

CD44 Is a Metastasis Suppressor Gene for Prostatic Cancer Located on Human Chromosome 11p13¹

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Abstract

We have used microcell fusion-mediated chromosomal transfer to introduce normal human chromosomes into highly metastatic rodent prostatic cancer cells to map the location of a metastasis suppressor gene(s). Using this approach, several chromosomal regions have been identified that harbor such metastatic suppressor genes, including human chromosome 11 between p11.2–13 (T. Ichikawa *et al.*, *Cancer Res.*, 52: 3486–3490, 1992, 54: 2299–2302, 1994; N. Nihei *et al.*, *Genes Chromosomes & Cancer*, 14: 112–119, 1995; C. W. Rinker-Schaeffer *et al.*, *Cancer Res.*, 54: 6249–6256, 1994). Using positional cloning, a metastatic suppressor gene, termed *KAI1*, was identified, which is located at human chromosome 11p11.2 (5). Overexpression of *KAI1* results in metastasis suppression in certain highly metastatic Dunning R-3327 rat prostatic cancer sublines, such as AT6.1, without metastasis suppression in other highly metastatic sublines, such as AT3.1. This suggests that an additional metastasis suppressor gene is located within the human chromosome 11p11.2–13 region. The *CD44* gene is located on human chromosome 11p13 and encodes an integral membrane glycoprotein that participates in specific cell-cell and cell-extracellular matrix interactions. Down-regulation of *CD44* expression both at the mRNA and protein levels correlates with metastatic potential within the Dunning system of rat prostatic cancer sublines. Transfection-induced enhanced expression of the *M*, 85,000 standard form of *CD44* in the highly metastatic AT3.1 rat prostatic cells greatly suppresses their metastatic ability to the lungs without suppression of their *in vivo* growth rate or tumorigenicity. These results suggest that *CD44* is a metastasis suppressor for prostatic cancer and that decreased expression of the standard form of *CD44* is involved in the progression of prostatic cancer to a metastatic state.

Introduction

In previous studies, we introduced a human chromosome 11 into several highly metastatic Dunning rat prostatic cancer sublines by microcell fusion-mediated chromosomal transfer. These studies demonstrated that suppression of metastatic ability is obtained without suppression of tumorigenicity of the hybrid cells when a specific region of human chromosome 11 is retained (1, 4, 5). Molecular and cytogenetic analyses demonstrated that metastasis suppressor gene(s) are located on human chromosome 11p11.2–13 (1, 4, 5). The metastatic ability of both Dunning R-3327 AT6.1 and AT3.1 rat prostatic cancer hybrids retaining this human chromosomal region is greatly suppressed without suppressing their tumorigenicity (1). Using positional cloning, a gene on human chromosome 11p11.2 was identified and termed *KAI1*, whose transfection-induced expression suppresses the metastatic ability of the AT6.1 subline but not the tumorigenicity (5). In contrast, transfection-induced re-expression of *KAI1* did not

suppress the metastatic ability of AT3.1 cancer cells.³ These results suggest that an additional metastasis suppressor gene(s) resides on human chromosome 11p11.2–13.

The *CD44* gene is known to be located on human chromosome 11 at p13 (6). *CD44* is a transmembrane glycoprotein encoded by 20 exons over a length of approximately 60 kb, at least 10 of which are variably expressed due to alternative splicing of the mRNA (7–9). *CD44* is involved in cell adhesion, serving as a receptor for the extracellular matrix component hyaluronic acid (10) and osteopontin (11). Although *CD44* appears to function in lymphocyte homing, lymphocyte activation, and extracellular matrix adhesion (6), the precise functions of each of the *CD44* isoforms are less clear. *CD44* has been proposed to play a major role in tumorigenicity or metastasis of different types of tumor cells (6). Individual isoforms differ in their ability to enhance (12–14) or decrease (15, 16) tumorigenicity or metastatic potential when overexpressed on tumor cells. Based upon its known functions and chromosomal location, *CD44* was tested as a candidate metastasis suppressor gene for the AT3.1 cells.

Materials and Methods

Cell Lines. A series of sublines (*i.e.*, AT1, AT2, G, AT6.1, AT3.1, CUB, and Mat-LyLu) have been developed from the androgen-responsive, slow-growing, nonmetastatic, well-differentiated Dunning R-3327H rat prostatic cancer. The developmental history and characteristics of each of these sublines have been described previously (17, 18). All of the sublines are grown in standard RPMI 1640 (M. A. Bioproducts, Walkerville, MD) containing 10% FCS (Hyclone, Logan, UT), 1 mM glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin (M. A. Bioproducts), and 250 nM dexamethasone (Sigma Chemical Co., St. Louis, MO). The cells were grown at 37°C in 5% CO₂ and 95% air.

Cloning of Human Standard CD44 Isoform (CD44s) cDNA and Construction of CD44 Expression Vectors. Human standard *CD44* cDNA was cloned by reverse transcription-PCR from the Dunning AT3.1-11-4 rat microcell-mediated chromosomal transfer hybrid, which retains a normal human chromosome 11 (1). Total RNAs were isolated by TRIZOL kit (Life Technologies, Gaithersburg, MD), and cDNAs were reverse transcribed by using first-strand cDNA synthesis reagents (Pharmacia Biotech, Inc., Piscataway, NJ), according to the manufacturer's instructions. A full open reading frame of the human *CD44* cDNA was isolated by PCR using primers designed as *CD44* 5' (5'-CTCCGGACACCATGGACAAGT) and *CD44* 3' (5'-CTCTTCTTATGCTATAACCTG) based on the *CD44* cDNA sequence (7). Briefly, 5 μ g of total RNA were reverse transcribed, and 2 μ l of the reverse transcription reaction mix were amplified. The reaction contained template, 15 mM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM MgCl₂, and 12.5 μ M of the *CD44* primers. The mixture was denatured for 5 min at 95°C and cooled to 72°C; 2.5 units AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT) were added, and the reaction was overlaid with mineral oil. It was then cycled for 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C for 35 cycles. The PCR products were cloned into pCR II vector (Invitrogen, San Diego, CA). The PCR products were digested with *EcoRI* and isolated by electrophoresis in low-melting temperature agarose. The *CD44 EcoRI* frag-

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³ J-T. Dong and J. T. Isaacs, unpublished results.

ment was cloned into the *EcoRI* site of pCDNA3.1⁺ (Invitrogen, San Diego, CA). The sense and antisense CD44 constructs were selected and confirmed by DNA sequencing using DNasequenasII according to the manufacturer's instructions (Amersham Corp., Arlington Heights, IL).

Transfections. AT3.1 cells were transfected by using lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. After 48 h, the cells were passaged into RPMI 1640 containing 500 $\mu\text{g/ml}$ of G418. After 1 week, colonies were isolated with the use of cloning rings and were grown as individual clones, which were maintained under G418 selection for all further experiments.

Northern Blot Analysis. Twenty μg of total RNA were electrophoresed on 1% agarose/formaldehyde RNA gels and transferred onto Hybond N nylon membrane (Amersham Corp.). The filters were prehybridized in Rapid-hyb buffer (Amersham Corp.) at 65°C for 30 min. Hybridization was performed with the respective cDNA probes at 65°C for 2 h. The filters were initially washed in 2 \times SSC at 65°C and then at higher stringency of 0.1% SSC/0.1%SDS at 65°C. The cDNA probes were radiolabeled with [α -³²P]dCTP by random primer method according to the manufacturer's instructions (Amersham Corp.).

Western Blot Analysis. Following washing of cells with PBS, 400 μl of protein extract solution were added, containing 20 mM Tris-Cl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 25 $\mu\text{g/ml}$ of aprotinin, 5 μM pepstatin, 25 $\mu\text{g/ml}$ of leupeptide, 1 mM of phenylmethylsulfonyl fluoride, and 1% SDS. Protein extract was resolved on 6% SDS-PAGE. Proteins were electroblotted onto Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat skin milk, 1% whole goat serum in PBS for 2 h at room temperature. After blocking, the membranes were rinsed in PBS/0.02% Tween 20 and incubated with 2 $\mu\text{g/ml}$ anti-CD44 monoclonal antibody (clone SFF-2; Bender MedSystems, Vienna, Austria) at room temperature for 1 h, and excess antibody was removed from the membrane by washing for 30 min in PBS/0.02% Tween 20. The membranes were then incubated with a 1:3000 dilution of horseradish peroxidase-conjugated, antimouse immunoglobulin (Bio-Rad Laboratories, Hercules, CA), followed by an additional 1 h at room temperature. After washing, the bound antibody complexes were detected using an ECL chemiluminescence reagent, as described by the manufacturer's instructions (Amersham Corp.).

Characterization of *in Vivo* Growth and Metastasis. To evaluate the *in vivo* growth rate and metastatic ability of the transfectant clones, 5-week-old male athymic nude mice (Charles River Laboratories, Frederick, MD) received injections s.c. in the flank with 4 \times 10⁵ cells. Thirty-one days later, the tumor-bearing animals were sacrificed. The tumors were weighted, and the lungs were inflated with Bouin's solution. Lung metastases were scored under a dissecting microscope.

Statistical Analysis. Values are expressed as the mean \pm SE. Statistical analysis of significance were calculated by one-way ANOVA, followed by the

Newman-Keuls test for multiple comparison, with a $P < 0.05$ being considered significant.

Results

CD44 Expression in Rat Prostatic Cells. We demonstrated previously that the metastatic ability of AT3.1 rat prostatic cancer cells was suppressed without tumor suppression by introduction of a human chromosome 11, and the putative location of metastasis suppressor gene(s) was mapped to 11p11.2–13 (1). One of the genes mapped to this region, CD44, which is a cell membrane glycoprotein involved in cell-cell and cell-matrix interaction, was chosen to study as a candidate suppressor gene. To examine whether CD44 is involved in metastasis suppression of AT3.1 cells, we initially determined the expression of the human CD44 protein by Western blot analysis using a human-specific anti-CD44 antibody that does not cross-react with rodent CD44 protein in the parental highly metastatic AT3.1 cells and hybrid clones produced by microcell fusion-mediated chromosomal transfer of human chromosome 11 (1). Some of these hybrids are metastasis suppressed (*i.e.*, AT3.1-11-4 and AT3.1-11-6), whereas the other hybrid (*i.e.*, AT3.1-11-3) is not suppressed (1). As seen in Fig. 1, the human-specific CD44 antibody detects the human CD44 standard M_r 85,000 isoform (CD44s) in the metastatically suppressed AT3.1-11-4 and AT3.1-11-6 hybrids, with no detection of the human CD44s in parental AT3.1 and nonmetastatically suppressed AT3.1-11-3 hybrid cells. These data demonstrate that enhanced CD44s expression by introduction of human chromosome 11 is consistently associated with the suppression of metastatic ability of the AT3.1 hybrid cells.

To examine further whether a critical level of expression of the CD44s is related to metastatic potential of prostatic cancer cells other than the AT3.1 cells, we have studied a large series of spontaneously developed Dunning rat prostatic cancer sublines that differ widely in metastatic ability (Table 1). We have compared CD44 mRNA and protein expression in cell lines with low (CUB, G, AT1, and AT2) or high (AT6.1, AT3.1, and Mat-LyLu) metastatic ability (Table 1; Fig. 2). The CD44 expression is 2–4-fold higher in the low metastatic CUB, G, AT1, and AT2 cell lines than that of the highly metastatic AT6.1, AT3.1, and Mat-LyLu cell lines. Collectively, these data demonstrate that down-regulation of CD44 expression within the Dunning R-3327 system of serially transplantable rat prostatic cancers

Fig. 1. Expression of CD44s in AT3.1 parental cells, AT3.1 hybrids containing a portion of the human chromosome 11, and in stable transfectants as assessed by Western blot analysis using anti-human CD44 antibody, which does not cross-react with rodent CD44. Fifty μg each of protein were subjected to 6% SDS-PAGE as described in "Materials and Methods."

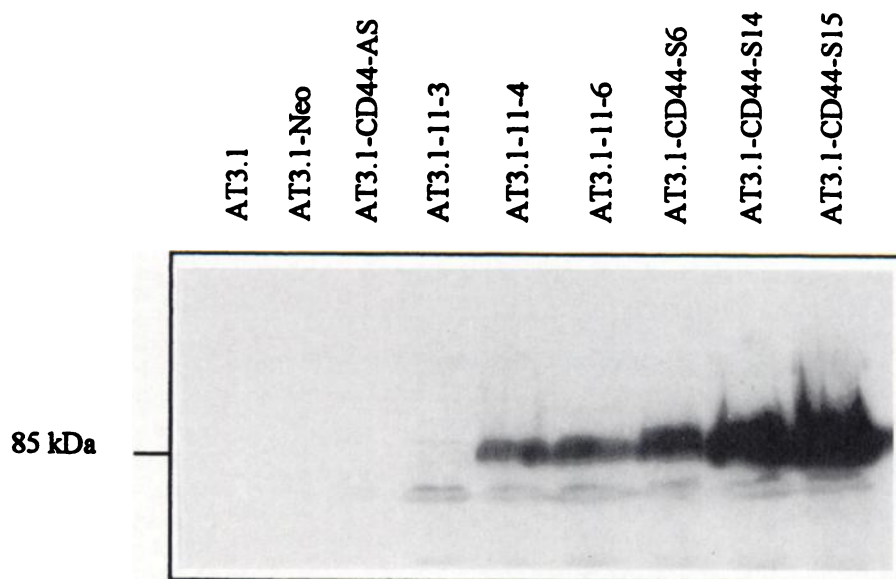


Table 1 *In vivo* characteristic of various Dunning R-3327 rat prostatic cancer sublines

Cell line	Growth rate (tumor doubling time in days)	Metastatic ability ^a	CD44s expression ^b	
			mRNA	Protein ^c
CUB	3.4 ± 0.6	Low	3	3
G	4.0 ± 0.2	Low	3	2
AT1	2.5 ± 0.2	Low	3	2
AT2	2.5 ± 0.2	Low to moderate	4	2
AT6.1	4.0 ± 0.3	High	1	1
AT3.1	1.8 ± 0.2	High	1	1
Mat-LyLu	1.7 ± 0.3	High	1.5	0.5

^a Low metastatic ability, <5% of s.c. inoculated rats develop distant metastases; moderate ability, >5%, <20%; high ability, >75% develop distant metastases.

^b The value for AT6.1 was standardized to 1, and the values for other sublines were adjusted accordingly.

^c Western blot analysis using a mouse anti-rat CD44 monoclonal antibody.

is consistently associated with the acquisition of highly metastatic ability.

Introduction of CD44s into Highly Metastatic Prostatic AT3.1 Cells. The correlation of the expression of CD44s with metastatic ability within the Dunning system of rat prostatic cancer sublines suggests that CD44s may act as a metastatic suppressor for prostatic cancer. To determine whether enhanced expression of CD44s can inhibit metastatic ability, cDNAs encoding CD44s in the sense and the antisense orientation were introduced into appropriate expression vectors and used for transfection into highly metastatic rat prostatic AT3.1 cells. Stable transfectants containing CD44s in the sense and the antisense orientation and vector-alone controls were selected in the presence of G418, subcloned, and tested for their expression of

Table 2 *In vivo* characteristic of AT3.1 parental, Neo control, and CD44 antisense and sense transfectants

Cell clone	Tumor weight (g) at excision ^a	Metastatic ability ^a (no. of lung metastases/animal)	Human CD44s protein expression ^b
AT3.1	8.4 ± 1.2	82 ± 10 (4) ^c	0
AT3.1-Neo	7.7 ± 1.4	72 ± 7 (4)	0
AT3.1-CD44-AS	7.1 ± 1.5	94 ± 6 (4)	0
AT3.1-CD44-S6	7.2 ± 1.7	26 ± 6 (4) ^d	7
AT3.1-CD44-S14	6.9 ± 1.4	23 ± 8 (3) ^d	9
AT3.1-CD44-S15	7.5 ± 1.8	16 ± 11 (5) ^d	10 ^e

^a Mean ± SE at 31 days postinoculation.

^b Antibody used for Western blot quantitation using an anti-human CD44 monoclonal antibody, which does not recognize rat CD44s.

^c Numbers in parentheses, number of animal/group.

^d $P < 0.05$ compared to the mean number of metastases with AT3.1-Neo transfectant.

^e The value for AT3.1-CD44-S15 was standardized to 10, and the values for other clones were adjusted accordingly.

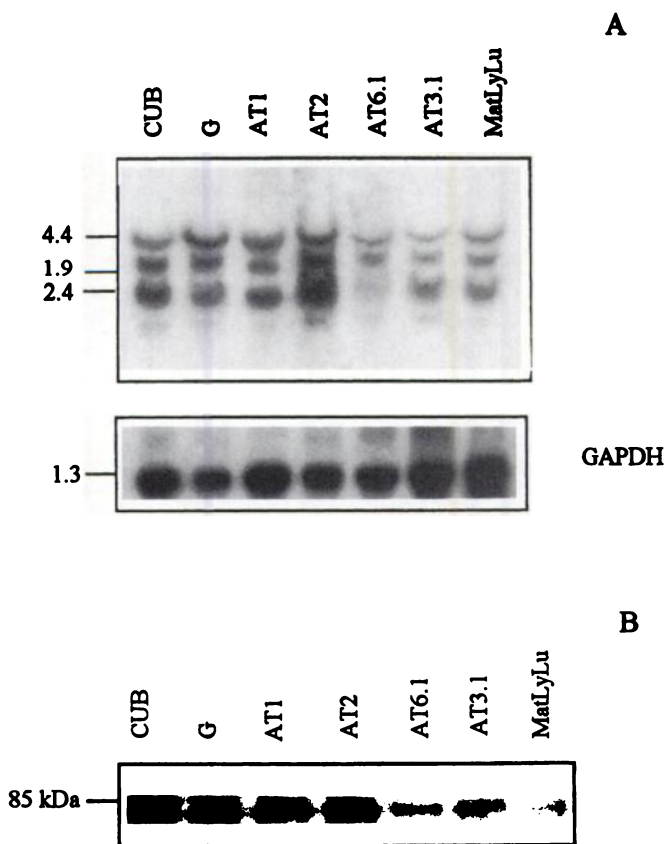


Fig. 2. Expression of CD44 in the Dunning rat prostatic cancer cell lines. A, expression of variant CD44 RNA. Twenty μ g each of total RNA isolated from the Dunning rat cells were subjected to RNA blot analysis, followed by hybridization to CD44 cDNA or a glyceraldehyde-3-phosphate dehydrogenase probe as described in "Materials and Methods." B, standard CD44 isoform protein expression. Fifty μ g each of protein isolated from the Dunning rat cells were subjected to Western blot analysis using mouse anti-rat CD44s monoclonal antibody. Gel electrophoresis and Western blotting were essentially done as described in "Materials and Methods."

CD44s by Western blot analysis. Expression of M_r 85,000 CD44s protein from representative clones is shown in Fig. 1 and summarized in Table 2.

To test the effect of overexpression of CD44s on *in vivo* behavior, 4×10^5 cells of parental AT3.1, AT3.1-Neo control transfectants, antisense (*i.e.*, AT3.1-CD44-AS), and sense (*i.e.*, AT3.1-CD44-S6, AT3.1-CD44-S14, and AT3.1-CD44-S15) transfectants were injected s.c. into the flanks of nude mice, respectively. All animals injected with the CD44s sense expression transfectants resulted in a more than 60% suppression of the number of lung metastases per mouse ($P < 0.05$) without any inhibition of the growth of the primary tumors (Table 2). In contrast, no metastasis suppression was obtained for the Neo control or the antisense transfectant (Table 2).

Discussion

We have demonstrated previously that human chromosome 11p11.2–13 contains metastasis suppressor genes for both highly metastatic Dunning AT6.1 and AT3.1 rat prostatic cancer cell lines (1). Several candidate metastasis suppressor genes have been identified within this region, including KAI1 (5). Transfection induced enhanced expression of KAI1 by the highly metastatic AT6.1 induced metastasis suppression, whereas such KAI1 expression did not suppress the metastatic ability of AT3.1 prostatic cancer cells. These results prompted us to identify additional gene(s) within chromosome 11p11.2–13 that are responsible for metastasis suppression in AT3.1 cells. In this report, we identified CD44, a cell surface glycoprotein involved in cell-cell and cell-matrix interaction, as a metastasis suppressor gene for prostatic cancer cells. CD44s was shown to suppress metastasis when re-expressed by AT3.1 prostatic cancer cells without suppression of the tumor growth.

Metastases are of major concern to oncologists, because cancer fatalities are rarely due to primary tumors but rather to widespread metastatic disease. Metastatic dissemination of cancer cells has been

shown to be a complex process involving an array of genes, such as adhesion molecules, including receptors for components of the extracellular matrix. Recently, there have been numerous reports concerning the role of CD44 variants in primary tumors and their metastases (6). In several animal models, experimental overexpression of specific CD44 isoforms on tumor cells has resulted in enhanced metastatic potential (12, 13). Whereas in other studies, repressed expression of the CD44 expression has been associated with malignant transformation of tumors of squamocellular origin (15), or overexpression of CD44s in colon carcinomas (16) has resulted in reduced tumorigenicity.

We assessed whether there is a possible correlation between the metastatic ability and decreased CD44s expression in the Dunning rat prostatic cancer system. We have demonstrated that a decreased expression of CD44s in a large series rat Dunning rat prostatic cancer sublines correlates with increased metastatic phenotype. MatLyLu and AT3.1 cell lines with the highest metastatic potential in the system expressed with least CD44s, whereas CUB and G sublines with the lowest metastatic ability expressed the highest CD44s levels. The metastatic ability is suppressed by more than 60% when AT3.1 cells are induced by means of transfection-mediated gene transfer to enhance their expression of CD44s protein.

While this work was in progress, Kallakury *et al.* (19) examined CD44 expression in a large series of human prostatic tissues and demonstrated that CD44s is normally expressed on the plasma membrane of prostatic glandular cells (*i.e.*, the cells of origin for prostatic cancer). These studies also demonstrated that CD44 expression is down-regulated in human prostatic cancer progression; down-regulation is correlated with high tumor grade, aneuploidy, and distant metastasis (19). These clinical observations are in agreement with the data presented in this report that enhanced expression of the standard CD44 isoform in prostatic cancer cells inhibits their *in vivo* metastasis ability, whereas down-regulation of CD44s protein expression in associated with acquisition of metastatic ability. Using immunohistochemical detection, we have demonstrated that a similar down-regulation of the cell surface KAI1 protein is correlated with acquisition of metastatic ability by human prostatic cancer (20). Combining our experimental findings with the clinical observations supports the idea that CD44 and KAI1 are metastasis suppressor genes for prostate cancer and that decreased expression of KAI1 and CD44 standard form protein may serve as useful diagnostic markers for predicting the metastatic ability of prostatic cancer cells.

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