

Inhibition of malignant activities of nasopharyngeal carcinoma cells with high expression of CD44 by siRNA

Y. SHI*, Y. TIAN*, Y.Q. ZHOU*, J.Y. JU, L. QU, S.L. CHEN, Z.G. XIANG, Y. LIU and L.P. ZHU

Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences,
School of Basic Medicine Peking Union Medical College, Beijing 100005, P.R. China

Received April 4, 2007; Accepted May 2, 2007

Abstract. Since CD44 was found to be significantly inhibited in the human nasopharyngeal carcinoma cell line CNE-2L2 with profound reduction of malignant activities caused by inhibition of α -mannosidase *Man2c1* gene expression and CD44 has been observed to be involved in many tumor-supporting functions, we studied the association of CD44 expression with the malignant activities of CNE-2L2 cells. Suppression of CD44 gene expression by RNA silencing technique resulted in profound reduction of malignant potential of the cells, including growth *in vitro*, colony formation, tumorigenesis and metastasis of tumors in nude mice. Direct injection of the adenoviruses harboring and producing siRNA to CD44 into the tumor inoculated with CNE-2L2 cells in nude mice caused inhibition of tumor growth. The data indicate a positive association of CD44 expression with the malignant activities of CNE-2L2 cells and suggest a possible therapeutic effect of direct introduction of siRNA to CD44 into tumors in some human solid tumors with high expression of CD44 gene.

Introduction

CD44 is a glycoprotein molecule on cell surface playing important roles in proliferation, differentiation, migration and survival of cells and angiogenesis through concentrating and presenting growth factors, chemokines and cytokines to corresponding receptors, coordinating with enzymes important in extracellular matrix degradation such as hyaluronidase and metalloproteinases, transmitting apoptotic, survival and differentiation signals after encountering relevant ligands

(1,2). CD44 is associated with both the physiological activities of normal cells and the pathological activities of cancer cells (2,3).

Since the involvement of CD44 in many tumor-supporting functions, suppressive effect of targeting agents specific for CD44 and its ligands, such as antibodies, antisense and CD44 soluble proteins, on tumor progression has been examined in animals (4-9). Marked reduction of malignant activities has been observed on various neoplasms, suggesting a therapeutic potential of anti-CD44 on tumors. However, progress of CD44-targeted therapy on tumors is still limited in humans. This is primarily due to the impracticability of carrying out these study approaches in the clinic. Thus, the studies on the association of CD44 expression with tumor progression in the clinic have been limited to the examination if the expression of CD44 is confined to the malignant tissues rather than the corresponding normal tissues and if the CD44 expression level is correlated with disease activity and/or with prognosis of a patient (2). The association of CD44 expression with tumor progression discovered in different laboratories was often confusing, even contradictory (2,10,11).

Tumor progression is a complicated process regulated by many factors (12,13). Factors which play primary parts in tumor progression vary from tumor to tumor. Up-regulation of CD44 might or might not be the major factor associated with the progression of a tumor. Therefore, it is important in selecting CD44-targeted procedures as a strategy of tumor therapy to determine if CD44 over-expression is positively associated with the malignant activities. For example the acute myeloid leukemia (AML) blasts strongly expressed CD44 cell surface antigen (14,15) and monoclonal antibodies to CD44 molecule have been found to be capable of triggering terminal differentiation of leukemic blasts (15,16). These anti-CD44 mAbs were also able to induce the differentiation of AML cell lines, inhibited their proliferation and, in some cases, induced apoptotic death (15,17). Hence, therapy of AML by targeting of CD44 with these mAbs has been conceived. The therapy has been tested in an animal model. It was observed that the transplanted human AML leukemic stem cells in nonobese diabetic-severe combined immune-deficient mice were efficiently eradicated by administration of an anti-CD44 antibody (18). In this study, effect of suppressed CD44 expression on malignant activities of human nasopharyngeal carcinoma CNE-2L2 cells with

Correspondence to: Dr L.P. Zhu, Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, P.R. China

E-mail: zhulp@pumc.edu.cn

*Contributed equally

Key words: siRNA, CD44, tumor, malignant activities, therapy

Table I. Primers for PCR and production of siRNA.

Primer		Sequence
CD44	Sense	5'caactccatctgtgcagcaaa3'
	Antisense	5'gtaacctcctgaagtgcgtc3'
β -actin	Sense	5'gatgatgatatcgccgcgt3'
	Antisense	5'tgggtcatttctcgccgtt3'
pXSN	Sense	5'agcctccgcctcctt3'
	Antisense	5'gaacctcgctgcaatccatctt3'
siCD44-1	Sense	5'tegagcgcagatcgattgaataaacgttatattcaaatcgatctgcgcgt3'
	Antisense	5'ctagtttgcgcagaatcgattgaataacgtttatattcaaatcgatctgcgc3'
siCD44-2	Sense	5'tcgaggcaggaagaaggatggatatgaacgcataccatcatttcctgcgt3'
	Antisense	5'ctagtttgcgcagaaggatggatatgcgttcatatccatcatttcctgcgt3'
siCD44-3	Sense	5'tcgagettcagcctactgcaaatccaacgggattgcagtaggcgtgaagcaaa3'
	Antisense	5'ctagtttgcgcgcctactgcaaatccgcgttgcagtaggcgtgaagca3'
siCD44-4	Sense	5'tcgaggacctttcaatgacaacgaacgcgttgcattgaaagaggtccaaa3'
	Antisense	5'ctagtttgcgcaccccttcaatgacaacgcgttgcattgaaagaggtcc3'
siegfp	Sense	5'tcgagcaagctgaccctgaagttacaacgttcagggtcagctgcgt3'
	Antisense	5'ctagtttgcgcgccttcaatgacaacgcgttgcattgaaagaggtcc3'

high expression of *CD44* gene was investigated. Suppression of *CD44* gene expression caused by RNA silencing technique resulted in profound reduction of the malignant activities. Treatment of the tumor by direct injection of Ad5-siCD44 into the tumor with CNE-2L2 cells inoculated subcutaneously was tested in nude mice. The therapy caused significant reduction of tumor growth. The data suggest that *CD44* targeted therapy might have potential on solid tumors with high expression of *CD44* gene by means of intra-tumor injection of siCD44 in the clinic.

Materials and methods

Cell line and animal. CNE-2L2 is a cloned cell line isolated from CNE-2Z, a human nasopharyngeal carcinoma (19). Tumors with CNE-2L2 cells inoculated subcutaneously into nude mice have high potential of lung metastasis. The cell is hereafter called the W cell in this report. A cell line with profound reduction of malignant activities derived from the W cell, called the AS cell was produced by means of inhibition of α -mannosidase *Man2c1* gene expression (20). Human embryonic kidney HEK293 cells were obtained from the Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, P.R. China). The cells were propagated in RPMI-1640 (Gibco-BRL, Rockville, MD) containing 10% fetal calf serum (Gibco-BRL). BALB/c athymic nude mice aged about 4-weeks were supplied by the Institute of Experimental Zoology, Chinese Academy of Medical Sciences (Beijing, P.R. China).

Construction of virus vector containing siRNA and generation of recombinant virus. pXSNhU6+27siCD44 and pXSNhU6+27siegfp were constructed following the procedures

introduced by Liu *et al* (21). Retrovirus vector pXSNhU6+27 was supplied by Dr De-Pei Liu, National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, which was derived from pLXSN (Clontech, Palo Alto, CA) and pAVU6+27 (Clontech). All the oligonucleotides were synthesized by the Shanghai Sangon Co. (Shanghai, P.R. China) and listed in Table I. The synthetic oligonucleotides were annealed and inserted into pXSNhU6+27 downstream of U6+27 promoter. The configuration of the constructs was verified by DNA sequencing. Generation of recombinant retroviruses was also carried out according to the procedures introduced by Liu *et al* (21).

The sense and antisense primers for siCD44-3 (Table I) were annealed and then ligated into the *Bam*H/*Hind*III sites of pDC316-EGFP-U6 shuttle plasmid (Gene Technology Co. Limited, Beijing, P.R. China) to create pDC316-EGFP-U6-siCD44. To generate recombinant adenoviruses, pDC316-EGFP-U6-siCD44 was cotransfected with genomic plasmid PBHglox_E1, 3Cre (Microbix Biosystems, Toronto) into 293 cells by calcium phosphate precipitation. The adenovirus (Ad5-siCD44) produced in 293 cells was purified by cesium chloride gradient ultracentrifugation. Viral titer was determined as infectious units (IFU) using TCIG50 end-point dilution. The final titer of the purified viral vectors was 5.7×10^9 IFU/ml. The viruses were aliquoted and stored at -80°C. Ad5-egfp was purchased from Gene Technology Co. Limited.

Virus infection of cells. When cell culture reached 70-80% confluence in 6-well plates, the culture medium was replaced by 3 ml flash DMEM (Gibco-BRL) containing 10% fetal calf serum. Retrovirus supernatant (1 ml) was added together with polybrene (6 μ g/ml final concentration) and the cells



SPANDIDOS bated at 37°C overnight. The next day, the medium iced with fresh medium supplemented with G418 (200 µg/ml, active components, Geneticin, Gibco-BRL). Seven to ten days later, G418-resistant cell pools were established.

Genomic PCR. Genotypes of all G418-resistant cell pools were determined by PCR analysis on genome DNA. PCR was carried out using the sense and antisense pXSN primers (Table I) to detect integration of pXSNhU6+27siRNA into genomic DNA. The PCR was performed using 1 µl of diluted template in a 50-µl reaction system at 94°C for 5 min, then 94°C for 45 sec, 58°C for 45 sec, 72°C for 90 sec for 30 cycles, followed by 72°C for 5 min for the final extension. PCR products were examined by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

RT-PCR. RNA was isolated from cells using TRIzol reagent (Gibco-BRL). Total-RNA was incubated with SuperScript II RNase H⁻ reverse transcriptase (Gibco-BRL) and oligo(dT)15 at 42°C for 90 min and then inactivated at 70°C for 15 min. After being treated with RNase H (Gibco-BRL) to remove the contaminating RNA, the synthesized first-strand cDNA was used as a template for gene amplification. The sense and antisense primers for *CD44* and *β-actin* are shown in Table I. The PCR was performed with 2 µl diluted template in a 50-µl reaction system at 94°C for 5 min, then 94°C for 30 sec, 59°C for 30 sec, 72°C for 30 sec, for 30 cycles, then 72°C for 10 min for the final extension. The primers were synthesized by the Sangon Biotech Co. (Shanghai). The PCR product was examined by electrophoresis.

Immunofluorescent staining and flow cytometric analysis. Cells were stained with an anti-CD44 mAb (Clone G44-26, BD Biosciences, Franklin Lakes, NJ) and next with FITC-labeled goat anti-mouse IgG (Sigma, St. Louis, MO). Mouse IgG2b (BD Biosciences) was used as a control. The immunofluorescence intensity on cells was detected by a flow cytometer (Coulter, Fullerton, CA).

Western blotting. Western blotting was performed following the procedures previously described (20). Anti-CD44 mAb (Clone BBA10, R&D, Minneapolis, MN) was used as the first antibody. β-actin was used as an internal control.

Cell proliferation assay. About 500 cells per well in 200 µl DMEM containing 10% fetal calf serum were seeded into 96-well plates (Corning Costar Corp., Corning, NY). At the end of days 1-6, 20 µl solution from CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega) was applied to each well and the plates were incubated at 5% CO₂, 37°C for 2.5 h. The supernatant in the wells was moved to new plates and determined for the spectrum absorbance at 490 nm. The assay was repeated 4 times.

Colony formation. Colony formation was carried out in soft agar according to the procedures previously described (20). Cells (1x10⁴) were seeded into the top agar. After 14 days incubation at 5% CO₂, 37°C, the number of colonies with a diameter >500 µm was counted and the colony formation rate was calculated. The experiment was performed in triplicate.

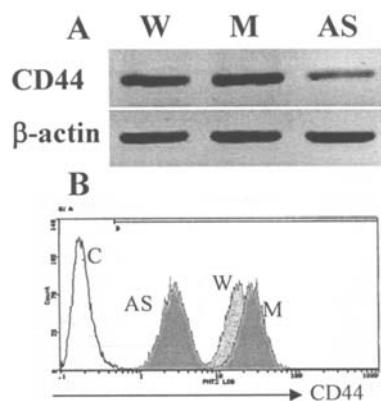


Figure 1. High expression of CD44 in W cells and down-regulation of *CD44* gene in AS cells. (A) RT-PCR; (B) Flow cytometric analysis. Cells were incubated with anti-CD44 antibody and then FITC-labeled goat anti-mouse IgG. W, M and AS represents wild-type CNE-2L2 cells, cells transduced with mock and cells transduced with reduced malignant activities resulted from transduction of antisense α-mannosidase *Man2c1*, respectively. C represents a control with mouse IgG2b as the first antibody.

DNA content analysis. DNA content analysis was performed following the procedures introduced in ref. (21).

Immunofluorescence microscopy. Cells grown on cover slips were rinsed with TBS-Ca²⁺ (1 mM) and then stained with mAb anti-human E-cadherin (Zymed, San Diago, CA). After incubation with FITC-conjugated goat anti-mouse IgG (Sigma), the fluorescence intensity of the cells was examined by a laser scan confocal microscope (Meridian, Austell, GA).

Tumor growth in nude mice. Cells in DMEM (5x10⁵) were subcutaneously inoculated into the right fore leg of nude mice with 8 mice in each group. The growth of the tumor at the inoculated sites was examined every week. The longest (a) and the shortest (b) diameters of the tumors were measured. The volume of the tumors was calculated according to the formula: volume = 4/3π a x b². Growth curves of the tumors were constructed. Eight weeks later, the mice were sacrificed and tumors were excised and weighed. The tumors and lungs of the mice were fixed in formaldehyde (pH 7.2), paraffin-embedded and sectioned. The lungs were sectioned, the sections were stained with hematoxylin and eosin and examined under a microscope.

Adenovirus-mediated gene therapy in nude mice. Each mouse was subcutaneously inoculated with 5x10⁶ wild-type CNE-2L2 cells at the left fore leg. The experiment was divided into 3 groups with 6 mice in each group. About 10 days later when the tumor grew to 50-100 mm³, the tumor in each mouse for group 1, 2 or 3 was injected with 0.1 ml PBS, 0.1 ml Ad5-egfp (4x10⁸ IFU) or 0.1 ml Ad5-siCD44 (4x10⁸ IFU) respectively. The injection was repeated 1 week later. After 2 weeks, the tumor size was measured and calculated according to the formula shown above.

Results

High expression of CD44 gene in the W cells and reduced expression of CD44 gene in the AS cells. Both RT-PCR

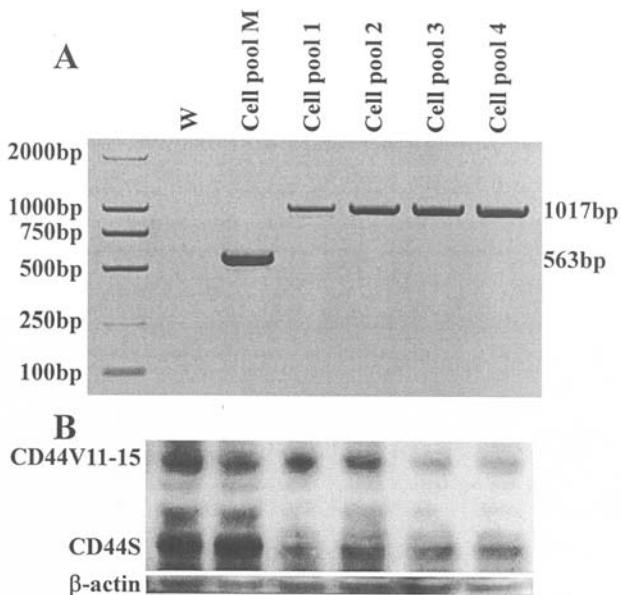


Figure 2. Inhibition of *CD44* gene expression in cells by siCD44. siCD44 was transduced into cells mediated by retrovirus. (A) Integration of siCD44 was detected by genomic PCR with primers pXSN F and pXSN R. (B) Expression of *CD44* was detected by Western blotting. W, wild-type CNE-2L2 cells; cell pool M, cells mock-transduced; cell pool 1, cells transduced with siCD44-1; cell pool 2, cells transduced with siCD44-2; cell pool 3, cells transduced with siCD44-3; cell pool 4, cells transduced with siCD44-4.

(Fig. 1A) and flow cytometric analysis (Fig. 1B) show high expression of *CD44* gene in wild-type CNE-2L2 cells (W cell). Our previous study on an mRNA differential display analysis revealed profound down-regulation of *CD44* gene in the CNE-2L2 cells with reduced malignant activities (AS cell) in comparison with that in W cells (19). Both RT-PCR (Fig. 1A) and flow cytometric analysis (Fig. 1B) confirmed the reduced expression of *CD44* gene in AS cells, suggesting an association of *CD44* gene expression with the malignant activities of the CNE-2L2 cells.

Development of cell pools with suppressed expression of *CD44* gene. We first determined if any association existed of down-regulation of *CD44* gene with reduced malignancy of the AS cells. We developed cell pools with suppressed expression of *CD44* gene by means of RNA silencing technique. Fig. 2A shows the integration of the target RNAs, including siCD44-1, siCD44-2, siCD44-3, siCD44-4 and siegfp into the genomic DNA of cells respectively. Western blotting using an anti-*CD44* mAb as a probe showed that two isoforms of *CD44* proteins, CD44s and CD44v11-15 were expressed in CNE-2L2 cells and both were suppressed in the 4 cell pools infected with the virus containing siCD44-1, siCD44-2, siCD44-3 or siCD44-4 (Fig. 2B). Since the cell pools siCD44-3 and siCD44-4 exhibited the most profound inhibition of *CD44* gene expression, they were used in the remaining studies.

Inhibition of proliferation in vitro of cell pools siCD44-3 and siCD44-4. Cell growth curves showed profound reduction of proliferation in vitro of cell pools siCD44-3 and siCD44-4 in comparison with that of cell pool M or that of W cells (paired t-test, cell pool siCD44-3 or cell pool siCD44-4 versus cell pool M or W cells, $p<0.001$) (Fig. 3A). Integration of siegfp

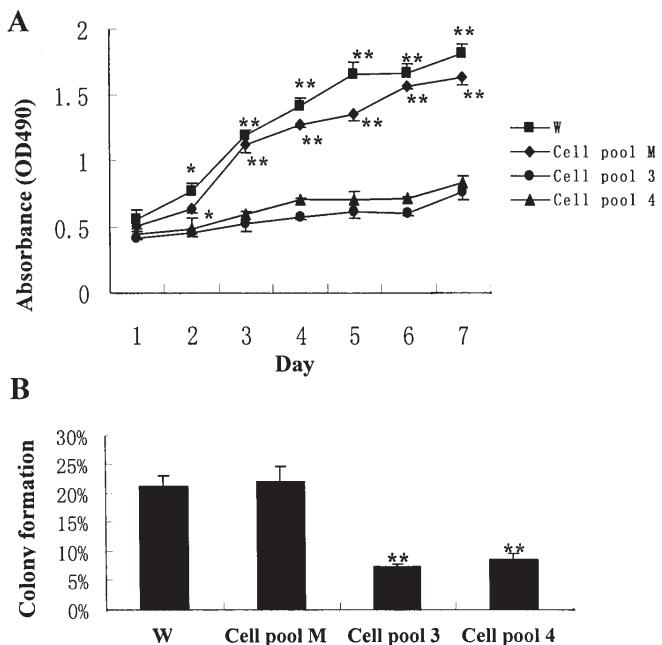


Figure 3. Growth Inhibition of cells with reduced *CD44* expression in culture. (A) Five hundred cells per well in culture medium were seeded into 96-well plates. Cell growth was continually detected using CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit. The test was repeated 4 times. The difference between cell pools 3 or 4, and W or cell pool M was significant ($*p<0.05$, $**p<0.001$). (B) Colony formation was assayed in soft agar. On day 14, colonies with a diameter of $\geq 500 \mu\text{m}$ were counted and the colony formation rate was calculated. The experiment was performed in triplicate. The difference between cell pools 3 or 4, and W cells or cell pool M was significant ($**p<0.001$).

did not affect cell growth. Colony formation test also showed cell growth inhibition of cell pools siCD44-3 and siCD44-4 (Fig. 3B). The Colony formation rate was $21.3\pm 0.7\%$, $22.1\pm 0.6\%$, $7.3\pm 0.5\%$ and $8.5\pm 0.5\%$ for W cells, cell pools M, siCD44-3 and siCD44-4, respectively. The inhibition of colony formation in cell pools siCD44-3 and siCD44-4 was significant (cell pools siCD44-3 or siCD44-4 versus W cells or cell pool M, paired t-test, $p<0.001$). The size of the colonies formed by cell pools siCD44-3 and siCD44-4 was much smaller than those by W cell and cell pool M (data not shown).

Suppression of tumorigenesis and tumor metastasis by inhibited expression of *CD44*. Tumor formation was observed in all of the 8 mice inoculated with W cells or cell pool M. In contrast, tumors formed only in 3 or 4 out of the 8 mice inoculated with cell pool siCD44-3 or with cell pool siCD44-4, respectively (Fig. 4A). Growth of the tumors from cell pools siCD44-3 or siCD44-4 was much slower than that of the tumors from W cells or cell pool M (Fig. 4B). The difference of the average size between the tumors from cell pools siCD44-3 or siCD44-4 and the tumors from W cells or cell pool M was significant on week 7 and 8 (paired t-test, $p<0.001$). The average weight of the tumors at the time of sacrifice was 1.56 ± 0.65 , 1.24 ± 0.86 , 0.22 ± 0.09 and 0.28 ± 0.13 g for W cells, cell pools M, siCD44-3 and siCD44-4, respectively (cell pools siCD44-3 or siCD44-4 versus W cells or cell pool M, paired t-test, $p<0.001$).

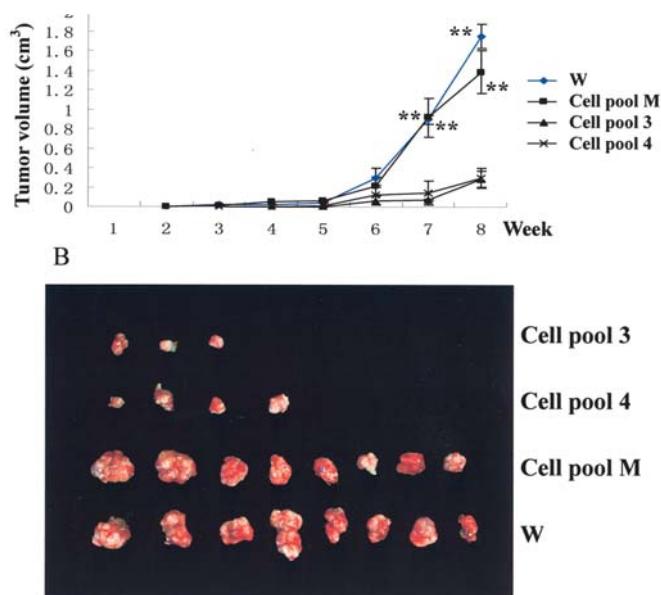


Figure 4. Growth inhibition of tumor in nude mice from inoculated cells with suppressed CD44 expression. A total of 5×10^5 cells were subcutaneously inoculated into the right fore leg of nude mice with 8 mice in one group. The longest (a) and the shortest (b) diameters of the tumor were measured every week for 8 weeks. The volume of the tumors was calculated according to the formula: volume = $4/3\pi a \times b^2$. (A) tumor growth curves. The difference between the average size of tumors from cell pool 3 or cell pool 4 and from W cells or cell pool M was significant (** $p < 0.001$). (B) Comparison of tumor size at sacrifice.

At the time of sacrifice, lung metastasis of tumors was examined. In the mice inoculated with W cells, 6/8 (62.5%) exhibited metastasis with 6 metastatic loci in 1 mouse, 3 metastatic loci in 2 mice, 2 metastatic loci in 2 mice and 1 metastatic locus in 1 mouse. In the mice inoculated with cell pool M, 6/8 (62.5%) mice suffered from metastasis with 4 metastatic loci in 1 mouse, 3 metastatic loci in 2 mice, 2 metastatic loci in 2 mice and 1 metastatic locus in 1 mouse. In sharp contrast, in the mice inoculated with cell pool siCD44-3 or with cell pool siCD44-4, only 1/8 (12.5%) of the mice showed lung metastasis with 4 metastatic loci in the former and 1 metastatic locus in the latter. The data indicate that suppression of CD44 gene expression results in reduction of metastasis potential of CNE-2L2 cells.

Suppression of CD44 gene expression resulted in G1 arrest and enhanced E-cadherin expression. Cell cycle analysis by a flow cytometer showed an increase of the cell percentage in G1 phase and a decrease of the cell percentage in S phase in cell pool siCD44-3 and cell pool siCD44-4 in comparison with those in W cells and cell pool M, indicating a G1 arrest of cell pool siCD44-3 and cell pool siCD44-4 (Fig. 5A). Fig. 5B shows an enhanced expression of E-cadherin on cell pool siCD44-3 and cell pool siCD44-4 with a belt formation at the cell junctions and a honeycomb-like appearance in sharp contrast to low expression of the molecule in W cells and cell pool M.

Adenovirus-mediated siCD44 therapy suppressed tumor growth. siRNA to CD44 was introduced into tumors inocu-

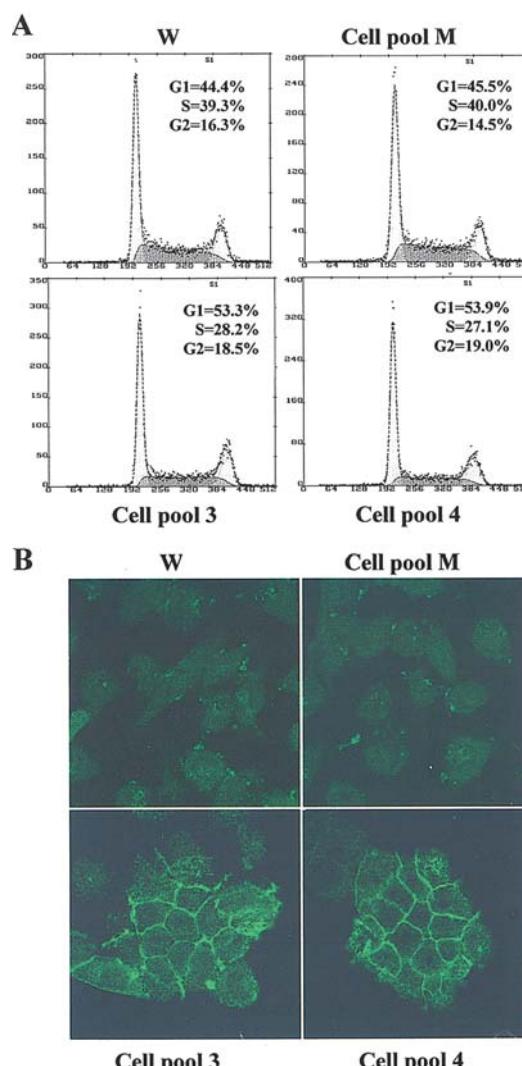


Figure 5. Suppression of CD44 gene expression resulted in G1 arrest and enhanced E-cadherin expression. (A) Cells were stained with propidium iodide and cell cycle was analyzed by a flow cytometer. (B) Cells grown on cover slips were rinsed with TBS-Ca²⁺ and then stained with anti-E-cadherin mAb. After incubation with FITC-conjugated goat anti-mouse IgG, the fluorescence intensity of cells was examined under a laser scan confocal microscope.

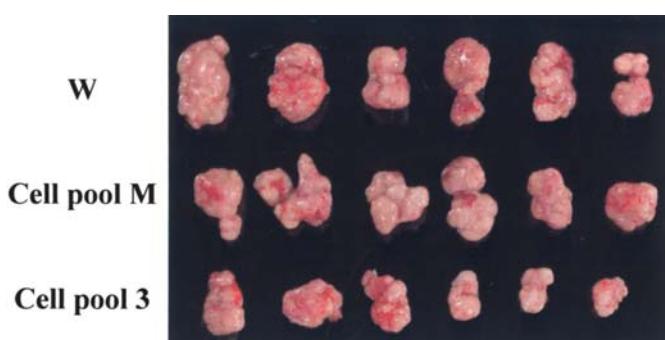


Figure 6. A therapeutic effect of siCD44 on the tumor from inoculated CNE-2L2 cells in nude mice. Wild-type CNE-2L2 cells (5×10^6) were subcutaneously inoculated into each mouse. When tumor grew to 50–100 mm³, PBS (0.1 ml), Ad5-egfp (4×10^8 IFU in 0.1 ml) or Ad5-siCD44 (4×10^8 IFU in 0.1 ml) was injected into the tumor in each mouse from group 1, 2 and 3 respectively. The injection was repeated one week later. Two weeks after the second injection, the mice were sacrificed and the tumors were excised, size-measured and weighed.

lated with W cells in nude mice by means of adenovirus mediation. Two weeks after the second virus injection, the average size of tumors was 3.139 ± 0.85 cm³ in the mice injected with PBS, 3.612 ± 0.888 cm³ in the mice injected with Ad5-egfp and 1.512 ± 0.742 cm³ in the mice injected with Ad5-siCD44. The difference between PBS or Ad5-egfp and Ad5-siCD44 was significant (paired t-test, $p < 0.05$). The average weight of the tumors was 2.28 ± 0.73 , 2.07 ± 0.40 and 1.195 ± 0.64 g for PBS, Ad5-egfp and Ad5-siCD44 respectively (PBS or Ad5-egfp versus Ad5-siCD44, paired t-test, $p < 0.05$). Fig. 6 shows comparison of the tumor sizes.

Discussion

In this study, we characterized the consequence of inhibition of *CD44* gene expression by RNA silencing technique on cellular proliferation *in vitro*, and *in vivo* tumorigenicity and metastasis potentiality of human nasopharyngeal carcinoma CNE-2L2 cells with high expression of *CD44* gene. As a consequence of the suppression of *CD44*, the CNE-2L2 cell displayed decreased cellular proliferation and colony formation, and G1 arrest in cell cycle, inhibition of tumor formation and lung metastasis in nude mice. Moreover, this study also showed an inhibitive effect of siCD44 therapy mediated by adenovirus on growth of the tumors inoculated with CNE-2L2 cells in nude mice.

Various anti-CD44 approaches including anti-CD44 antibody, soluble CD44-immunoglobulin fusion protein and anti-sense CD44 have been successfully used to suppress malignant activities in experimental animals. A number of malignant activities were effectively suppressed when anti-CD44 antibody was administered to animals. These malignant activities were intracerebral progression of glioblastoma in rat (24), metastasis of pancreatic adenocarcinoma in rat (24), lymph node invasion of T cell lymphoma in mice (5), lung metastasis of mouse melanoma cell B16F10 (25), intra-abdominal spread of a human ovarian cancer xenograft in nude mice (6), and metastasis of human melanoma inoculated into SCID mice (26). Since anti-CD44 antibody might affect nonmalignant cells as well, Avin *et al* developed a bispecific antibody that bound simultaneously to CD44 molecule and to an idiotypic determinant of the murine B cell lymphoma, 38C-13. These anti-Id x anti-CD44 bispecific Abs blocked 38C-13 cell adhesion to hyaluronic acid, while not affecting adhesion of Id-negative cells. *In vivo* studies demonstrated that the bispecific Abs inhibited lymphoma cell dissemination to the lymph nodes, bone marrow, and spleen, and prolonged survival of tumor-bearing mice (27). Malignant activities of tumors could also be suppressed by soluble CD44-immunoglobulin fusion protein or antisense CD44. For example, injection of CD44s- or CD44v10-immunoglobulin fusion protein into mice markedly reduced melanoma metastasis in the lung (25). Tumor formation by human lymphoma Namalwa cells, stably transfected with CD44s, in nude mice was suppressed by a soluble human CD44s-immunoglobulin fusion protein (7). Colorectal carcinoma cells transfected with V6 antisense and injected into the spleen neither developed liver metastasis nor grew at the abdominal incision site of nude mice (9). SCID mice inoculated with colon carcinoma cells transfected with CD44s antisense showed reduced tumor growth and liver metastasis (8). These data suggest that CD44

might be a promising target molecule for the therapy of some human tumors.

However, anti-CD44 antibody and soluble CD44-immunoglobulin fusion protein would have side effects on the normal cells and tissues with CD44 expression *in vivo* and the difficulty of introducing antisense cDNA into target cells has not been solved yet. Therefore, these approaches are impracticable in the clinic. Nevertheless, possibilities of performing therapy of anti-CD44 for human tumors are still being tested. Based on strong expression of CD44 on human acute myeloid leukemia blasts and the capacity of the monoclonal antibody in triggering terminal differentiation, inhibiting proliferation and in some cases inducing apoptotic death of leukemia blasts (14-17), Jin *et al* injected an activating mAb specific to CD44 (H90) into nonobese diabetic-severe combined immune-deficient mice transplanted with human AML cells and found that leukemia repopulation in the mice was markedly reduced by eradication of AML LSCs (leukemia stem cells) through blocking LSC trafficking to supportive environments and altering their stem cell fates. The authors speculated that the study might provide a generalized therapeutic strategy to eradicate cancer stem cells by CD44 targeting approaches, since cancer stem cells that maintain some solid tumors might also strongly express CD44 (18). In our study, we first examined if reduced expression of *CD44* gene exhibited any suppressive effect on the malignant activities of human nasopharyngeal carcinoma CNE-2L2 cells with high expression of *CD44*. Having obtained positive results, we tested the therapeutic effect of the siRNA to *CD44* on the tumor inoculated with CNE-2L2 cells in nude mice. Significant inhibition of tumor growth was observed. Although the inhibition was not strong enough in this study, the therapeutic efficacy could be modulated by increasing the dose of virus and frequency of injection. The approach of introducing siCD44 into tumor by direct injection of viruses harboring and producing the siRNA into tumor is practicable in clinic. Thus, our study suggests a new therapeutic strategy of some human solid tumors with high expression of *CD44* gene, especially those appearing on the body surface.

Inhibition of *CD44* gene expression caused up-regulation of E-cadherin in CNE-2L2 cells. E-cadherin plays very important parts in adhesion among epithelial cells and has been considered to be a tumor suppressor gene (28). Loss or reduction of E-cadherin expression was reported to be correlated with enhanced aggressiveness and dedifferentiation of many carcinomas (28-30). Thus, contribution of enhanced expression of E-cadherin resulted from down-regulation of *CD44* to the reduction of malignant activities of CNE-2L2 cells could be implicated.

Acknowledgements

The study was supported by The National Basic Research Program of P.R. China (2001CB510004) and The National High Technology Research and Development Program of P.R. China (2002BA711A03).

References

- Ponta H, Sherman L and Herrlich PA: CD44: from adhesion molecules to signaling regulators. Nat Rev Mol Cell Biol 4: 33-45, 2003.



- SPANDIDOS[®], Nedvetzki S, Golan I, Melnik L and Faitelson Y: CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol* 35: 211-231, 2004.
3. Maniñá R and Zoller M: CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol* 35: 211-231, 2004.
 4. Breyer R, Husein S, Radu DL, Putz KM, Gunia S, Hecker H, Sammi M, Walter F and Stan AC: Disruption of intracerebral progression of C6 rat glioblastoma by *in vivo* treatment with anti-CD44 monoclonal antibody. *J Neurosurg* 92: 140-149, 2000.
 5. Zahalka MA, Okon E, Gossler U, Holzmann B and Naor D: Lymph node (but not spleen) invasion by murine lymphoma is both CD44- and hyaluronate-dependent. *J Immunol* 154: 5345-5355, 1995.
 6. Strobel T, Swanson L and Cannistra SA: *In vivo* inhibition of CD44 limits intra-abdominal spread of a human ovarian cancer xenograft in nude mice: a novel role for CD44 in the process of peritoneal implantation. *Cancer Res* 57: 1228-1232, 1997.
 7. Sy MS, Guo YJ and Stamenkovic I: Inhibition of tumor growth *in vivo* with a soluble CD44-immunoglobulin fusion protein. *J Exp Med* 176: 623-627, 1992.
 8. Harada N, Mizoi T, Kinouchi M, Hoshi K, Ishii S, Shiiba K, Sasaki I and Matsuno S: Introduction of antisense CD44S cDNA down-regulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells. *Int J Cancer* 91: 67-75, 2001.
 9. Reeder JA, Gotley DC, Walsh MD, Fawcett J and Antalis TM: Expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis and tumor growth in a wound environment. *Cancer Res* 58: 3719-3726, 1998.
 10. Bankfalvi A, Terpe HJ, Breukelmann D, Bier B, Rempe D, Pschadka G, Krech R and Bocker W: Gains and losses of CD44 expression during breast carcinogenesis and tumour progression. *Histopathology* 33: 107-116, 1998.
 11. Friedrichs K, Franke F, Lisboa BW, Kugler G, Gille I, Terpe HJ, Holzel F, Maass H and Gunthert U: CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. *Cancer Res* 55: 5424-5433, 1995.
 12. Yokota J: Tumor progression and metastasis. *Carcinogenesis* 21: 497-503, 2000.
 13. Cairns RA, Khokha R and Hill RP: Molecular mechanisms of tumor invasion and metastasis: an integrated view. *Curr Mol Med* 39: 659-671, 2003.
 14. Ghaffari S, Dougherty GJ, Eaves AC and Eaves CJ: Altered patterns of CD44 epitope expression in human chronic and acute myeloid leukemia. *Leukemia* 10: 1773-1781, 1996.
 15. Gadhoun Z, Delaunay J, Maquarre E, Durand L, Lancereaux V, Qi J, Robert-Lezenes J, Chomienne C and Smadja-Joffe F: The effect of anti-CD44 monoclonal antibodies on differentiation and proliferation of human acute myeloid leukemia cells. *Leuk Lymphoma* 45: 1501-1510, 2004.
 16. Charrad RS, Li Y, Delpech B, Balirrand N, Clay D, Jasmin C, Chomienne C and Smadja-Joffe F: Ligation of the CD44 adhesion molecule reverses blockage of differentiation in human acute myeloid leukemia. *Nat Med* 5: 669-676, 1999.
 17. Charrad RS, Gadhoun Z, Qi J, Glachant A, Allouche M, Jasmin C, Chomienne C and Smadja-Joffe F: Effects of anti-CD44 monoclonal antibodies on differentiation and apoptosis of human myeloid leukemia cell lines. *Blood* 99: 290-299, 2002.
 18. Jin L, Hope KJ, Zhai Q, Smadja-Joffe F and Dick JE: Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat Med* 12: 1167-1174, 2006.
 19. Yu DN and Gao J: Study on the invasive properties of cloned human nasopharyngeal carcinoma CNE-2L2 variants using organ culture *in vitro*. *Acta Acad Med Sinicae* (in Chinese) 16: 317-321, 1994.
 20. Yue W, Jin YL, Shi GX, Liu Y, Gao Y, Zhao FT and Zhu LP: Suppression of 6A8 α -mannosidase gene expression reduced the potentiality of growth and metastasis of human nasopharyngeal carcinoma. *Int J Cancer* 108: 189-195, 2004.
 21. Liu CM, Liu DP, Dong WJ and Liang CC: Retrovirus vector-mediated stable gene silencing in human cell. *Biochem Biophys Res Commun* 313: 716-720, 2004.
 22. Chen SL, Shi Y, Jin YL, Liu Y, Zhao FT and Zhu LP: Differential gene expression in nasopharyngeal carcinoma cell with reduced and normal expression of 6A8 α -mannosidase. *Acta Acad Med Sinicae* (in Chinese) 27: 305-310, 2005.
 23. Breyer R, Husein S, Radu DL, Putz KM, Gunia S, Hecker H, Sammi M, Walter F and Stan AC: Disruption of intracerebral progression of C6 rat glioblastoma by *in vivo* treatment with anti-CD44 monoclonal antibody. *J Neurosurg* 92: 140-149, 2000.
 24. Seiter S, Arch R, Reber S, Komitovski D, Hofmann M, Ponta H, Herrlich P, Matzku S and Zoller M: Prevention of tumor metastasis formation by anti-variant CD44. *J Exp Med* 177: 443-455, 1993.
 25. Zawadzki V, Perschl A, Rosel M, Hekele A and Zoller M: Blockade of metastasis formation by CD44-receptor globulin. *Int J Cancer* 75: 919-924, 1998.
 26. Guo Y, Ma J, Wang J, Che X, Narula J, Bigby M, Wu M and Sy MS: Inhibition of human melanoma growth and metastasis *in vivo* by anti-CD44 monoclonal antibody. *Cancer Res* 54: 1561-1565, 1994.
 27. Avin E, Haimovich J and Hollander N: Anti-idiotype x anti-CD44 bispecific antibodies inhibit invasion of lymphoid organs by B cell lymphoma. *J Immunol* 173: 4736-4743, 2004.
 28. Pecina-Slaus N: Tumor suppressor gene E-cadherin and its role in normal and malignant cells. *Cancer Cell Int* 3: 17-23, 2003.
 29. Takeishi M: Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 5: 806-811, 1993.
 30. Beavon IRG: The E-cadherin-catenin complex in tumor metastasis: structure, function and regulation. *Eur J Cancer* 46: 247-256, 2000.