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The *Drg-1* Gene Suppresses Tumor Metastasis in Prostate Cancer

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Abstract

***Drg-1* was previously identified (N. van Belzen *et al.*, Lab. Investig., *77:* 85–92, 1997) as a gene that was up-regulated by the induction of differentiation in a colon carcinoma cell line *in vitro*. Subsequently, this gene was found to be regulated by several factors including hypoxia, androgen, p53, and N-myc. Recently, Drg-1 has also been shown to be involved in tumor progression in animals, although the clinical significance of its involvement remains to be investigated. To clarify the functional role of Drg-1 in prostate cancer, we examined a clinical archive of cancer specimens for the expression of Drg-1 by immunohistochemistry. We found that the expression of Drg-1 had a significant inverse correlation with the Gleason grading and the overall survival rate of patients. In particular, the gene expression in patients with lymph node or bone metastasis was significantly reduced as compared with those with localized prostate cancer, suggesting that the function of Drg-1 is negatively involved in metastatic progression of the disease. To further clarify the function of this gene in the advancement of prostate cancer, a spontaneous metastasis assay was performed in a severe combined immunodeficient (SCID) mouse model. We found that Drg-1 almost completely inhibited lung colonization of highly metastatic prostate cancer cells without affecting the growth of the primary tumors. These results strongly suggest that *Drg-1* is a candidate metastasis suppressor gene for prostate cancer and may serve as a useful prognostic marker.**

Introduction

The *Drg-1* gene was originally found to be induced *in vitro* by cellular differentiation and, hence, was named as *Differentiation-Related-Gene-1* (1). Since then, three more genes, namely, *Drg-2*, *-3*, and *-4*, that encode proteins highly related to Drg-1 have been identified. These genes constitute the *NDRG* gene family, although the members vary in the pattern of tissue-specific expression and possibly in function (2, 3). Drg-1 is identical to the human *RTP*, *cap43*, and *rit42*, and homologous to the mouse genes *TDD5* and *Ndr1* and to the rat *Bdm1* (4–9). The protein encoded by the *Drg-1* gene has a molecular weight of M_r 43,000 and possesses three unique 10-amino-acid tandem repeats at the COOH-terminal end. Analysis of the amino acid sequence predicted that there were seven or more phosphorylation sites, and Drg-1, indeed, has been shown to be phosphorylated by protein kinase A (10). *Drg-1* mRNA is detected in most of the organs, and the level of expression is particularly high in prostate, ovary, intestine, and kidney. It was shown that the expression of this gene was repressed by c-myc and N-myc/Max complex *in vitro* (8). On the other hand, p53 was found to be able to induce expression and nuclear translocation of *Drg-1* in response to DNA-damaging agents (6). The expression of the gene was also augmented by hypoxia and *PTEN* although the clinical significance of these regulations is yet to be clarified (11, 12). In addition, the *Drg-1* gene has been shown to be up-regulated by hormones such as androgen (7) and by various chemical agents including homocysteine, β-mercaptoethanol, tunicamycin (4), lysophosphatidyl choline (13), nickel compounds (5), and synthetic retinoids (14). Therefore, the *Drg-1* gene is controlled by multiple factors and responsive to various stimuli. However, little is known about the biochemical function and the exact downstream target of this gene.

Recently, Kurdistani *et al.* (6) showed that the introduction of *Drg-1* cDNA in human bladder cancer cells suppressed tumorigenicity in nude mice. On the other hand, overexpression of Drg-1 in colon cancer cells in nude mice has been shown to have no effect on tumorigenesis, although significantly suppressing *in vivo* liver metastasis (15). Therefore, the results of these two animal studies raise a possibility of a differential organ-specific function of *Drg-1*, and several *in vitro* studies indicated this gene to be the target of multiple regulatory pathways. However, little clinical data are available regarding the functional characterization of this gene in the context of tumor progression. Immunohistochemical analysis of a few colon cancer specimens showed that the expression of *Drg-1* was reduced in poorly differentiated adenocarcinoma as compared with the normal or well-differentiated colonic epithelium (1). In an *in situ* hybridization study with a limited number of samples, *Drg-1* expression was found to be reduced in breast and prostate tumors (6). However, there are no statistical data to date that evaluate the functional involvement of this gene in tumor progression in any type of cancer.

The present study was aimed at clarifying the function of Drg-1 in a clinical setting and in an animal model of prostate cancer. Here, we show for the first time that the expression of the *Drg-1* gene has significant inverse correlation with grades of prostate cancer, particularly with the metastatic progression of the disease, and that the expression also strongly correlates with patient survival. Our studies also indicate that *Drg-1* suppresses the ability of prostate cancer cells to invade through extracellular matrix *in vitro* and that this gene strongly suppresses tumor metastasis without affecting primary tumor growth in animal.

Materials and Methods

Tumor Specimens. Formaldehyde-fixed and paraffin-embedded human prostate tissue specimens from 62 patients were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan) and the Cooperative Human Tissue Network (United States), dating from 1988 to 2001. The specimens consisted of 28 cases of needle biopsy, 9 cases of transurethral resection, and 25 cases of radical prostatectomy. The ages of patients ranged from 53 to 89 years with a mean of 72 years. Complete five-year follow-up data were available for 43 patients. For each case, a representative paraffin block was selected that contained both tumor and benign prostatic tissue.

Immunohistochemical Staining. Four-μm-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were baked at 60°C for 1 h, deparaffinized in xylene, and

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rehydrated. For antigen retrieval, the sections were treated with 25 mM sodium citrate buffer (pH 9) at 80°C for 20 min followed by treatment with 3% H_2O_2 solution to block endogenous peroxidase activity. The slides were incubated overnight at 4°C with anti-Drg-1 rabbit polyclonal antibody at 1:100 dilution. The specificity of the antigen-antibody reaction was confirmed by using preimmune serum with the same dilution. The sections were then incubated with horseradish peroxidase-conjugated antirabbit IgG for 30 min at room temperature, and 3,3′-diaminobenzidine substrate chromogen solution (Envision-plus kit; DAKO Corp., Carpentaria CA) was applied, followed by counterstaining with hematoxylin.

Analysis of Immunohistochemical Staining. Most of the patients presented heterogeneous tumor grade; therefore, each patient sample was assigned two separate Gleason grades, corresponding to the two predominant histological patterns. The nuclear grades were determined according to the WHO criteria, and the degree of differentiation was classified into "well," "moderate," or "poor." Results of the immunohistochemistry were judged based on the intensity of staining, and the grading of the Drg-1 expression was done by two independent persons (S.B. and K.W.) without prior knowledge of the grade, stage, or patient survival. The cases were then divided into those that showed positive staining and those that showed reduced expression of Drg-1.

Cell Lines, Expression Plasmids, and Transfection. Dunning-rat and human prostatic carcinoma cell lines, AT6.1 and ALVA41, were kindly provided by Drs. C. Rinker-Schaeffer (University of Chicago, Chicago, IL) and W. Rosner (Columbia University, New York, NY), respectively. All of the cell lines were cultured in RPMI 1640 supplemented with 10% FCS, streptomycin (100 μg/ml), penicillin (100 units/ml), and 250 nM dexamethasone (Sigma, St. Louis, MO) and were grown at 37°C in a 5% CO_2 atmosphere. Drg-1 cDNA was a generous gift from Dr. S. W. Lee (Beth Israel Deaconess Medical Center, Boston, MA). To create a mammalian expression plasmid, the cDNA was amplified by PCR for which the forward primer included the Kozak sequence and *Eco*R1 linker and the reverse primer included a *Xho*I linker. The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) using standard techniques. The Drg-1 expression plasmid or the vector alone was transfected into the AT6.1 and ALVA cell lines by LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and the G418-resistant clones were selected. The expression of Drg-1 in the cloned cells was tested by reverse transcription-PCR and was further confirmed by Western blot.

Western Blot. To examine the Drg-1 protein expression, the cells were washed with PBS and lysed by 4× sample buffer [10% 1 M Tris (pH 6.8), 30% SDS 20%, 30% glycerol, 30% β-mercaptoethanol]. The lysates were boiled for 5 min, resolved by SDS-PAGE on a 10% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membrane was blocked with 5% powdered skim milk in TTBS [100 mM Tris (pH 7.5), 0.9% NaCl, 0.1% (v/v) Tween 20] for 1 h at room temperature, followed by overnight incubation with the anti-Drg-1 rabbit polyclonal antibody, diluted 1:1000 with blocking solution at 4°C. The membrane was washed with TTBS and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The antigen antibody complex was detected by the ECL system (Amersham Life Sciences) according to the manufacturer's protocol.

***In Vitro* Cell Growth and Anchorage-independent Growth Assay.** For *in vitro* growth assay, AT6.1, ALVA, and the respective Drg-1-expressing clones were seeded at an initial concentration of 10^3 cells/well in a 12-well plate. At regular intervals, cells were trypsinized and resuspended, and the cell numbers were counted by hemocytometer. The anchorage-independent growth of AT6.1, ALVA, and their Drg-1-expressing derivatives were examined by clonogenic cell growth on soft agar as described previously. Briefly, each well of a 12-well plate was first layered with 0.6% agarose in growth medium (RPMI 1640 supplemented with 10% FCS). The cell lines to be tested were trypsinized, and 10^3 cells were resuspended in growth medium containing 0.3% agarose and then were poured as a top layer in the 12-well plates. The plates were incubated at 37°C for 2 weeks, and the number of colonies was counted under microscope.

***In Vitro* Motility and Invasion Assay.** For the motility assay, 10^5 cells were added to the cell culture inserts with microporous membrane without any extracellular matrix coating (Becton Dickinson, Bedford, MA). RPMI medium (700 μl), containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37°C, and the upper chamber was removed. The cells in the lower chamber were incubated for an additional 72 h and were stained with tetrazolium dye, and the number of colonies was counted under a microscope. For *in vitro* invasion assay, the working method was similar to that described above, except that the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson).

Spontaneous Metastasis Assay. To examine the growth rate and metastatic ability of the Drg-1 clones in animals, 0.5×10^6 cells in 0.2 ml of PBS were injected s.c. into the dorsal flank of 5-week-old SCID[2] mice (Harlan Sprague–Dawley, Indianapolis, IN). Mice were monitored daily, and the tumor volume was measured as an index of the growth rate. Tumor volume was calculated using the equation, Volume = ([Width + Length]/2) × W × L × 0.5236. The doubling time of tumor during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Statistical Analysis. The association between Drg-1 and other prognostic markers was calculated by χ^2 analysis. The Kaplan-Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log-rank test. Univariate analysis for the prognostic value of Drg-1 was performed by the Cox proportional hazard-regression model. For *in vitro* experiments and animal studies, one-way ANOVA was used to calculate the *P*s. In all cases, SPSS software was used.

Results

Drg-1 Expression Is Significantly Reduced with Advancement of Human Prostate Cancer. To examine the involvement of Drg-1 in the progression of human prostate cancer, the expression of Drg-1 in clinical samples was analyzed by immunohistochemistry in 62 prostate cancers with various Gleason grades. As shown in Fig. 1*A*, the Drg-1 protein was shown to be highly expressed in the epithelial cells of normal glands and ducts. The basal cell layers also showed a high level of Drg-1, whereas the stroma did not have a detectable level of expression. The protein appeared to be localized mostly in the cytoplasm, but the expression was also detected in cell membranes. The nuclear stain was not observed frequently except in two cases in which the signal strongly shifted from cytoplasm to nucleus in tumor cells. The endothelial cells and nerve bundles frequently showed Drg-1 expression. The protein was detected equally in all cases of normal prostate tissue as well as prostatic intraepithelial neoplasia and benign prostatic hyperplasia, whereas the expression was significantly reduced with the progression of carcinoma, suggesting down-regulation of the *Drg-1* gene with advancement of the disease. The level of *Drg-1* expression was statistically analyzed with respect to known prognostic factors (Fig. 1*B*). When the cases were subdivided into two groups, those with Gleason score of ≤ 7 (38 cases) and those with a Gleason score of >7 (24 cases), the reduction in *Drg-1* expression correlated significantly with the Gleason grade ($P = 0.015$). Although the age at diagnosis did not have any correlation with expression level of this gene, the nuclear grades of the samples were positively correlated ($P = 0.044$) with the expression of *Drg-1*. In addition, the *Drg-1* expression inversely correlated with the degree of differentiation overall ($P < 0.001$). However, one-way ANOVA test with Tukey's W procedure indicated that the down-regulation of *Drg-1* is not significant between well- and moderately differentiated tumors, although it is highly significant between moderately and poorly differentiated tumors. These results are in agreement with the existing idea of this gene being up-regulated by the induction of cellular differentiation *in vitro*, and they suggest that *Drg-1* suppression may be more important in a late stage of tumor progression. It should be noted that a significant level of differential expression of *Drg-1* was observed between the patients with organ-confined disease and those with metastasis to lymph node or bone. Whereas 28 (70%) of 40 localized prostate cancer cases were positive for *Drg-1*, only 5 (25%)

[2] The abbreviations used are: SCID, severe combined immunodeficient; MKK4, mitogen-activated protein kinase kinase 4.

(A)

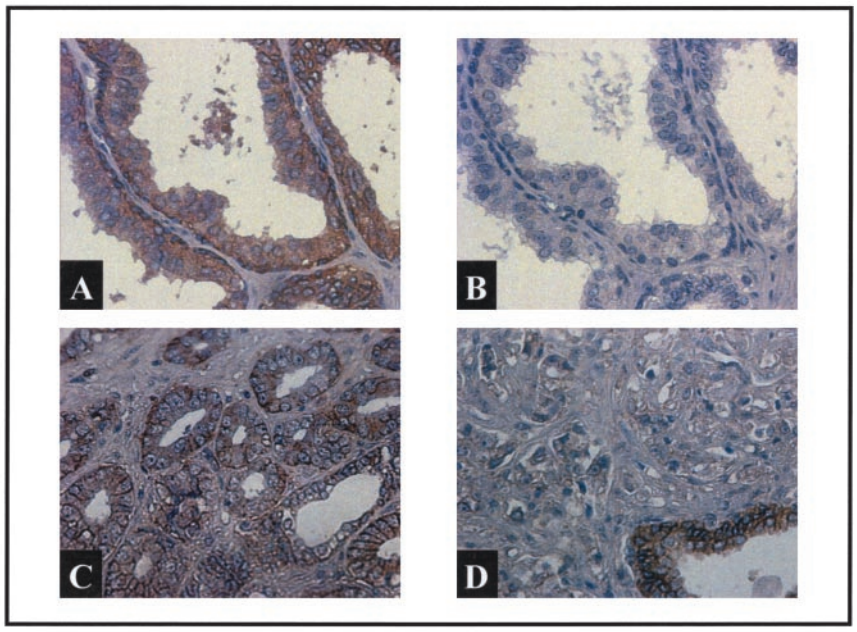


Fig. 1. Immunohistochemical analysis of Drg-1 in human prostate cancer. *A, Drg-1* expression is reduced with increasing Gleason grade. Immunohistochemistry was performed on paraffin tissue sections from prostate cancer patients of various grades. *A*, normal prostate tissue, *C–D*, grades 3 and 5 prostate carcinoma, treated with anti-*Drg-1* antibody. *B*, normal prostate tissue treated with preimmune serum as negative control. *B*, association of Drg-1 with other clinical parameters. In each case, χ^2 test was performed to test the significance of association. *, statistically significant correlation ($P < 0.05$).

(B)

	Drg-1 expression			P value
	All (62)	positive (34)	reduced (28)	
Age (mean +/- S.E. yrs.)	72.0 +/- 1.0	72.6 +/- 1.3	71.2 +/- 1.6	0.9
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015*
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001*
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044*
Metastasis status				
Organ confined	40	28	12	
Lymph node metastasis	20	5	15	0.003*
Bone metastasis	19	5	14	0.006*

of each of the 20 and 19 cases with lymph node and bone metastasis, respectively, were positive for *Drg-1* expression. Thus, the negative correlation of Drg-1 with metastatic spread to lymph node and bone is highly significant ($P = 0.003$ and 0.006, respectively) and, in fact, is much stronger than the positive correlation with Gleason scores. These results strongly suggest a negative involvement of Drg-1 in the metastatic process in prostate cancer.

To evaluate the importance of the *Drg-1* gene as a prognostic marker, we chose 43 patients dating from 1988 to 1997, and examined whether the down-regulation of Drg-1 with the advancement of prostate carcinoma correlates with survival. As shown in Fig. 2, patients with Drg-1 positive expression had significantly more favorable prognosis than those with reduced expression (log rank *P*, 0.002). In Cox regression analysis in univariate mode, the *Drg-1* gene expression had a significant predictive value ($P = 0.0256$), although it was less predictive than lymph node or bone metastasis ($P < 0.001$). Nevertheless, the reduced expression of *Drg-1* can be a strong predicator of lymph node and bone metastasis and, in turn, of survival. Therefore, these data underscore the clinical relevance of this gene in the advancement of prostate cancer.

Drg-1 Suppresses Invasiveness of Prostate Cancer Cells *in Vitro*. To understand the functional role of the *Drg-1* gene in tumor progression, we first established permanent cell lines expressing Drg-1 using the human prostate carcinoma cell line ALVA and highly metastastic Dunning-rat prostate carcinoma cell line AT6.1. These cell lines were examined for the rate of *in vitro* proliferation and anchorage-independent colony formation. As shown in Fig. 3, *A* and *B*, the growth rate of cloned cells did not show a significant difference from the parent cell in either assay. These cell lines also did not show any notable morphological changes. These results suggest that Drg-1 does not affect the growth or morphology of prostate tumor cells. Because the expression of the *Drg-1* gene is inversely correlated with metastasis in clinical samples, we examined the effect of Drg-1 on cell motility and invasiveness. As shown in Fig. 3*C*, the motile ability of the clones was virtually identical to the parental cell lines. However, expression of Drg-1 significantly reduced *in vitro* invasion (up to 90%

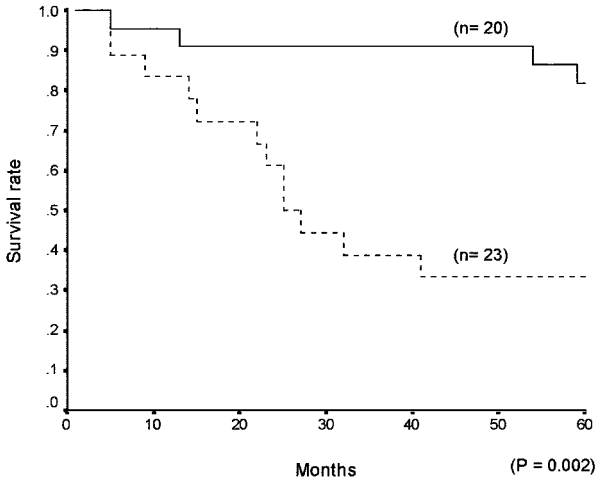


Fig. 2. Drg-1 expression is correlated with overall survival rate. Overall survival rate over a period of 5 years was measured in 43 patients with prostate cancer, in relation to Drg-1 expression. Solid line, Drg-1 positive patients; *dotted* line, patients with reduced expression of Drg-1. *P* was determined by log-rank test.

in AT6.1 cell line and 20–30% in ALVA) when they were tested by an *in vitro* Matrigel assay (Fig. 3*D*). These data suggest that Drg-1 suppresses the invasive ability of prostate cancer cells *in vitro*, which is consistent with the results of the immunohistochemical analysis of clinical specimens.

Drg-1 Suppresses Spontaneous Lung Metastasis of Prostate Cancer Cells *in Vivo*. To further investigate the role of Drg-1 in primary tumor growth as well as invasion and metastasis *in vivo*, the metastatic cell line AT6.1, AT6.1 transfected with vector only, and five Drg-1-transfected clones (Drg-1 numbers 4, 7, 8, 10, and 12) were individually injected s.c. into the dorsal flanks of SCID mice. As shown in Fig. 4*A*, Western blot analysis indicated that only the clones 4, 7, 8, and 10 expressed Drg-1 protein, whereas AT6.1, the vector-only clone, and the clone number 12 did not have a detectable level of Drg-1 expression. Therefore, the vector-only clone and clone number 12 served as negative controls. The mice were monitored for the formation and the growth rate of tumors and were then sacrificed at 4 weeks after the inoculation of the cells. At the experimental end point, their lungs were removed, and the number of metastatic lesions was grossly counted. As shown in Fig. 4*C*, all of the clones and the parental cells formed primary tumors in the animals with similar growth rates during the 4-week period, suggesting that Drg-1 does not have an effect on tumorigenesis and tumor growth. On the other hand, the four clones (numbers 4, 7, 8, and 10) that were positive for *Drg-1* expression showed a significantly lower incidence of lung metastases compared with the parental cell line AT6.1, the vector-only clone, and clone number 12. These results strongly suggest that Drg-1 can suppress the metastatic process of prostate cancer cells without affecting tumorigenicity *in vivo*.

Discussion

The *Drg-1* gene was previously reported by two researcher groups (6, 15) to play an important role in colon and bladder cancer, based on the results of animal experiments. However, their results are inconsistent with regard to the step at which *Drg-1* is involved. It appears that *Drg-1* suppresses the tumorigenesis of bladder cancer cells, whereas it interrupts the distant metastasis of colon cancer cells

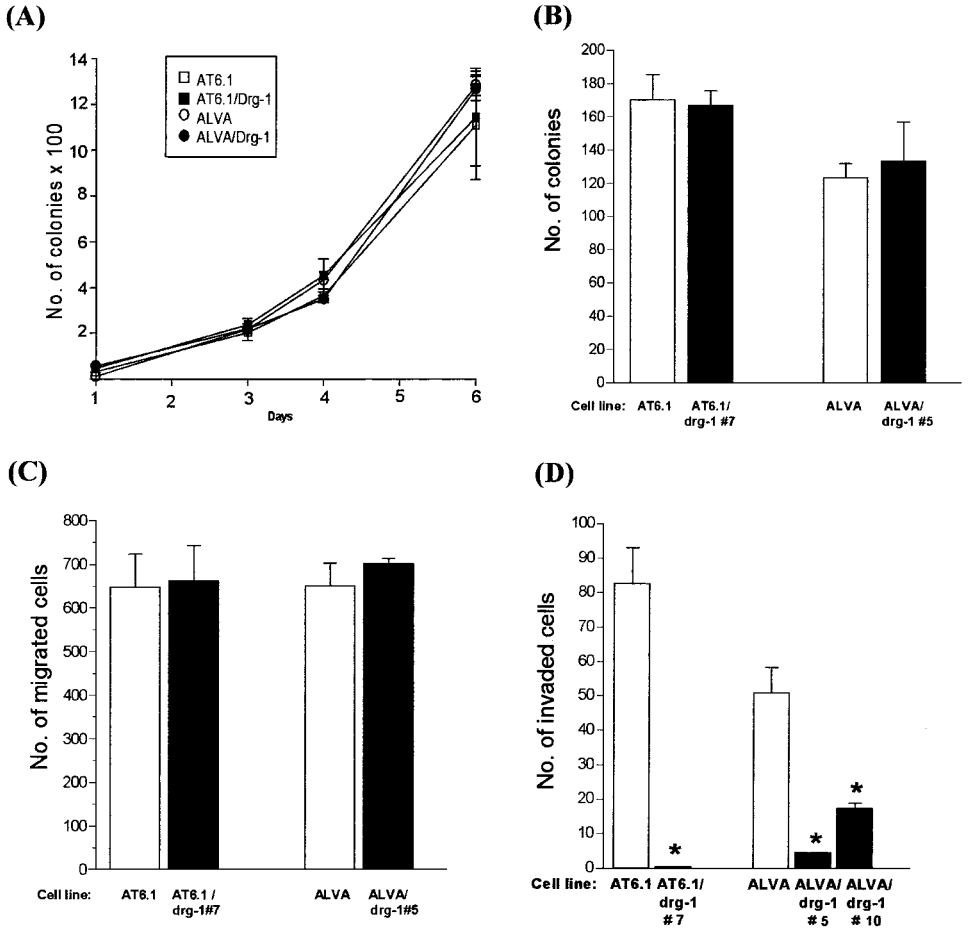
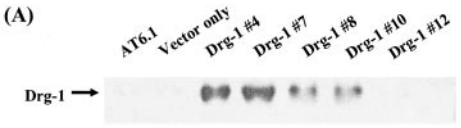
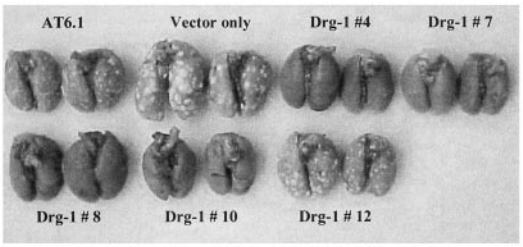


Fig. 3. *In vitro* characteristics of Drg-1. Drg-1 cDNA was transfected into AT6.1 and ALVA cell lines, and clones were established; these clones were studied in comparison with the parental lines. *A*, *in vitro* cell growth assay. *B*, anchorage-independent growth assay in soft agar. *C*, motility assay using cell culture inserts without any reconstituted extracellular matrix. *D*, invasion assay using Matrigel-coated invasion chamber. All of the assays were done in triplicate. *, statistically significant difference ($P < 0.05$).

(A)



(B)



(C)

Cell line	Drg-1[a]	Tumor incidence[b]	In vivo doubling time	Lung metastases Mean+/-SE[d]	Median[d]	(Range)[e]	P value
AT6.1	-	5/5	3.4 +/- 0.3	153.7 +/- 22.0	150	(116-195)	
Vector only	-	5/5	3.3 +/- 0.4	134.5 +/- 22.8	110	(78-240)	0.7
Drg-1 #4	+	5/5	3.4 +/- 0.3	13.7 +/- 6.6	10	(3-32)	<0.001*
Drg-1 #7	+	5/5	3.0 +/- 0.2	5.8 +/- 2.5	2	(2-14)	<0.001*
Drg-1 #8	+	5/5	2.9 +/- 0.4	11.4 +/- 5.5	9	(0-11)	<0.001*
Drg-1 #10	+	5/5	3.2 +/- 0.5	1.0 +/- 0.5	1	(0-3)	<0.001*
Drg-1 #12	-	5/5	3.4 +/- 0.4	176 +/- 33.1	180	(80-280)	0.7

a. Drg-1 expression was examined by western blot.
b. No. of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.
c. No. of metastatic lesions on lungs were counted macroscopically after 4 weeks of subcutaneous inoculation.
d. No. of metastatic lesions on lungs per SCID mouse.
e. No. of metastatic lesions on lungs per group of SCID mice.

Fig. 4. *Drg-1* suppresses spontaneous lung metastasis without affecting growth of primary tumor. *A*, the parental cell line (*AT6.1*), cells transfected with vector (*Vector only*), and Drg-1-transfected clones (*#4, #7, #8, #10,* and *#12*) were tested for Drg-1 protein expression by Western blot using anti-Drg-1 rabbit polyclonal antibody. Each of these cell lines was injected s.c. into SCID mice (5 mice/group). After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from two mice from each group are shown as examples (*B*). The number of tumor-bearing mice, primary tumor growth rate and metastases formation are summarized (*C*).

without affecting their growth rate at the site of primary tumor. More importantly, the clinical significance of the *Drg-1* gene has not been clarified by statistical analysis in any type of cancer. In this report, we have shown that the expression of the *Drg-1* gene is inversely correlated with the Gleason grading, which is the most well-established pathological criterion for judging the clinical stage and malignant progression in prostate cancer. We have also shown that *Drg-1* expression is strongly correlated with patients' five-year survival, suggesting a potential usefulness of Drg-1 as a prognostic marker. In particular, we observed that the down-regulation of Drg-1 expression was more significant in patients with metastasis to lymph nodes. Consistent with these observations, the results of our animal experiment indicate that Drg-1 was capable of suppressing lung metastasis of prostate cancer cells, whereas it did not affect the growth of primary tumor. Therefore, Drg-1 appears to function as a metastasis suppressor in prostate cancer.

The molecular mechanism underlying the metastasis suppression by Drg-1 is of considerable interest, although this remains to be investigated. However, it should be noted that Drg-1 was previously shown to be up-regulated by the tumor suppressors, *PTEN* and *p53*, and that these tumor suppressors are known to be able to down-regulate metastasis-related genes such as *MMP-1*, *-2*, and *-13* (16–18). Therefore, it is possible that Drg-1 is a part of the pathway of down-regulation of those protease genes by PTEN and p53, which may account for the metastasis suppressor function of the *Drg-1* gene. In fact, the results of our *in vitro* experiments indicate that Drg-1 suppressed the invasiveness of prostate cancer cells, which is also in good agreement with the results reported by Guan *et al.* (15), who found that overexpression of Drg-1 suppressed *in vitro* invasiveness of colon cancer cells. PTEN and p53 are also known as negative regulators of angiogenesis, which is a critical factor for both tumor growth and metastasis (19, 20). Activation of both genes lead to the inhibition of *VEGF* gene expression and, hence, suppression of angiogenesis. Therefore, it is conceivable that the down-regulation of Drg1 by PTEN and p53 may also be a part of a pathway of suppression of angiogenesis which leads to metastasis suppression.

Drg-1 has been known to be a stress-response gene, and the expression of this gene is induced by various chemical agents and antineoplastic drugs such as tunicamycin, meracptoethanol, homocysteine, and CPT-11. Motwani *et al.* recently found that the activation of Drg-1 by CPT-11, a topoisomerase I inhibitor, resulted in the suppression of apoptosis of cancer cells, which, in turn, became drug resistant (21). Therefore, Drg-1 appears to be involved not only in tumor progression but also in drug resistance. It is noteworthy that these chemical agents are able to induce stress-activated protein kinase (JNK/SAPK) through the activation of MKK4. Interestingly, MKK4 was recently shown to suppress metastasis of prostate cancer *in vivo* without affecting primary tumor growth (22). This raises a possibility that Drg-1 may lie in the down-stream of the MKK4 pathway, which leads to metastasis suppression, although this possibility needs to be examined in detail.

Metastasis remains the ultimate and major cause of mortality in cancer-related deaths. The process of metastasis is complex, involving multiple steps, and is, therefore, poorly understood as yet at the cellular and molecular level. There was a significant advancement in our understanding of this process with the recent discovery of the metastasis suppressor genes that, by definition, suppress the formation of metastases without affecting the growth rate of the primary tumor. To date, only five genes have been identified that meet this criterion, namely *NM23*, *KAI1*, *KISS1*, *BRMS1*, and *MKK4* (23). Our data presented in this report clearly indicate that the *Drg-1* gene belongs to this category. Further investigation on the molecular mechanism of the function of this gene would provide information about rational therapeutic approaches for metastatic disease.

References

1. van Belzen, N., Dinjens, W. N., Diesveld, M. P., Groen, N. A., van der Made, A. C., Nozawa, Y., Vlietstra, R., Trapman, J., and Bosman, F. T. A novel gene which is up-regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms. Lab. Investig., *77:* 85–92, 1997.
2. Okuda, T., and Kondoh, H. Identification of new genes *ndr2* and *ndr3* which are related to *Ndr1/RTP/Drg1* but show distinct tissue specificity and response to N-*myc*. Biochem. Biophys. Res. Commun., *266:* 208–215, 1999.
3. Zhou, R. H., Kokame, K., Tsukamoto, Y., Yutani, C., Kato, H., and Miyata, T. Characterization of the human NDRG gene family: a newly identified member, *NDRG4*, is specifically expressed in brain and heart. Genomics, *73:* 86–97, 2001.
4. Kokame, K., Kato, H., and Miyata, T. Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis. J. Biol. Chem., *271:* 29659–29665, 1996.
5. Zhou, D., Salnikow, K., and Costa, M. *Cap43*, a novel gene specifically induced by Ni^{2+} compounds. Cancer Res., *58:* 2182–2189, 1998.
6. Kurdistani, S. K., Arizti, P., Reimer, C. L., Sugrue, M. M., Aaronson, S. A., and Lee, S. W. Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. Cancer Res., *58:* 4439–4444, 1998.
7. Lin, T. M., and Chang, C. Cloning and characterization of *TDD5*, an androgen target gene that is differentially repressed by testosterone and dihydrotestosterone. Proc. Natl. Acad. Sci. USA, *94:* 4988–4993, 1997.
8. Shimono, A., Okuda, T., and Kondoh, H. N-myc-dependent repression of *ndr1*, a gene identified by direct subtraction of whole mouse embryo cDNAs between wild type and N-myc mutant. Mech. Dev., *83:* 39–52, 1999.
9. Yamauchi, Y., Hongo, S., Ohashi, T., Shioda, S., Zhou, C., Nakai, Y., Nishinaka, N., Takahashi, R., Takeda, F., and Takeda, M. Molecular cloning and characterization of a novel developmentally regulated gene, *Bdm1*, showing predominant expression in postnatal rat brain. Brain Res. Mol. Brain Res., *68:* 149–158, 1999.

10. Agarwala, K. L., Kokame, K., Kato, H., and Miyata, T. Phosphorylation of RTP, an ER stress-responsive cytoplasmic protein. Biochem. Biophys. Res. Commun., *272:* 641–647, 2000.
11. Park, H., Adams, M. A., Lachat, P., Bosman, F., Pang, S. C., and Graham, C. H. Hypoxia induces the expression of a 43-kDa protein (PROXY-1) in normal and malignant cells. Biochem. Biophys. Res. Commun., *276:* 321–328, 2000.
12. Unoki, M., and Nakamura, Y. Growth-suppressive effects of *BPOZ* and *EGR2*, two genes involved in the PTEN signaling pathway. Oncogene, *20:* 4457–4465, 2001.
13. Sato, N., Kokame, K., Shimokado, K., Kato, H., and Miyata, T. Changes of gene expression by lysophosphatidylcholine in vascular endothelial cells: 12 up-regulated distinct genes including 5 cell growth-related, 3 thrombosis-related, and 4 others. J. Biochem. (Tokyo), *123:* 1119–1126, 1998.
14. Piquemal, D., Joulia, D., Balaguer, P., Basset, A., Marti, J., and Commes, T. Differential expression of the *RTP/Drg1/Ndr1* gene product in proliferating and growth arrested cells. Biochim. Biophys. Acta, *1450:* 364–373, 1998.
15. Guan, R. J., Ford, H. L., Fu, Y., Li, Y., Shaw, L. M., and Pardee, A. B. *Drg-1* as a differentiation-related, putative metastatic suppressor gene in human colon cancer. Cancer Res., *60:* 749–755, 2000.
16. Sun, Y., Sun, Y., Wenger, L., Rutter, J. L., Brinckerhoff, C. E., and Cheung, H. S. p53 down-regulates human *matrix metalloproteinase-1* (*Collagenase-1*) gene expression. J. Biol. Chem., *274:* 11535–11540, 1999.
17. Koul, D., Parthasarathy, R., Shen, R., Davies, M. A., Jasser, S. A., Chintala, S. K., Rao, J. S., Sun, Y., Benvenisite, E. N., Liu, T. J., and Yung, W. K. Suppression of *matrix metalloproteinase-2* gene expression and invasion in human glioma cells by MMAC/PTEN. Oncogene, *20:* 6669–6678, 2001.
18. Sun, Y., Cheung, J. M., Martel-Pelletier, J., Pelletier, J. P., Wenger, L., Altman, R. D., Howell, D. S., and Cheung, H. S. Wild type and mutant p53 differentially regulate the gene expression of *human collagenase-3* (*hMMP-13*). J. Biol. Chem., *275:* 11327–11332, 2000.
19. Koul, D., Shen, R., Garyali, A., Ke, L. D., Liu, T. J., and Yung, W. K. *MMAC/PTEN* tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer. Int. J. Oncol., *21:* 469–475, 2002.
20. Dachs, G. U., and Tozer, G. M. Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. Eur. J. Cancer, *36(13 Spec No):* 1649–1660, 2000.
21. Motwani, M., Sirotnak, F. M., She, Y., Commes, T., and Schwartz, G. K. Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells. Cancer Res., *62:* 3950–39555, 2002.
22. Kim, H. L., Griend, D. J., Yang, X., Benson, D. A., Dubauskas, Z., Yoshida, B. A., Chekmareva, M. A., Ichikawa, Y., Sokoloff, M. H., Zhan, P., Karrison, T., Lin, A., Stadler, W. M., Ichikawa, T., Rubin, M. A., and Rinker-Schaeffer, C. W. Mitogen-activated protein kinase kinase 4 metastasis suppressor gene expression is inversely related to histological pattern in advancing human prostatic cancers. Cancer Res., *61:* 2833–2837, 2001.
23. Yoshida, B. A., Sokoloff, M. M., Welch, D. R., Rinker-and Schaeffer, C. W. Metastasis-suppressor genes: a review and perspective on an emerging field. J. Natl. Cancer. Inst. (Bethesda), *92:* 1717–1730, 2000.