal pathway was inhibited after silencing CD44.

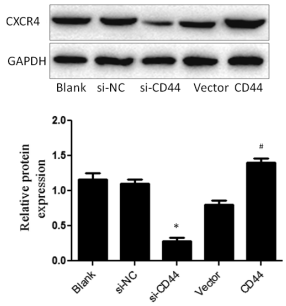

Figure 3. Effect of CD44 expression on the expression levels of CXCR4.
The expression of CXCR4 in Huh7 cells was detected by western blot. *$P<0.05$ *vs.* si-NC group; #$P<0.05$: *vs.* Vector group.

Inhibition of CD44 inhibits the expression levels of Wnt/β-catenin signal proteins

The expression of β-catenin decreased significantly in the si-CD44 group compared to the si-NC group. On the other hand, GSK-3β expression increased significantly in the si-CD44 group compared to the si-NC group (Fig. 4). Thus confirming an association between Wnt/β-catenin pathway activation and CD44.

DISCUSSION

Wnt signal is divided into the typical Wnt pathway and two atypical Wnt pathways (Reya & Clevers, 2005). The typical Wnt signal pathway is currently the most widely studied in clinical practice. Studies have demonstrated that nearly 50% of currently known tumors show an association with abnormal Wnt/β-catenin signal pathways, such as intestinal cancer (Barker *et al.*, 2009), breast cancer (Shackleton *et al.*, 2006; Teissedre *et al.*, 2009), etc. Abnormal expression of proteins such as GSK-3β (Cho *et al.*, 2010), β-catenin (Clements *et al.*, 2002), and MMPs (Conlon & Murray, 2019) in the pathway triggers sustained cell proliferation, ultimately leading to cancer (MacDonald *et al.*, 2009). Meanwhile, it performs a crucial role in cellular invasion and metastasis (Nguyen *et al.*, 2009b; Stein *et al.*, 2006). Therefore, the present study investigated whether CD44 could mediate invasion and metastasis in HCC cells by regulating the Wnt/β-catenin signal pathway.

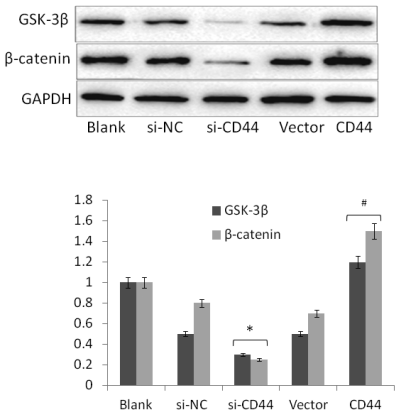

Figure 4. Effect of CD44 expression on Wnt/β-catenin signal pathway.
The expression of GSK-3β and β-catenin in Huh7 cells was detected by western blot. *$P<0.05$ *vs.* si-NC group; #$P<0.05$: *vs.* Vector group.

In the present study, cellular proliferation and invasion (Huh7 cells) reduced after silencing of CD44. However, the proliferative and invasive capacity of cells increased after overexpressing CD44 and thus confirmed that CD44 is involved in the progression of HCC. In addition, protein levels of MMP-2, MMP-9, and β-catenin were decreased; the expression of GSK-3β was increased after CD44 silencing in Huh7 cells. However, the opposite results were presented after over-expression of CD44. These findings suggest that down-regulation of CD44 inhibits the Wnt/β-catenin signal pathway and gradually inhibit invasion and metastasis of Huh7 cells.

The activated Wnt pathway stimulates CXCL12 release, a key paracrine molecule that controls different biological processes like cellular activation and migration, influences inflammation, and angiogenesis (Giordano *et al.*, 2019; Meng *et al.*, 2018). The earlier connection between CXCL12 expression and the Wnt/-catenin pathway has been reported in fibrosis, particularly in liver fibrosis (Akcora *et al.*, 2018). But no study involving CD44/CXCL12/Wnt/β-Catenin Axis has been reported to date. To support our current findings, additional in vivo research is necessary. Additionally, this study was conducted using a hepatocellular carcinoma cell line, which does not reflect a real-world scenario. However, this study offered the first proof-of-concept data indicating CD44/Wnt/CXCL12 signaling axis in hepatocellular carcinoma cells.

CONCLUSION

In summary, by silencing CD44 expression, invasion and metastasis of HCC cells could be inhibited. This result could possibly be obtained by mediating the Wnt/β-

catenin signal pathway. This provides the more comprehensive role of CD44 as a therapeutic target in patients with HCC.

Declarations

Author Contributions. XS, FD and WL contributed to the study conception and design. Data collection and analysis were performed by JT and KY, while analysis and interpretation of the results were performed by QL and ZZ. The manuscript was written by XS. FD, WL, JT, KY, QL and ZZ read, interpreted and revised the manuscript critically for important intellectual content. All authors approved the final manuscript.

Conflict of interest. No conflict of interest is associated with this work.

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