Original Article

Inhibition of CD44 Expression by Small Interfering RNA to Suppress the Growth and Metastasis of Ovarian Cancer Cells *in Vitro* and *in Vivo*

(small interfering RNA (siRNA) / CD44 / ovarian tumour / *in vitro* / *in vivo*)

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Abstract. Since ovarian cancer cells express CD44, which causes very strong cell adhesion to peritoneal mesothelium and an unfavourable prognosis, we designed small interfering RNA (siRNA) targeting the *CD44* gene to analyse the functional consequences of this inhibition in human ovarian cancer. We transfected ovarian cancer cell line SKOV-3 with well-designed CD44 siRNA or control siRNA. Western blot analysis was used to assess the CD44 expression. Following stable transfection, significant inhibition of CD44 expression with 66.13 ± 4.21 % ($P < 0.05$) in CD44 siRNA1 cells and 62.01 ± 3.97 % ($P < 0.05$) in CD44 siRNA2 cells was detected. We performed *in vitro* experiments including cellular adhesion to hyaluronan and human peritoneal mesothelial cells, etoposide-induced apoptosis, and Boyden chamber invasion assays. The adhesion percentages of CD44 siRNA1 and CD44 siRNA2 cells were significantly lower than those of the control siRNA cells in adhesion both to hyaluronan and to human peritoneal mesothelium. The CD44 siRNA transfectants showed significant inhibition of *in vitro* invasion and loss of resistance to apoptosis than the control siRNA cells. *In vivo* study with BALB/c mice was applied to compare the tumour growth and peritoneal dissemination. Nude mice treated with CD44 siRNA cells revealed significantly lower tumour volume and less peritoneal dissemination compared to mice treated with the control siRNA cells. In conclusion, down-regulation of CD44 expression by siRNA inhibits the *in vitro* adhesion, invasion and resistance to apoptosis of ovarian cancer cells, suppresses tumour growth and peritoneal dissemination of human ovarian cancer xenograft in nude mice.

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Abbreviations: BCECF-AM – 3'-O-acetyl-2',7'-bis(carboxyethyl)--4 or 5-carboxyfluorescein diacetoxymethyl ester, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulphoxide, EDTA – ethylenediaminetetraacetic acid, ELISA – enzyme-linked immunosorbent assay, FBS – foetal bovine serum, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OD – optical density, PBS – phosphate-buffered saline, RNAi – RNA interference, siRNA – small interfering RNA.

Introduction

Ovarian cancer cells are characterized by their ability to freely invade the peritoneal cavity, which is consistent with the well-known aggressiveness and high morbidity rate (Greenlee et al., 2000). A number of studies have aimed at identifying specific molecules expressed in ovarian cancer that correlate with tumour cell invasive behaviour. Among such candidate molecules is CD44, which belongs to a family of multifunctional transmembrane glycoproteins expressed in ovarian cancer tissues (Ponta et al., 2003). CD44 as a ubiquitously expressed cell adhesion molecule is unique in that it regulates both cell-cell and cell-matrix interactions in normal and transformed epithelial cells (Ponta et al., 2003). A large number of CD44 isoforms generated by alternative splicing have been implicated in tumour growth and metastasis (Günthert et al., 1991; Hofmann et al., 1991).

Based on this observation, a number of studies have employed sequences against CD44 to inhibit cancer cell invasion (Roscic-Mrkic et al., 2003; Bourguignon et al., 2004; Draffin et al., 2004; Omara-Opyene et al., 2004; Ghatak et al., 2005). However, the effect of down-regulation of *CD44* gene expression by CD44 small interfering RNA (siRNA) on growth and metastasis in human ovarian cancer has not been reported. In this study, we designed siRNA targeting sequences of the human *CD44* gene to analyse the functional consequences of this inhi-

bition in human ovarian cancer cell line SKOV-3, which was reported to express a high level of CD44 by Yang et al. (2005) and Köbel et al. (2006).

Material and Methods

Cell culture

SKOV-3 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Corporation, Carlsbad, CA), supplemented with 10% foetal bovine serum (FBS, Invitrogen) with penicillin/streptomycin antibiotics (100 units of penicillin, and 100 μg of streptomycin per ml).

CD44 siRNA

CD44 siRNA were designed from sequences of human CD44 from the NCBI database (AH003670, AY101193, U40373, M59040, and M24915) using programs available on-line. After a BLAST search, the CD44 siRNA are as follows.

siRNA1: sense 5′-GCAGAUCGAUUUGAAUAUATT-3′
antisense 5′-UAUAUUCAAAUCGAUCUGCTT-3′;
siRNA2: sense 5'-GUAUGACACAUAUUGCUUCTT-3′;
antisense 5'-GAAGCAAUAUGUGUCAUACTT-3′.

siRNA1 and siRNA2 were corresponding to *CD44* exon 3 and exon 4, respectively, which was reported to cause 70–90% inhibition of CD44 expression (Ghatak et al., 2005). They did not show homology to any other known human genes. The siRNA that was used as a non-specific control (non-silencing) siRNA was directed against the sequence 5'-AAUUCUCCGAACGUGUCACGU UU-3' (Cat. No. 80-11310; Qiagen, Hilden, Germany).

Plasmid CD44 siRNA

The pSilencer 4.1-CMV hygromycin vector was purchased from Ambion (Austin, TX). CD44 siRNA with *Bam*HI/*Not*I sites were synthesized and cloned into the above-mentioned vector as per the manufacturer's protocol. Sticky-end ligation was performed using T4 DNA ligase (New England Biolabs, Ipswich, MA). Plasmid DNA (Maxi-prep kit, Qiagen) of clones obtained after transformation was tested for the presence of the CD44 siRNA insert and orientation by double digestion of the clones with *Bam*HI and *Not*I enzymes (New England Biolabs, Ipswich, MA).

Transfection of SKOV-3 cells with CD44 siRNA

For stable transfection, SKOV-3 cells were trypsinized and resuspended in fresh DMEM medium without antibiotics. The amount of 3×10^4 cells (500 μl/well) was plated in 24-well plates a day prior to transfection, corresponding to a cell density of 30–50%. Transfections were performed with lipofectamine and plasmid DNA vector with and without the CD44 siRNA insert according to the manufacturer's protocol (Ambion, Austin, TX) with 1000 μg/ml of hygromycin for the selection of stable clones. After the selection process, the clones including untransfected cells and cells transfected with CD44 siRNA1, CD44 siRNA2 and control siRNA were maintained in 10% serum medium containing 500 μg/ml of hygromycin, respectively.

Western blot analysis

For Western blot analysis, the post-transfection cells were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN_3, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, and 1% Triton-X-100). Samples containing equal amounts of protein were separated using non-reducing conditions on acrylamide gels and transferred to a nitrocellulose filter with electroblotting at 4 °C. The filters were blocked for 1 h in phosphate-buffered saline (PBS) containing 10% dry milk, washed in PBS containing 1% dry milk and 0.2% Tween 20, and then incubated with mAb BU52 (Binding Site, Inc., San Diego, CA) for 1 h at room temperature. Filters were again washed and then incubated with horseradish-peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h. β-Actin was used as internal control. The Western blot analysis was repeated three times.

Cellular adhesion to hyaluronan assays

The adhesion assays were performed as described by Harada et al. (2001). Ninety-six-well plates were coated with 2 mg/ml of potassium hyaluronan from rooster comb (Sigma-Aldrich, St. Louis, MO) overnight. The amounts of 1×10^6 CD44 siRNA1 cells, utransfected cells, control siRNA cells and CD44 siRNA2 cells were suspended in 1 ml of PBS and incubated with 5 mg of the fluorescent dye 3'-O-acetyl-2',7'-bis(carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM; Biotium, Inc. Hayward, CA) at 37 °C for 15 min. After washing twice with PBS, the cells were added to the 96-well plates at a density of 5×10^4/well and incubated for 30 min at 4 °C. The percentage of cell adhesion was determined as mean fluorescence intensity of experimental wells/mean fluorescence intensity of total cells plated. The experiment was repeated three times.

To examine the CD44-dependency of the adhesion, CD44 siRNA1 cells, utransfected cells, control siRNA cells and CD44 siRNA2 cells were preincubated with mouse anti-human CD44 mAb BU52 (Binding Site) at a concentration of 5 μg/1×10^6 cells in 1 ml PBS for 30 min at 4°C. The cells were washed three times with PBS and then applied to the adhesion assay for hyaluronan as described above. The adhesion assays were performed three times, respectively.

Adhesion assay to human peritoneal mesothelial cells

Primary cultures of human peritoneal mesothelial cells were obtained by enzymatic degradation of omental samples obtained from patients undergoing elective abdominal surgery. Local ethics committee approval had been obtained prior to the start of the study and all patients gave informed consents. The cells were isolated

as Stylianou et al. (1990) described. Briefly, 3 cm × 3 cm of the greater omentum was washed with PBS twice and incubated with 0.125% trypsin and 0.04% ethylenediaminetetraacetic acid (EDTA) in PBS for 20 min at 37 °C. Then the undissolved omentum was removed and the isolated mesothelial cells were collected. The mesothelial cells were seeded onto a 96-well plate at 5×10^4 cells/well and cultured in RPMI 1640 with 20% FBS, 10 ng/ml epidermal growth factor, 0.1 μM insulin and 0.4 μg/ml hydrocortisone for two days. After reaching confluence, 5×10^4 of BCECF-AM-labelled cancer cells were added to each well. The cells were incubated for 90 min at 37°C and the percentage of cell adhesion was determined as mean fluorescence intensity of experimental wells/mean fluorescence intensity of total cells plated. The experiment was repeated three times.

Apoptosis assay

The apoptosis assay was performed as described by Lakshman et al. (2005). Briefly, to induce apoptosis, etoposide (Sigma-Aldrich) was dissolved in dimethyl sulphoxide (DMSO, Gaylord Chemical, Bogalusa, LA) at 25 μg/μl and used at 10 μg/ml. The amount of 1×10^6 cells was plated on 35 mm culture plates and 10 μg/ml of etoposide was added. DMSO was used as a control. After various time intervals, cell suspension in DMEM medium without serum was prepared and apoptosis was quantified using the cell death detection enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Roche, Laval, QC, Canada). Cell death was calculated by measuring the optical density (OD) of the sample with etoposide-OD of the corresponding DMSO control. The experiment was repeated three times.

In vitro invasion assay

This experiment was performed using the Boyden chamber assay described by Li and Zhu (1999) and Chen (2005). Transwell chambers with polycarbonate membranes (8-μm pores in 6-well tissue culture plates, Costar, Cambridge, MA) were coated with Matrigel (Becton Dickinson, Heidelberg, Germany), diluted in RPMI (1 mg/ml, 675 μl/4.7 cm^2), and incubated for 60 min at 37°C. For each milliliter of serum-free DMEM, 2×10^5 of CD44 siRNA1 cells, utransfected cells, control siRNA cells and CD44 siRNA2 cells were added to the upper compartment of the transwell chambers, respectively. After incubation for 72 h, viable cells were stained with 0.5 mg/ml 3-(4,5-dimethylthiazol-2--yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). After further incubation for 4 hours, the cells on the upper and lower surfaces in the transwell chambers were removed separately and lysed. MTT was solubilized with DMSO and absorbance was measured at 560 nm and calculated relative to the control siRNA cells. The experiment was repeated three times.

Animal studies

Five to six weeks old female BALB/c mice were purchased from the Institute of Experimental Zoology of Chinese Academy of Medical Science (Beijing, China). All animal studies were carried out according to the Institutional Animal Care and Use Committee of our institute. Prior to injection, cells were rinsed with 1x PBS, trypsinized and resuspended in DMEM. The cell number and viability were determined by trypan blue exclusion (>90% viability). A total of 96 mice were used in our studies. For each study, 48 mice were randomly assigned to four groups (12 mice/group) corresponding to group CD44 siRNA1, CD44 siRNA2, untransfected and control siRNA.

For measurement of the inhibition of *in vivo* growth by CD44 siRNA, 2×10^6 cells per mouse were injected subcutaneously in the flanks. The size of the tumour at the injected sites was measured every week. The longest (a) and the shortest (b) diameters of the tumour were recorded. The volume of the tumour was calculated according to the formula: $V = 4/3\pi\ a \times b^2$ (Shi et al., 2007). Growth curves of the tumour were obtained according to the mean size of the tumour. The mice were sacrificed after six weeks of the injection, and the subcutaneous tumours were excised and weighed.

To assess the effect of CD44 siRNA transfection on peritoneal dissemination, 2×10^6 cells in 200 μl of PBS were administered into the peritoneal cavity of the mice. The mice were sacrificed after four weeks, and the amounts of ascites were measured. All of the metastatic nodules larger than 2 mm in diameter were excised for weighing and comparing. The nodules were sectioned and the sections were stained with haematoxylin and eosin and examined under a microscope.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SigmaStat Version 3.0 (Systat software, Point Richmond, CA). Individual comparisons were made with Student's *t*-test. The criterion for significance was $P < 0.05$ for all comparisons.

Results

Down-regulation of CD44 expression by siRNA in human ovarian cancer cells

Following transfection with plasmid encoding CD44 siRNA, Western blot showed that the CD44 expression of CD44 siRNA1 cell line and CD44 siRNA2 cell line was significantly decreased compared to the control siRNA cell line (Fig. 1A, 1B), with 66.13 ± 4.21 % ($P < 0.05$) by CD44 siRNA1 and 62.01 ± 3.97% ($P < 0.05$) by CD44 siRNA2.

Adhesion of CD44 siRNA transfectants to hyaluronan

The binding of CD44 siRNA1 and CD44 siRNA2 cells to hyaluronan was significantly lower than that of the untransfected cells and control siRNA cells (Fig. 2). This adhesion to hyaluronan was CD44-dependent, since the adhesion of utransfected cells and control

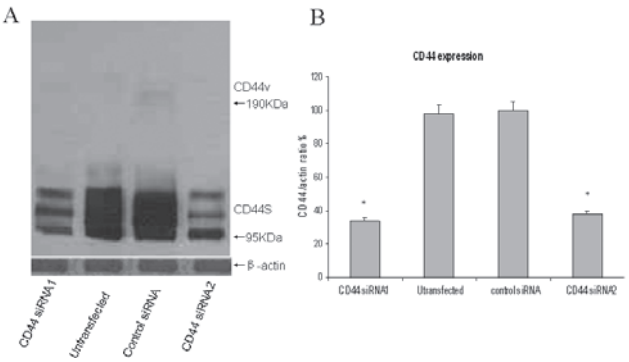

Fig. 1. Western blot analysis of SKOV-3 cell line CD44 expression after transfection by CD44 siRNA. Human ovarian cancer SKOV-3 cells were transfected by CD44 siRNA1, control siRNA or CD44 siRNA2. **A:** CD44 expression was analysed by Western blotting. **B:** Quantitative values represent the CD44 expression value/actin ratio, after normalized CD44 level in control siRNA cells to 1. Compared to control siRNA cells, CD44 expression decreased to 66.13 ± 4.21 % ($P < 0.05$) in CD44 siRNA1 cells and 62.01 ± 3.97 % ($P < 0.05$) in CD44 siRNA2 cells.

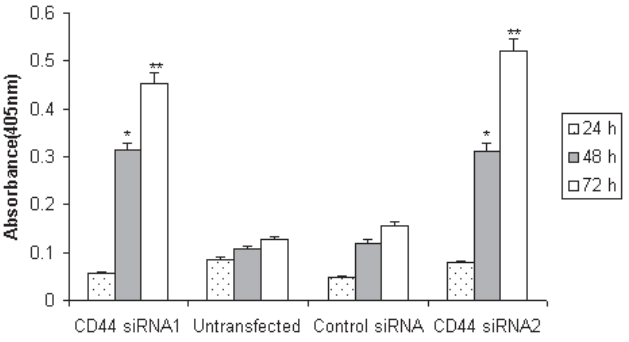

Fig. 3. Etoposide-induced apoptosis was detected by ELISA assay. The amount of 1 × 10^6 cells was plated on 35 mm culture plates and 10 μg/ml of etoposide were added. DMSO was used as a control. Cell death was calculated by measuring the OD of the sample with etoposide-OD of the corresponding DMSO control. The experiment was repeated three times and the mean ± SD was shown in the figure. CD44 siRNA1 and CD44 siRNA2 cells exhibited significantly increased levels of apoptosis at 48 h ($P < 0.05$) and 72 h ($P < 0.01$) compared to the control siRNA cells. * $P < 0.05$; ** $P < 0.01$.

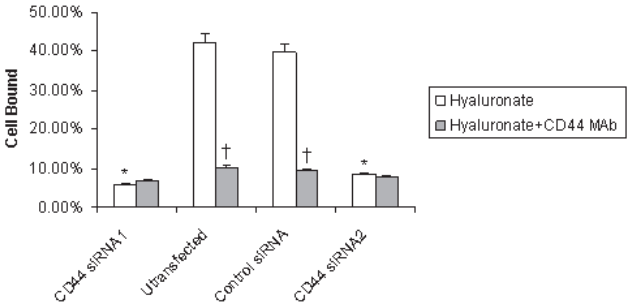

Fig. 2. *In vitro* adhesion to immobilized hyaluronan. The amount of 1 × 10^6 of cells preincubated with BCECF-AM was added to the 96-well plates coated with 2 mg/ml of potassium hyaluronan at a density of 5 × 10^4/well and incubated for 30 min at 4 °C. The percentage of cell adhesion was determined as mean fluorescence intensity of experimental wells/mean fluorescence intensity of total cells plated. The experiment was repeated three times and data were shown as mean ± SD. Percentages of adhesion to hyaluronan were significantly lower in CD44 siRNA1 cells ($P < 0.05$) and CD44 siRNA 2 cells ($P < 0.05$) than those in the control siRNA cells. When cancer cells were pretreated with anti-CD44 mAb, the adhesion of utransfected cells and control siRNA cells were significantly reduced ($P < 0.05$) when compared with the same group without anti-CD44 mAb, whereas the adhesion of CD44 siRNA1 cells and CD44 siRNA2 cells was not affected ($P > 0.05$). * $P < 0.05$; † $P < 0.05$.

siRNA cells was almost abolished by the pretreatment with anti-CD44 mAb (Fig. 2). We can also see that the adhesion in CD44 siRNA1 cells and CD44 siRNA2 cells was not affected by anti-CD44 mAb (Fig. 2).

In vitro adhesion of CD44 siRNA transfectants to peritoneal mesothelium

The attachment of CD44 siRNA1 cells (12.8 ± 2.7 %) and CD44 siRNA2 cells (11.7 ± 2.1 %) to human peritoneal mesothelial cells was significantly lower than that of the control siRNA cells (41.5 ± 5.4 %, $P < 0.05$). No significant difference was detected between the untransfected cells (39.7 ± 4.3 %) and the control siRNA cells ($P > 0.05$).

Loss of resistance to apoptosis in CD44 siRNA cells

Compared to control siRNA cells, CD44 siRNA1 cells and CD44 siRNA2 cells showed significant reduction in their resistance to etoposide-induced apoptosis at 48 h ($P < 0.05$) and 72 h ($P < 0.01$), but no significant increase in apoptotic induction was observed at 24 h (Fig. 3, $P > 0.05$).

CD44 siRNA inhibits in vitro invasion

We performed a Boyden chamber assay to evaluate whether CD44 inhibition by siRNA had any consequences on tumour cell invasion. The result showed that CD44 siRNA1 and CD44 siRNA2 reduced invasion by 59.37 ± 3.42 % ($P < 0.05$) and 50.91 ± 2.89 % ($P < 0.05$), respectively, compared to control siRNA cells (Fig. 4).

Transfection of CD44 siRNA reduces tumour development in vivo

One mouse in the control siRNA1 group died on the 31st day, and the tumour volume after its death and weight of the tumour were excluded from the study. The tumour sizes of the CD44 siRNA1 group and CD44 siRNA2 group were significantly smaller than those of

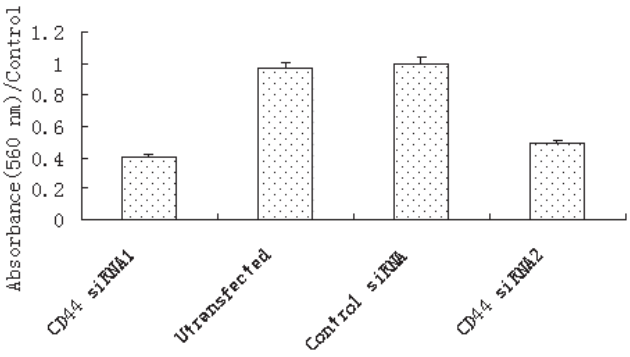

Fig. 4. Boyden chamber assay. Invasion of SKOV-3 cells after inhibition with siRNA. Columns represent percentages of invaded cells (mean and SD). Student's *t*-test was used with significance $P < 0.05$ from three independent experiments. *Significant differences compared with control siRNA cells, $P < 0.05$

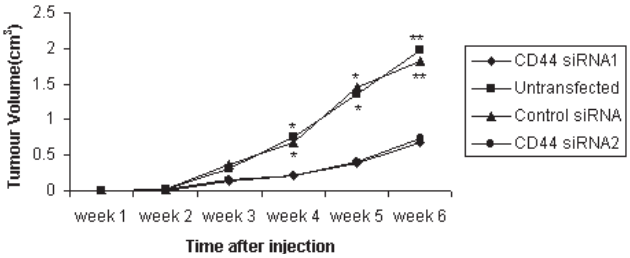

Fig. 5. CD44 siRNA inhibits the *in vivo* tumour growth. The amount of 2×10^6 cells per mouse was injected subcutaneously in the flanks of 12 mice in each group. Growth curves showed that the mean tumour size of the control siRNA group was significantly higher than that of the CD44 siRNA1 and the CD44 siRNA2 group (at the end of the 4th and 5th week, $P < 0.05$; at the end of the 6th week, $P < 0.01$). * $P < 0.05$; ** $P < 0.01$

the control siRNA group (Fig. 5). At the end of the first and second week, the tumour sizes of the four groups were almost similar. At the end of the third week, the tumour sizes in the CD44 siRNA1 group and CD44 siRNA2 group were smaller than the control siRNA group and the untransfected group, but not significantly ($P > 0.05$). The mean tumour size of the control siRNA group was significantly higher than that of the CD44 siRNA1 and the CD44 siRNA2 group at the end of the 4th and 5th week ($P < 0.05$) and the 6th week ($P < 0.01$). The average tumor weight of the CD44 siRNA1 group (0.77 ± 0.06 g, $N = 12$) and CD44 siRNA2 group (0.89 ± 0.11 g, $N = 12$) was significantly lower than that of the control siRNA group (2.14 ± 0.27 g, $N = 11$), $P < 0.05$.

Transfection of CD44 siRNA reduces peritoneal dissemination

It was reported that SKOV-3 cells rapidly colonized the peritoneum in BALB/c mice when injected into the peritoneal cavity (McDonnel et al., 2005). In our study, extensive disseminated tumours were formed in most of the mice four weeks after intraperitoneal inoculation of 2×10^6 cancer cells per mouse in the untransfected group and the control siRNA group. In the CD44 siRNA1 group and CD44 siRNA2 group, the weight of nodules and the amount of ascites were significantly lower than those of the control siRNA group (Table 1).

Table 1. Peritoneal dissemination in BALB/c mice

Group	Weight of nodules (g)	Amount of ascites (ml)
CD44 siRNA1	0.621 ± 0.202*	1.88 ± 0.49*
Utransfected	1.876 ± 0.491	4.88 ± 1.71
Control siRNA	1.630 ± 0.549	5.02 ± 1.54
CD44 siRNA2	0.890 ± 0.410*	1.27 ± 0.74*

Twelve mice in each group. Data were shown as mean ± SD.
* $P < 0.05$ compared to the control siRNA group

Discussion

The CD44 proteoglycan, a member of the hyaladherin family, is expressed by a wide variety of cell types, including epithelial cells and haematopoietic cells, and plays an important role in cell-cell and cell-matrix adhesion (Skubitz, 2002). CD44 has been found to interact with its ligand hyaluronan at the N terminus of its extracellular domain (Liao et al., 1995). The binding of hyaluronan to CD44 is known to be involved in the onset of a variety of biological activities, including Ca^{2+} mobilization, receptor redistribution, cell adhesion, proliferation, migration, and aggregation, gene expression, angiogenesis and tumour metastasis (Bourguignon et al., 2005). The presence of high levels of CD44 is emerging as an important metastatic tumour marker in a number of carcinomas and is also associated with an unfavourable prognosis for a variety of cancers, including ovarian cancer (Yeo et al., 1996). Of course, there exist carcinomas (like urothelial carcinoma) in which disappearance of CD44 is related to high-grade malignancy. It has been shown by many experiments that ovarian cancer cells express CD44 isoforms that cause very strong cell adhesion to hyaluronan-enriched peritoneal mesothelium (Yeo et al., 1996). Various anti-CD44 approaches including anti-CD44 antibody, soluble CD44-immunoglobulin fusion protein and anti-sense CD44 have been used to suppress malignant activities in experimental animals (Seiter et al., 1993; Guo et al., 1994; Strobel et al., 1997). However, anti-CD44 antibody and soluble CD44-immunoglobulin fusion protein would have a side effect on the normal cells and tissues with CD44 expression *in vivo*, although bispecific antibodies were used (Avin et al., 2004). The difficulty of introducing antisense cDNA into target cells has not been solved yet (Shi et al., 2007). These problems make the anti-CD44 approaches impracticable in future research and clinical work.

RNA interference (RNAi) has been rapidly adopted as a functional genomics tool in a wide range of species

and as allowing transient or stable knockdown of gene expression generation in cell lines and animals. With an increasing list of genes successfully knocked-down by RNAi in mammalian cells and improvements in the delivery of siRNAs to cells, including *in vivo* delivery to mice, the siRNA technology has thus greatly widened its scope to be considered in therapeutic approaches, including cancer (Avin et al., 2004). Previous studies using siRNA targeting the human *CD44* gene have successfully inhibited the tumour growth and metastasis in prostate cancer (Draffin et al., 2004; Omara-Opyene et al., 2004), breast cancer (Bourguignon et al., 2004; Draffin et al., 2004), nasopharyngeal cancer (Shi et al., 2007) and colon cancer (Subramaniam et al., 2007).

In our study, it was revealed that CD44 siRNA suppressed CD44 expression in human ovarian cancer cell line SKOV-3 *in vitro* and *in vivo*. We confirmed the successful transfections of CD44 siRNA by inhibition of CD44 expression with 66.13 ± 4.21 % ($P < 0.05$) in CD44 siRNA1 cell line and 62.01 ± 3.97 % ($P < 0.05$) in CD44 siRNA2 cell line (Fig. 1). Adhesion assays showed that the adhesion percentages of CD44 siRNA1 and CD44 siRNA2 cells were significantly lower than those of the control siRNA cells in both adhesion to hyaluronan (Fig. 2) and adhesion to human peritoneal mesothelium. We also found the adhesion to hyaluronan was CD44-dependent, since the adhesion of utransfected cells and control siRNA cells was almost abolished by the pretreatment with anti-CD44 mAb (Fig. 2). These data suggested that CD44 was a major cell surface receptor for hyaluronan in ovarian cancer, which was in accordance with studies reviewed by Skubitz (2002).

Bourguignon et al. (2005) reported that the binding of hyaluronan and CD44 played a pivotal role not only in cell adhesion, but also in activating oncogenic signalling and leading to ovarian tumour cell function. Lakshman et al. (2005) reported that CD44 expression was accompanied by resistance to apoptosis in colonic epithelium. Subramaniam et al. (2007) reported that inhibition of human colon cancer CD44 expression could cause loss of resistance to apoptosis. There was no report on the relationship between CD44 expression and apoptosis in ovarian cancer. Consequently, we investigated whether depletion of CD44 by siRNA affected apoptosis in human ovarian cancer cells. We found that CD44 siRNA1 cells and CD44 siRNA2 cells showed significant reduction in their resistance to etoposide-induced apoptosis at 48 h ($P < 0.05$) and 72 h ($P < 0.01$). We also demonstrated that inhibition of CD44 expression in ovarian cancer cell line SKOV-3 could reduce *in vitro* invasion. These results were also confirmed by our *in vivo* tumour growth study, in which significant differences of the tumour size (Fig. 5) and weight were shown between the CD44 siRNA1 group or CD44 siRNA2 group and the control siRNA group.

Contamination of the abdominal cavity by tumour cells is a crucial step in the progression of invasive ovarian cancer because it transforms the disease to an incurable state. In this situation, tumour cells interact with the peritoneal mesothelial cells, which strongly express hyaluronan on their surface (Jones et al., 1995), and one important system mediating this interaction is the CD44/hyaluronic acid complex (Skubitz, 2002). In our *in vivo* peritoneal dissemination study, extensive disseminated tumours were formed in most of the mice four weeks after intraperitoneal inoculation of 2×10^6 cancer cells per mouse in the untransfected group and the control siRNA group. In the CD44 siRNA1 group and CD44 siRNA2 group, the weight of nodules and amount of ascites were significantly lower than those of the mice treated with the control siRNA cells (Table 1). Our data in this study revealed that CD44 not only interacted with hyaluronan to play a crucial role in adhesion to peritoneal mesothelium, but also associated with the apoptosis and invasion of the ovarian cancer cells.

Taken together, this study suggested that down-regulation of CD44 expression by siRNA inhibited the *in vitro* adhesion, invasion and resistance to apoptosis of ovarian cancer cells, as well as suppressed tumour growth and peritoneal dissemination of human ovarian cancer xenograft in nude mice. As most human ovarian cancer cells express multiple CD44 isoforms (Yeo et al., 1996; Skubitz, 2002), it is practicable to apply CD44 siRNA for the understanding of ovarian cancer progression and the development of experimental therapeutic strategies.

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