CD44v6 promotes β-catenin and TGF-β expression, inducing aggression in ovarian cancer cells

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Abstract. A high expression of CD44v6 has been reported in numerous malignant cancers, including stomach, prostate, lung and colon. However, the pathological role and the regulatory mechanisms of CD44v6 have yet to be elucidated. In the present study, the expression levels of CD44v6 were shown to be significantly higher in ovarian cancer tissues, as compared with adjacent normal tissues. Furthermore, the upregulated expression levels of CD44v6 were correlated with disease recurrence and poor survival in patients. The expression of CD44v6 was knocked down in the CAOV3 ovarian cell line, by transfection of a specific small hairpin RNA. The present study showed a correlation between the aggression, viability, invasion and migration of the ovarian cancer cells, with the expression of CD44v6. In addition, the expression of CD44v6 was positively correlated with the expression levels of β-catenin and tumor growth factor-β, which indicates that the effects of CD44v6 on ovarian cancer cell aggression may be mediated by these two signaling pathways. In conclusion, the present study provides a novel insight into the association between CD44v6 expression and ovarian cancer. CD44v6 may provide a novel target for the prognosis and treatment of ovarian cancer.

Introduction

Ovarian cancer is one of the most common female cancers and is associated with a high mortality rate, due to its high malignancy and the difficulties associated with its treatment. It remains to be elucidated how ovarian cancer metastasizes and regulates migration, survival, and growth (1). Previous studies have reported that cancer stem cells may participate in cancer development (2). Furthermore, it has been identified that several genes may be indicators of cancer stem cells, and regulate tumor propagation and invasion (3).

CD44 has been identified as an indicator of cancer stem cells. It is a widely expressed protein that binds hyaluronic acid, resulting in cell-cell and cell-matrix adhesion (4,5). This function of CD44 may provide information regarding its association with tumor progression. Previous studies have suggested that numerous tumor types and cancer stem cells express CD44 (6,7). Notably, CD44 transcripts have complex alternative splice forms that contribute to different isoforms and are associated with different functions (8,9). In the intestine, CD44v has been shown to induce gut adenoma differentiation, whereas CD44s showed no such function (10). The expression of the CD44s isoform has been widely studied; however, the functions of other isoforms, including CD44v in tumor progression, remain unknown. Among all of the CD44v isoforms, it has previously been shown that CD44v6 may bind hepatocyte growth factor (HGF), osteopontin and cytokines, which are key factors of the tumor microenvironment (11). In addition, CD44v6 has a central role in cell migration and invasion. Orian-Rousseau *et al* (12,13) showed that CD44v6 mediated the MET and HGF signaling pathway. CD44v6 has also been identified as a downstream factor of Wnt signaling, that may stimulate the β-catenin/Tcf-4 signaling pathway (14,15). Todaro *et al* (16) showed that CD44v6 is a marker of constitutive and reprogrammed cancer stem cells associated with colon cancer metastasis. These studies suggest that CD44v6 may be a marker for predicting cancer metastasis. However, whether the mechanism of CD44v6 is universal in different tumor types remains to be determined.

Transforming growth factor (TGF)-β is a factor that is expressed in numerous developmental pathways, and has been shown to have key roles in apoptosis, the cell cycle and the immune system (17). Inhibition of TGF-β expression may be a prospective method for cancer therapy, since TGF-β stimulation has been shown to induce tumorigenesis and metastasis (18). Therefore, understanding the regulation of the TGF-β signaling pathways may provide novel ways for the treatment of malignant tumors. Previously, TGF-β has

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been considered to be a protein which promotes carcinogenic progression. Simultaneously, Wnt/β-catenin, a signaling pathway upstream of CD44v6, has also been shown to participate in tumor progression (19). Inhibition of the Wnt/β-catenin pathway may stimulate tumorigenesis and angiogenesis. This may be due to the stabilization of β-catenin by Wnt, which results in the activation of TCF/LEF family transcription factors (20). Determining the effects of CD44v6 on TGF-β and Wnt/β-catenin may provide further understanding of the mechanism, and give indicators of therapeutic targets, in ovarian cancer.

In the present study, it was hypothesized that an abnormal CD44v6 expression may result in ovarian cancer aggression. By transfection of a small hairpin (sh)RNA specifically targeting CD44v6 expression, it was demonstrated that CD44v6 promoted β-catenin and TGF-β expression and induced aggression in ovarian cancer cells. These results may provide a potential therapeutic target for ovarian cancer.

Materials and methods

Materials. The clinical samples used in the present study were supplied by the Xiangya School of Medicine, Central South University (Changsha, China). The samples were obtained during surgery and the characteristics of the patients are detailed in Table I. Informed consent was provided and approval was obtained from the Institutional Review Board of the Xiangya School of Medicine Research Ethics Committee (Changsha, China). The CAOV3 ovarian cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA).

Rabbit antibodies against human CD44v6 (HPA005785), β-Catenin (C2206), TGF-β (SAB4502954) and β-actin (AV40173) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The secondary antibodies, conjugated with horseradish peroxidase, against rabbit Immunoglobulin G (sc-2030) were purchased from Santa Cruz Biotechnology Inc., (Dallas, TX, USA).

A CD44v6 interference vector was constructed to analyze and compare the biophysical properties of knockdown and over-expression of CD44v6, in ovarian cancer cells. The recombinant expression CD44v6 shRNA plasmid was purchased from Santa Cruz Biotechnology Inc. (sc-62576-SH). The recombinant expression plasmid expressing CD44v6 was then constructed. The CD44v6 fragments were inserted into the plasmid pcDNA3.1(t) (Invitrogen Life Technologies, Carlsbad, CA, USA) between the *Xho*I and *Bam*HI restriction sites, and the recombinant plasmids pc3.1(t)-CD44v6 were constructed, according to the manufacturer's instructions. The cells were transfected with pcDNA3.1(t)-CD44v6 and/or CD44v6 shRNA plasmid using Lipofectamine® 2000 (Invitrogen Life Technologies). The cells were randomly divided into three groups (five parallel treatments for each group): Control, non-treated group; pcDNA3.1 control group, no CD44v6 fragment was inserted into the plasmid; and CD44v6 interference group, 1 μg CD44v6 shRNA plasmid transfection. Following a 24 h transfection, the cells were harvested and used for the following experiments.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the ovarian cancer and adjacent normal tissues using TRIzol® reagent (Invitrogen Life Technologies), according to the manufacturer's instructions, and stored at -80˚C until further use. A total of 1 μg RNA was reverse transcribed into first-strand cDNA using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). The qPCR was performed using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Grand Island, NY, USA) in a 50 ml reaction volume, containing iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA), 100 nM primers and 20 ng cDNA template. The parameters of the PCR reactions were as follows: 94˚C for 3 min for one cycle, then 94˚C for 30 sec, 60˚C for 30 sec, 72˚C for 45 sec for 40 cycles, and 72˚C for 5 min for one cycle. Following the amplification, the PCR products were assayed using a dissociation curve, to verify single product generation. The relative gene expression was calculated with the SDS 1.3 software on the Applied Biosystems 7500 Real-Time PCR System, using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$).

Western blot analysis. Following incubation, the cells of each group were collected by centrifugation at 15,000 rpm for 15 min and then lysed in radioimmunoprecipitation assay buffer on ice. The extracted protein samples were loaded onto 12% SDS-PAGE gels, separated by electrophoresis and then transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). The membranes were blocked with 5% skimmed milk for 30 min, and then incubated with the primary antibodies overnight, at 4˚C. The membranes were washed with phosphate-buffered saline three times and then incubated with the horseradish peroxidase conjugated secondary antibody for 1 h, at room temperature. The protein bands were detected using the chemiluminescence system SuperSignal West Pico Chemiluminescent Substrate (GE Healthcare Life Sciences, Piscataway, NJ, USA). Three independent experiments were repeated to assess the relative protein levels.

Cell invasion assay. A fluorescence Transwell assay was used to determine cell invasion. The cells were inserted into an 8 μm pore-size PET membrane (GE Healthcare Life Sciences), and cultured at 4˚C overnight. The cells were then harvested and labeled with the fluorescent dye Calcein AM (GE Healthcare Life Sciences). The results were measured using a Beckman DU-8 UV-spectrophotometer (Eppendorf, Hamburg, Germany), at a wavelength of 494 nm/517 nm.

Migration assay. The number of BrdU-positive cells per 1 mm length was examined and used to define the cell density. The initial dissector frame was randomly positioned in the cell and the mean of 10 areas was recorded as the number of migrated cells.

Statistical analysis. The data is expressed as the means ± standard error of the mean. To determine differences between the groups, the data were analyzed by a one-way analysis of variance. The normality and constant variance for experimental data were tested by the Levene's test. A Kaplan-Meier survival curves were produced to examine survival rates. Kaplan-Meiers was defined as the time from randomization to mortality from any cause. The data underwent logarithmic transformation to meet the necessary assumptions of analysis of variance, if the data did not have homogenous variance. Fisher's exact test was

Table I. Correlation between CD44v6 expression levels and clinicopathological factors of the 60 patients with ovarian cancer.

Characteristics	CD44v6 expression		
	Low (0,1)	High (2,3)	P-value[a]
Age			
Years, mean ± SD	59.23 ± 7.80	55.42 ± 10.30	0.75
Gender			
Male	14	14	0.53
Female	16	16	
Smoking status			
Yes	13	18	0.48
No	13	16	
Stage			
I + II	18	10	0.01
III + IV	7	25	
Tumor status			
T1-T2	15	18	0.01
T3-T4	15	12	
Lymph node metastasis			
N0	15	5	0.03
N1-N3	9	21	
Distal metastasis status			
M0	23	10	0.02
M1	11	16	
Recurrence status			
Yes	8	24	0.00
No	23	5	

[a]Statistical significance of the differences between the characteristics of the two groups, except for 'Histological type', was analyzed by a two-sided Fisher's exact test. The P-value for 'Histological type' was analyzed by χ^2 test. SD, standard deviation.

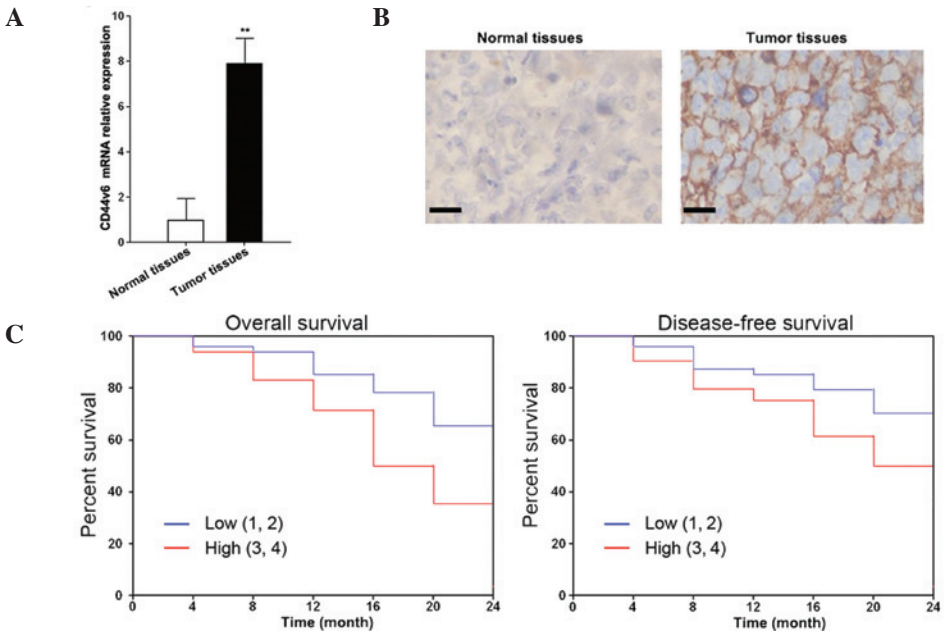

Figure 1. CD44v6 expression in ovarian cancer tissues and its correlation with survival rates. (A) Relative mRNA expression levels of CD44v6 ovarian cancer and adjacent normal tissues. The data represent the means ± standard error of the mean. *P<0.05. (B) Immunohistochemical analysis of CD44v6 expression in normal and ovarian cancer tissues. (C) Overall and disease-free survival rates in low (relative expression between 1 and 2) and high (relative expression between 3 and 4) expression samples.

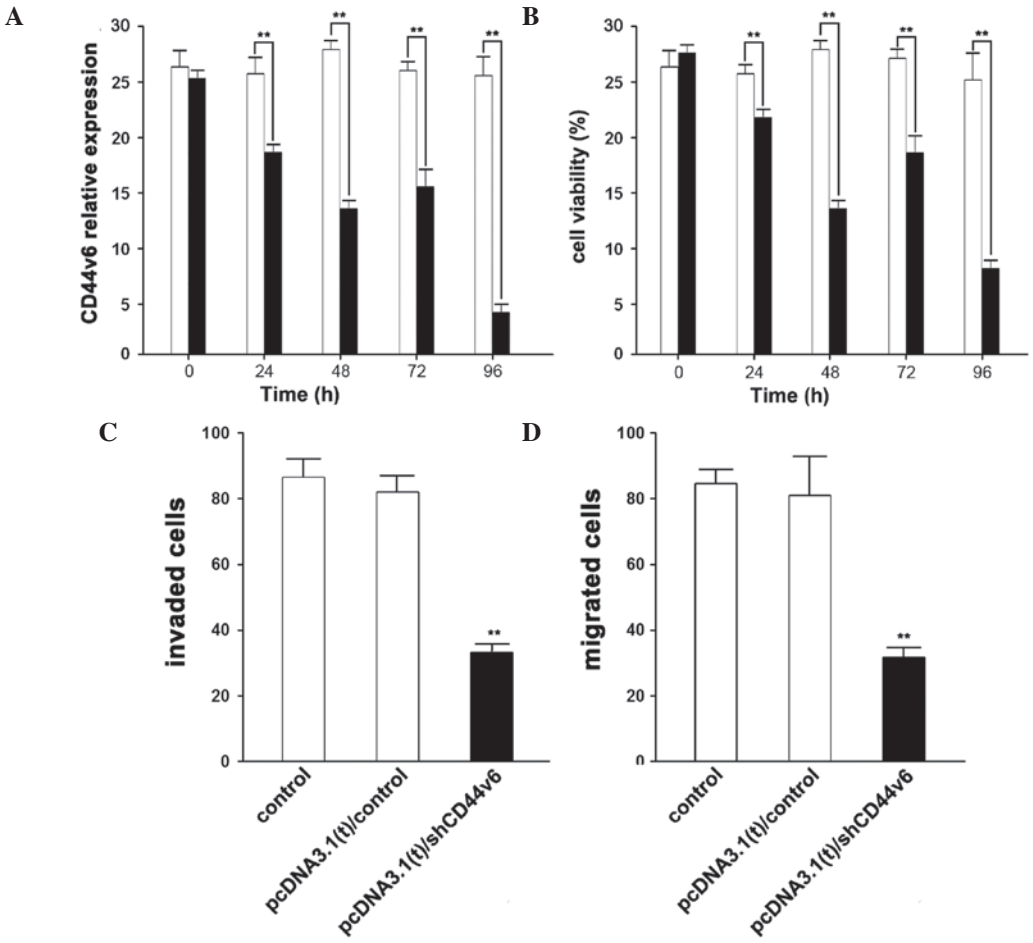

Figure 2. Cell biological activites following small hairpin (sh)CD44v6 vector transfection. (A) Relative CD44v6 mRNA expression levels at different time points, following shCD44v6 vector transfection. (B) Cell viability at different time points following shCD44v6 vector transfection. The percentage of (C) invaded cells and (D) migrated cells following shCD44v6 vector transfection. The data represent the means ± standard error of the mean. **Significant difference between the control and the cells transfected with the shCD44V6 vector (P<0.05). H, hour.

used to analyze data. The statistical tests were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). A P<0.05 was considered to indicate a statistically significant difference.

Results

The expression levels of CD44v6 are higher in ovarian cancer tissues. The relative mRNA expression levels of CD44v6 in ovarian cancer tissues were determined by qPCR. A total of 60 tumor samples and their respective adjacent normal tissues were tested. Out of the 60 tumor samples, 55 had significantly higher expression levels of CD44v6, as compared with the adjacent normal tissues (Fig. 1A). Furthermore, the upregulated expression levels of CD44v6 were correlated with metastasis and recurrence (Table I). The protein expression levels of CD44v6 were higher in the ovarian cancer tissues, as compared with the adjacent normal tissues (Fig. 1B). The Kaplan-Meier survival curves showed that CD44v6 expression was negatively correlated with overall and disease-free survival rates (Fig. 1C). The high expression CD44v6 samples, with relative expression levels between 3 and 4, had higher overall and disease-free survival rates, as compared with the low expression CD44v6 samples, with relative expression levels between 1 and 2.

Knockdown of CD44v6 expression in ovarian cancer cells. The higher expression levels of CD44v6 may induce ovarian cancer; therefore, the present study aimed to determine whether knockdown of CD44v6 expression had an effect on ovarian cancer progression. Following the knockdown of CD44v6 expression by shCD44v6 vector transfection, the expression levels of CD44v6 were significantly suppressed, in a time-dependent manner (Fig. 2A).

Simultaneously, following shCD44v6 vector transfection, the cell-growth and invasive abilities of CAOV3 cells were reduced(Fig. 2B,C,D). The cell viability decreased in a time-dependent manner, which is similar to the reduction in the expression levels of CD44v6. The cells were assayed 96 h post-transfection with the shCD44v6 vector, and the invasive and migratory abilities of the CAOV3 cells were shown to decrease significantly.

Depression of Wnt/β-catenin and TGF-β by CD44v6 expression knockdown in ovarian cancer cells. The β-catenin and TGF-β expression levels were also assayed 96 h post-transfection. The qPCR showed that the mRNA expression levels of both β-catenin and TGF-β were significantly decreased, following a knockdown of CD44v6 expression (Fig. 3A). In addition, the protein expression levels of β-catenin and TGF-β, following a

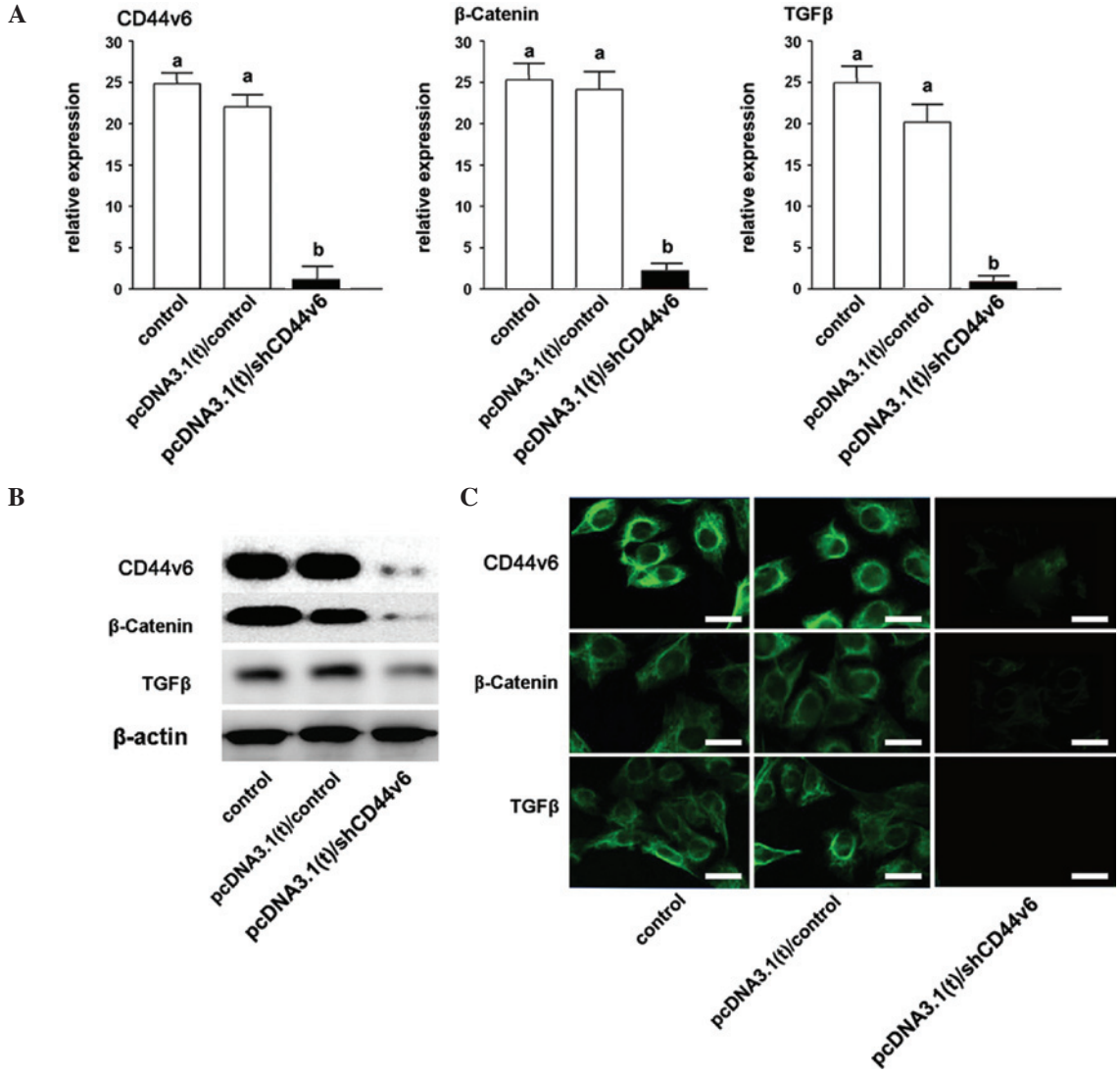

Figure 3. Correlation of β-catenin and transforming growth factor (TGF)-β expression in CAOV3 ovarian cancer cells. (A) Relative mRNA expression levels among the control, pcDNA 3.1(t)/control and pcDNA 3.1(t)/shCD44v6 groups. The data represent the means ± standard error of the mean. Different characters represent significant differences among the groups ($P<0.05$). Protein expression levels assayed by (B) western blotting and (C) immunofluorescence; scale bar 10 μm.

knockdown of CDv446 expression, were shown to be reduced, as determined by western blotting and immunofluorescence.

Discussion

The results of the present study suggest that the expression of CD44v6 is increased in ovarian cancer tissues obtained from patients, and the knockdown of CD44v6 expression may result in depression of tumor metastases and cell invasion. Previously, CD44v6 has been shown to be involved in the progression of numerous types of cancer, including stomach, prostate, lung and colon (21-26). In the present study, the CD44v6 expression levels were increased in ovarian cancer tissues, which indicates that it may have a potential effect on ovarian cancer development and aggression. In addition, the CD44v6 expression levels were correlated with overall and disease-free survival rates, suggesting that CD44v6 may be an indicator and a potential therapeutic target of ovarian cancer. Notably, other isoforms of CD44, including CD44s and CD44v3 have converse expression levels, as compared with CD44v6 (13,27). The aim of the present study was to determine how the expression of CD44v6 affects the progression and metastasis of ovarian cancer. Therefore the viability, invasion and migration of CAOV3 cells were determined, to understand the effects of CD44v6 on ovarian cancer cells.

Following a shCD44v6 vector transfection into CAOV3 cells, the CD44v6 expression levels were significantly inhibited. Furthermore, the metastasis of the CAOV3 cells was depressed. These results suggest that the knockdown of CD44v6 expression may affect ovarian cancer cell growth and metastatic ability. The invasive ability of CAOV3 also decreased significantly, following knockdown of CD44v6 expression. It has previously been reported that CD44v6 expression may promote the aggression of ovarian cancer cells. Shi *et al* (28) demonstrated that CD44v6, but not CD44s, had a higher expression in ovarian serous cancer, as compared with primary tumor tissues. Todaro *et al* (16) showed that colorectal cancer stem cells expressed CD44v6 and the expression of CD44v6 was necessary for the migration and metastasis of cancer stem cells. It was also indicated that CD44v6 expression levels were higher in cancer

stem cells, as compared with primary tumor cells. These results suggest that the metastatic process of ovarian and colorectal cancer cells may be induced by CD44v6, and it may be required for maintenance of cancer stem cells, which support cancer progression (29). In the present study, the expression levels of CD44v6 were shown to promote the occurrence and development of ovarian cancer. Therefore, it may be hypothesized that the metastatic process of ovarian cancer is initiated by cancer stem cells, via CD44v6. CD44v6 may be a potential indicator of diagnosis and prognosis as well as a therapeutic target. However, the mechanisms of CD44v6 promotion remain to be elucidated.

TGF-β has previously been identified as a key factor in early and late tumor development (30). The results of the present study suggest that TGF-β signaling may be regulated by CD44v6 in ovarian cancer cells. In previous studies, TGF-β has been suggested as a promoter for increasing epithelial-mesenchymal transition and metastasis in numerous types of cancer, such as colon, ovarian, stomach, prostate and lung (30,31). These results indicate that TGF-β may induce tumor progression at both early and late stages. Besides the TGF-β signaling pathway, β-catenin is another pathway associated with cancer cell invasion. β-catenin is located in the plasma membrane and binds E-cadherin; it also has a crucial role in adherens junctions (32,33). β-catenin ,when located in the nucleus, also participates in the Wnt signaling pathway. The translocation of β-catenin at the subcellular level may be activated by the Wnt signaling pathway (19). The present study reported that knockdown of CD44v6 expression resulted in the decreased expression levels of both TGF-β and β-catenin.

In conclusion, the present study indicated that the upregulated expression levels of CD44v6 in ovarian cancer cells may contribute to its pathology. Furthermore, knockdown of CD44v6 expression affected the progression of CAOV3 ovarian cancer cells. The knockdown of CD44v6 expression may downregulate the expression of β-catenin and TGF-β. These results indicate that CD44v6 may be a potential therapeutic target in ovarian cancer.

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