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13. ABSTRACT (Maximum 200 Words)

Prostate cancer is the most frequently diagnosed cancer among men in the United States. It represents approximately 7% of all cancer deaths and ranks as the second leading cause of cancer death in males. The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene. Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test this hypothesis, we used gp-Fy knockout mice and injected prostate tumor cells that do or do not express KAI1 gene. Our preliminary results indicate that the cancer cells expressing KAI1 are capable of attaching to vascular endothelial cells through direct interaction of KAI1 and gp-Fy proteins, and this interaction leads to inhibition of tumor cell proliferation. Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

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INTRODUCTION

The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (1). Ample evidence from both clinical data and the results of in vitro as well as animal experiments overwhelmingly support the notion that KAI1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis (2). Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test our hypothesis, we will examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo. We also plan to identify specific peptide sequences that activate KAI1 and to assess the efficacy of the peptides on tumor growth in an animal model. Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

BODY

Task 1. To examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo.

We previously presented our preliminary data showing that KAI-expressing tumor cells attach to endothelial cells that express gp-Fy, and this interaction appears to results in tumor cell growth inhibition. To further corroborate the notion of growth arrest of tumor cells upon interaction with gp-FY on endothelial cell surface, GFP-tagged prostate carcinoma cells, AT6.1 and their KAI1 overexpressing derivatives, were mixed with HBME or HUVEC cells (gp-FY$^+$) in suspension, and plated followed by selection for GFP$^+$ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when KAI1$^+$ cells (AT6.1/Flag-KAI1), as compared to KAI1$^-$ cells (AT6.1), interacted with HBME or HUVEC (Fig. 1). In order to confirm whether this effect is mediated by gp-Fy in the endothelial cells, similar experiment was performed where the tumor cells with or without KAI1 expression (AT6.1/Flag-KAI1 or At6.1) were mixed with cells with or without gp-Fy expression (AT6.1/Flag-Fy or At6.1). We found that the prostate tumor cells expressing KAI1 (AT6.1/Flag-KAI1) formed significantly fewer

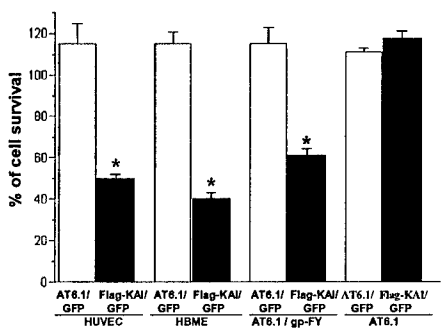

Fig. 1. Interaction between KAI1 and gp-Fy causes growth arrest in prostate cancer cells. Prostate cancer cells expressing both KAI1 and GFP genes (AT6.1/Flag-KAI1, closed bars) or cells expressing only GFP (AT6.1, open bars) were mixed with cells expressing gp-Fy (HUVEC, HBME or AT6.1/Flag-Fy) or not expressing gp-Fy (AT6.1). They were incubated for 1 hour at 37°C. The mixture was then plated in the presence of hygromycin which allowed growth of only AT6.1/Flag-KAI1 cells. After 5 days, the number of colonies was counted under a fluorescent microscope.

number of colonies than the KAI1$^-$ cell line (AT6.1) when mixed with cells expressing gp-FY (Fig. 1). On the other hand, there was no significant difference in the number of colonies between the two cell lines when allowed to interact with AT6.1(gp-FY$^-$).

To examine whether the interaction between KAI1 and gp-FY is essential for the metastasis suppressor function of KAI1 *in vivo*, we utilized the gp-Fy$^{-/-}$ mice which was previously developed by targeting the exon1, 2 and intron of the gp-Fy gene[3]. These mice were indistinguishable from wild-type and heterozygous littermates in terms of size, development, and health, anatomy of various organs, or immune system except for neutrophil migration in peritoneal inflammations induced by lipopolysaccharide and thioglycolate. Since blastocysts of C57BL/6J mice were used to establish the chimera for the generation of the gp-Fy$^{-/-}$ mice, the syngenic metastatic tumor cell line, B16BL6, was chosen in order to establish xenograft tumors in these mice. Flag-tagged KAI1 gene or the empty vector was overexpressed in B16BL6 cells, and several clones were obtained. We subcutaneously injected various clones with or without KAI1 overexpression into the gp-Fy$^{-/-}$ mice as well as its heterozygote and wild-type littermates. The primary growth rates of the mice were monitored and the pulmonary metastases were examined at autopsy. Primary tumor developed in 100% of the mice, and the growth rate or final volume of the tumors did not significantly vary with the KAI1 level in the grafted tumor cells or with gp-Fy status of the mice (Table 1 . However, the KAI1-positive clones developed significant number of pulmonary metastases in the gp-Fy$^{-/-}$ mice, while metastasis was found to be almost completely abrogated when the same clones were injected in the heterozygote and wild-type littermates (Table 1). The tumor cells lacking KAI1 (B16BL6/vector), however, equally metastasized in all the three groups of mice. Thus, in the absence of the gp-Fy gene, even the tumor cells expressing high level of KAI1 recapitulated the metastatic phenotype of downregulation of the KAI1 gene. These results of our animal experiment strongly support our notion that gp-Fy plays a critical role in the metastasis suppressor function of the KAI1 gene *in vivo*.

Clone #	KAI1 expression	Tumor volume (mean+/- S.E.)			Incidence of pulmonary metastasis			
		Fy+/+	Fy-/-	Fy+/-	Fy+/+	Fy-/-	Fy+/-	P value
2	positive	4.9+/-0.03	4.5+/-0.02	4.5+/-0.01	2/15(13.3%)	9/15(60%)	1/15(6.7%)	0.02*, 0.008**
empty vector	negative	4.9+/-0.05	4.8+/-0.05	4.9+/-0.03	6/15(40%)	5/14(35.7%)	5/14(35.7%)	0.8*, 0.89**

Table 1. B16BL6 cells expressing KAI1 (B16BL6/KAI1 #2) or empty vector (B16BL6/vector) was injected subcutaneously into gp-Fy knockout (gp-Fy$^{-/-}$) mice, the heterozygote (gp-Fy$^{-/+}$) and wild-type (gp-Fy$^{+/+}$) littermates. The tumor volumes of the mice were measured, and the lungs were observed for macroscopic metastatic lesions after euthanasia. The results of the animal experiments are summarize.

Task 2. To identify specific peptide sequences that activate KAI1 and to assess the efficacy of the peptides on tumor growth in an animal model.

In order to localize the domains that are essential for the interaction of KAI1 and gp-Fy, we tested several individual domains as well as serial deletions from the N-terminus of the KAI1 gene against full length gp-Fy target and vice versa (Fig. 2). Our results indicate that the first 32 amino acids from the N-terminus of KAI1, spanning the first intracellular and transmembrane domains, are dispensable for the interaction, but the conformation of the protein as a whole may be important as none of the other fragments yielded a positive interaction. On the other hand, deletion of the first extracellular domain of gp-Fy at N-terminus completely abrogated the interaction, suggesting that the N terminus of gp-Fy is essential for binding to KAI1.

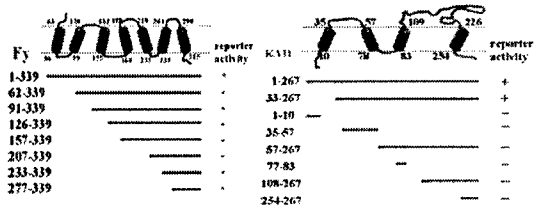

Fig. 2. Analysis of interacting domains of KAI1 and gp-Fy. Regions of KAI1 and gp-Fy, as indicated by the amino acid sequence numbers were tested as baits in the yeast mating assay for interaction with full length gp-Fy and KAI1, respectively. '+' indicates positive interaction and '-' indicates lack of interaction.

KEY RESEARCH ACCOMPLISHMENTS

1. We have obtained the evidence showing that the cancer cells that expresses the KAI1 gene bind to vasculature endothelial cells through specific interaction between KAI1 on the cancer cells and gp-Fy protein expressed on endothelium.
2. We have shown that this interaction result in growth arrest of tumor cells.
3. We have established gp-Fy kockout mice colony.
4. Using the knockout mice, we have obtained preliminary data to show that the gp-Fy gene is necessary for the suppressor function of KAI1.
5. We localized the essential region of gp-FY which interacts with Kai1.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Pai, S.K., Hirota, S., Hosobe, S., Takano Y., Saito, K., Piquemal, D., Commes, T., Watabe, M., Gross, S.C., Wang, Y., Ran S., and Watabe K. (2004) Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression. *Oncogene*, 23, 5675-81
2. Bandyopadhyay, S., Pai, S.K., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Takano Y., Saito, K., Piquemal, D., Commes, T., Watabe, M., Gross, S.C., Wang, Y., Huggenvik,

J.and Watabe K (2004) PTEN up-regulates the tumor metastasis suppressor gene Drg-1 in prostate and breast cancer. *Cancer Research.* 64(21):7655-60.

Abstract/presentation

1. Bandyopadhyay S, Watabe M, Goodarzi G, Gross SC, Pai SK, Hirota S, Hosobe S, Miura K, Saito K, Wang Y, and Watabe K. Tumor metastasis suppressor gene Drg-1 is controlled by PTEN. (2004) American Association for Cancer Research. Orlando, FL.
2. Bandyopadhyay, S and Watabe, K . Roles of tumor metastases suppressor gene Kai1 in prostate and breast cancer (2004). Southern Illinois University, School of Medicine, Cancer Institute seminar

Employment

1. Dr. Sucharita Bandyopadhaya (Postdoc) was supported by the current grant.
2. Rui Zhan (currently in Ph.D. program) was partly supported by the current grant.

CONCLUSIONS

Our results indicate that KAI1 indeed specifically interacts with gp-Fy protein which is predominantly expressed on vascular endothelial cells. This notion is also supported by our preliminary data generated by using gp-Fy knockout mice. We are still in the process of gathering more data to solidify our conclusions by conducting both in vitro and in vivo experiments as proposed. Overall, our project is in good progress although it is little slower than expected. This is mainly due to the initial difficulty of expanding the gp-Fy knockout colony. However, the colony is now fully established and the experiments are underway. We also have a good progress in identifying the region of gp-Fy which interacts with Kai1. If we can further narrow down the region, we will synthesize various peptides and test them for anti-proliferation activity.

"So what"

Our results so far strongly support our hypothesis that KAI1 exert it's suppressor function through direct interaction with go-Fy. If proven to be true, it will open a new avenue to develop a therapeutic method for prostate cancer.

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Oncogene (2004) 23, 5675–5681

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SHORT REPORTS

Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression

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The differentiation-related gene-1 (Drg-1) was first identified as a gene strongly upregulated by induction of differentiation in colon carcinoma cells *in vitro*, and later the same gene was shown to suppress tumorigenicity of human bladder cancer cells *in vivo*. On the other hand, we and others have demonstrated that the Drg-1 gene suppresses prostate and colon cancer metastases in mouse models. In the context of such potential organ-specific differential function of the Drg-1 gene, the present study was designed to clarify the expression status, regulation and function of Drg-1 in the case of human breast cancer. We found that the expression of the Drg-1 protein was significantly reduced in breast tumor cells, particularly in patients with lymph node or bone metastasis as compared to those with localized breast cancer. Drg-1 expression also exhibited significant inverse correlation with the disease-free survival rate of patients and emerged as an independent prognostic factor. The downregulation of the Drg-1 gene appeared to be largely at the RNA level, and the DNA methylation inhibitor, 5-Azacytidine, significantly elevated the Drg-1 gene expression in various breast tumor cell lines. Furthermore, we found that overexpression of the Drg-1 gene suppresses the invasiveness of breast cancer cells *in vitro*, and this suppression was also achieved by treatment of cells with 5-Azacytidine. Together, our results strongly suggest functional involvement of the Drg-1 gene in suppressing the metastatic advancement of human breast cancer.
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Keywords: marker; metastasis; invasion; methylation

The differentiation-related gene-1 (Drg-1) gene was originally identified by differential display as being significantly upregulated by induction of differentiation in colon carcinoma cells *in vitro* (van Belzen *et al.*, 1997). The gene was mapped to human chromosome 8q24.2, and Drg-1 mRNA was found to be expressed almost ubiquitously with the highest level of expression in kidney, prostate, ovary and intestine (Guan *et al.*, 2000). Drg-1 encodes a 43 kDa protein that possesses three unique 10-amino-acid tandem repeats at the C-terminal end (van Belzen *et al.*, 1997). However, the biochemical function of the protein remains largely unknown. Interestingly, using the differential display technique, several groups independently identified Drg-1 as a target of various stimuli, including hypoxia, androgen, homocysteine, Ni^{2+}, N-Myc and PTEN (Kokame *et al.*, 1996; Lin and Chang, 1997; Zhou *et al.*, 1998; Okuda and Kondoh, 1999; Ulrix *et al.*, 1999; Park *et al.*, 2000; Unoki and Nakamura, 2001). In addition, the tumor suppressors p53 and von Hippel–Lindau factor have been shown to modulate Drg-1 gene expression *in vitro* (Kurdistani *et al.*, 1998; Masuda *et al.*, 2003). Recently, we have shown that overexpression of the Drg-1 gene almost completely suppressed lung metastasis of prostate tumor cells in a SCID mouse model without affecting primary tumor growth (Bandyopadhyay *et al.*, 2003). The metastasis suppressor activity of Drg-1 has also been observed in colon cancer cells *in vivo* (Guan *et al.*, 2000). On the other hand, introduction of Drg-1 cDNA in human bladder cancer cells has been shown to suppress tumorigenicity in nude mice (Kurdistani *et al.*, 1998). Therefore, the results of these studies raise an interesting possibility of differential organ-specific function of Drg-1, while several *in vitro* studies indicate this gene to be the target of multiple regulatory pathways. Despite such intriguing function of Drg-1, relatively few data are available to assess the role of this gene in clinical setting of human cancer. Recently, as the first statistical evaluation of Drg-1 protein expression in the context of tumor progression, we have shown that the expression of the Drg-1 gene has statistically significant inverse correlation with advancement of prostate cancer and with patient survival (Bandyopadhyay *et al.*, 2003). In this report, considering the differential organ-specific function of Drg-1 in tumor progression, we sought to clarify the role of Drg-1 in human breast cancer progression. For the present study, 85 surgically resected breast tumor specimens were randomly selected from

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surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). The specimens dated from 1993 to 1997 and the ages of the patients ranged from 29 to 79 with a mean of 54 years. Histological grades of the tumors were determined by pathologists according to Bloom and Richardson criteria. Complete 5-year follow-up data were available for all the patients, and those who died of other causes were eliminated from the study. In order to examine the status of Drg-1 gene expression in clinical setting, immunohistochemical analysis of Drg-1 expression was performed on the above set of breast cancer patient samples. As shown in Figure 1A, we found that the Drg-1 protein was highly expressed in the epithelial cells of normal mammary duct–lobular units while the protein expression was generally reduced in the tumor cells. In all cases, the protein was detected at high level in the cytoplasm of normal mammary epithelial cells, and some of the normal ducts and lobules also had strong membranous expression of Drg-1. In addition, the basal cell layers showed high level of Drg-1, while the stroma did not have any detectable level of expression. For each breast cancer case, the level of Drg-1 expression was quantitatively judged by calculating the average staining intensity for all tumor cells in a given specimen following a previously used scoring criterion (Davol *et al.*, 2003). Staining intensities of the tumor cells were scored on a 0–3 scale (Figure 1B, a–d) by two independent persons blinded to the patients' outcomes and clinicopathological characteristics. These intensities were multiplied by the percentage of tumor cells at each intensity to reflect accurately the heterogeneity of the tumor staining. The results were then summed and divided by 100 to achieve a scale of 0–3. All the tumor specimens blinded to clinical characteristics were then randomized into 'Instructing' and 'Validating' groups of 40 and 45 patients, respectively, and a validation analysis was performed. Among all the potential cutoff points, 1.6 was found to be the best cut point in terms of predictivity. Accordingly, patients were divided into 'positive' ($\geqslant 1.6$) and 'reduced' (<1.6) with respect to Drg-1 expression. The two scorers had a 95% concordance in assignment of patients to positive *versus* reduced Drg-1 score. The Drg-1 protein was detected consistently in the normal mammary gland cells in all the cases, while the Drg-1 expression was significantly reduced in the tumor cells of nearly 30% patients (Figure 1C). Most importantly, a significant level (overall P-value $=0.01$) of differential expression of Drg-1 was observed between the patients with organ-confined disease ($T_xN_0M_0$) and those with metastasis to lymph node or bone ($T_xN_{1-3}M_0$ and $T_xN_{1-3}M_1$). As shown in Figure 1C, while only three patients (10.3%) exhibited reduced Drg-1 expression out of 29 localized breast cancer cases, 19 (37.3%) and three (60%) patients were negative for Drg-1 expression among the 51 and five cases with different degrees of metastases respectively ($P=0.02$ and 0.04). Thus, the expression level of Drg-1 had a significant negative correlation with metastatic spread to lymph node and bone, but did not show any significant correlation with the size or the histological grade of the primary tumor (Figure 1C). These results strongly suggest the negative involvement of Drg-1 in the process of metastasis to lymph node and bone in breast cancer.

Recently, Drg-1 mRNA expression has been shown to increase in a p53-dependent manner in certain bladder and breast cancer cell lines *in vitro* (Kurdistani *et al.*, 1998). Drg-1 expression has also been suggested to be modulated by androgen in prostate cancer cell lines, although there is some controversy (Lin and Chang, 1997; Ulrix *et al.*, 1999). To evaluate whether there is any relation between Drg-1 and p53 or estrogen receptor (ER) *in vivo*, we examined the status of these markers in the present set of breast cancer samples. For p53 and ER immunostaining, percentage of the tumor cells with positive nuclear staining was determined. In addition, 10 fields on each slide were randomly chosen and the expression of each of Drg-1, p53 and ER was comparatively observed in the same field. However, no significant correlation of Drg-1 protein expression was observed with either p53 or ER status (Figure 1C), suggesting that Drg-1 is predominantly regulated by pathways independent of p53 and estrogen signaling *in vivo*.

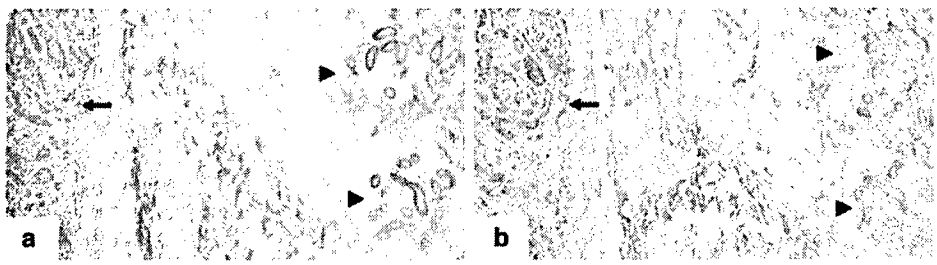
In order to determine whether the reduction of Drg-1 protein in tumor cells corresponded to the reduction at the mRNA level, RT–PCR analysis was performed. Four representative clinical samples were chosen that exhibited clear downregulation of Drg-1 in tumor cells by immunohistochemistry, and paired tumor and nontumor mRNA was analysed from each patient. As shown in Figure 1D, in three out of the four cases, there was significant reduction of Drg-1 mRNA in the tumor cells compared to the normal counterparts, while equal

Figure 1 Expression of Drg-1 in human breast cancer. (**A–C**) Immunohistochemical staining was performed on formaldehyde-fixed, paraffin-embedded human breast tissue sections as described before (Bandyopadhyay *et al.*, 2003). The following antibodies were used for this study: anti-Drg-1 rabbit polyclonal antibody (1 : 150); anti-p53 mouse monoclonal antibody (clone DO-7, Dako Corp., Carpentaria, CA, USA) (1 : 100); and anti-estrogen receptor mouse monoclonal antibody (clone 1D5, Dako Corp., Carpentaria, CA, USA) (1 : 70). (**A**) Cancer and normal tissues treated with Drg-1 antibody (**a**). Note that strong expression of Drg-1 is detected in normal mammary lobules (arrowhead), while the expression is found to be significantly reduced in the invasive tumor mass (arrow) in the same section. The same tissue section is treated with preimmune serum (**b**). (**B**) Drg-1 staining of four different breast tumors chosen to represent the four levels of staining intensity. (**a**), (**b**), (**c**) and (**d**) correspond to intensities 0, 1, 2 and 3, respectively. (**C**) Drg-1 expression with respect to other clinicopathological parameters was statistically analysed by standard χ^2 test using SPSS software. *Statistically significant difference ($P<0.05$). (**D**) Drg-1 expression is reduced at the RNA level in mammary tumor cells. Paired tumor (T1–T4) and normal cells (N1–N4) were isolated from the paraffin-embedded tissue sections of four selected subjects by microdissection under a microscope and total RNA was isolated using the Pinpoint RNA isolation system (Zymo Research, Orange, CA, USA). Total RNA was reverse-transcribed and the cDNA was then amplified with a pair of 5′ and 3′ primers for the Drg-1 gene (5′ GCGAATTCGCCACCATGTCTCGGGAGATGCAGGATG and 5′-ATGGTAGGTGAGGATGACAGG) or human β-actin (Stratagene). PCR was performed for 45 cycles using the following profile: 94°C, 1 min; 55°C, 1 min; 72°C, 1 min

amount of β-actin expression was observed in all the samples. This result suggests that the significant down-regulation of the Drg-1 gene in cancer cells is for the most part at the RNA level.

In order to evaluate the prognostic value of the Drg-1 gene, univariate analysis for disease-free survival over a 5-year period was performed on the 85 patients. As shown in Figure 2a and b, patients positive for Drg-1

A



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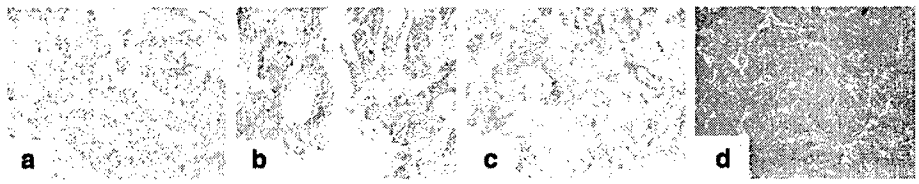
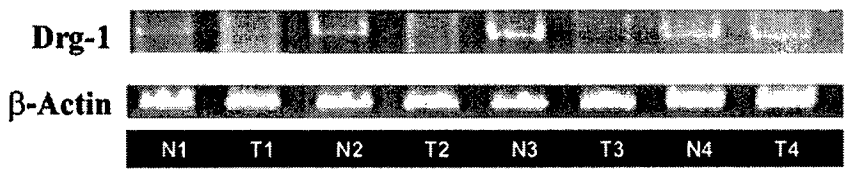


C

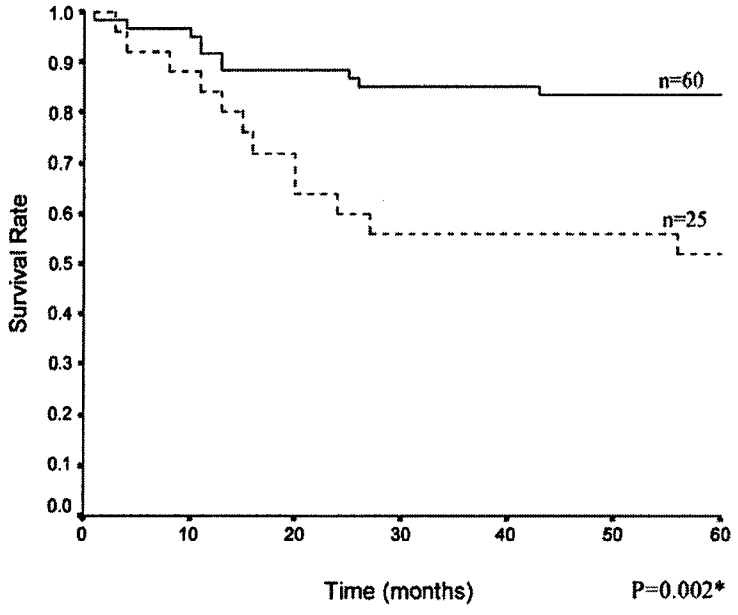
Relationship between Drg-1 and other clinical parameters

Factor	Total (85)	Drg-1 expression positive	Drg-1 expression reduced	P value
Age				
< 51	33	25 (75.8%)	8 (24.2%)	
>51	52	35 (67.3%)	17 (32.7%)	0.56
Histological grade				
I/II	30	24(80.0%)	6(20.0%)	
III	55	36(65.5%)	19(34.5%)	0.16
P53				
Wild type	57	40 (70.2%)	17 (29.8%)	
mutant	28	20 (71.4%)	8 (28.6%)	0.99
ER				
Positive	40	27 (67.5%)	13 (32.5%)	
Negative	45	33 (73.3%)	12 (26.7%)	0.73
Tumor status				
$T_{1-2}N_XM_X$	64	46 (71.9%)	18 (28.1%)	
$T_{3-4}N_XM_X$	21	14 (66.7%)	7 (33.3%)	0.86
Metastasis status				
$T_XN_0M_0$	29	26 (89.7%)	3 (10.3%)	
$T_XN_{1-3}M_0$	51	32 (62.7%)	19 (37.3%)	0.02*
$T_XN_{1-3}M_1$	5	2 (40.0%)	3 (60%)	0.04*

D



a



b

Univariate and multivariate analyses of DFS in breast cancer patients

		Univariate (log rank)	Multivariate (Cox regression analysis)		
Variables	**reference level**	**P**	**Hazard ratio**	**95% CI**	**P**
Drg-1	**positive**	**0.002***	**2.435**	**1.030 – 5.760**	**0.043***
Histological grade	**I/II**	**0.03***	**-**	**-**	**0.189**
Metastases	**organ-confined ($T_xN_0M_0$)**	**0.006***	**4.538**	**1.024 – 20.117**	**0.046***

Figure 2 Drg-1 expression is correlated with survival rate in breast cancer. (**a**) Disease-free survival (DFS) rate over a period of 5 years was analysed in 85 breast cancer patients in relation to Drg-1 expression by the Kaplan–Meier method, and *P*-value was determined by log-rank test. The solid and dotted lines indicate Drg-1-positive patients and patients with reduced expression of Drg-1, respectively. (**b**) Univariate survival analysis by log-rank test and multivariate analysis using Cox proportional hazards regression model were conducted to assess the independent contribution of the indicated factors to disease prognosis. In all cases, SPSS software was used

had significantly more favorable prognosis than those with reduced expression of the gene ($P = 0.002$, log-rank test). Among the various other parameters tested in univariate analysis, histological grade and metastasis status exhibited significant correlation with patient survival (Figure 2b), while levels of p53, ER, tumor size and patient's age did not have any significant correlation (data not shown). In multivariate Cox regression analysis involving Drg-1 expression, histological grade and metastasis status, Drg-1 emerged as a statistically significant independent prognostic factor (Figure 2b). The odds ratio for Drg-1 is 2.435 (95% CI 1.030–5.760, $P = 0.043$), implying that the death risk of patients with reduced Drg-1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with Drg-1 positivity. Thus, the reduced expression of Drg-1 can be a strong predicator of lymph node and bone metastases and, in turn, of patient survival. Therefore, these data underscore the clinical relevance of the Drg-1 gene in advancement of breast cancer.

Because the low levels of Drg-1 mRNA and protein expression strongly correlate with poor clinical outcome, the regulatory mechanism of this gene is of significant interest. One notable mechanism of transcriptional inactivation that has been defined in different types of human cancer is aberrant methylation of cytosines located 5′ to guanosines (CpG) in the promoter region of tumor suppressor and metastasis suppressor genes. Scanning the 5′ upstream region of the Drg-1 gene revealed two potential CpG islands, at positions −24 to −687 and −837 to −1494 upstream to the transcription start site, thereby suggesting that DNA methylation may contribute to the regulation of this gene. To test the possibility that alterations in DNA methylation could influence Drg-1 expression, cells from human breast carcinoma cell lines MDA-MB-435, MDA-MB-231, MDA-MB-468 and MCF-7 were treated for 4 days with DNA methylation inhibitor, 5-Azacytidine. In all the cell lines tested, demethylation resulted in a notable increase in the expression of Drg-1 at both mRNA and protein levels to different extents

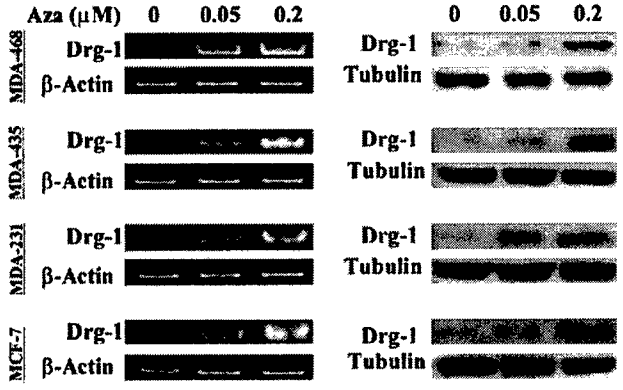


Figure 3 Drg-1 expression is modulated by DNA methylation. Human breast carcinoma cell lines MDA-MB-468, MDA-MB-435, MDA-MB-231 and MCF-7 were cultured in RPMI-1640 medium supplemented with 10% FCS, streptomycin (100 μg/ml), penicillin (100 U/ml) and 250 nM dexamethasone at 37°C in a 5% CO_2 atmosphere. The cells were treated with increasing concentrations of the methyltransferase inhibitor, 5-Azacytidine, or media alone, as indicated, for 4 days, and the media containing the inhibitor was refreshed each day. Expressions of Drg-1 and β-actin were examined by semiquantitative RT–PCR (left panel). Briefly, RNA was extracted by the RNeasy kit (Qiagen), and RT–PCR was performed using the same set of primers as described in Figure 1D legend. Expression of Drg-1 was also analysed by Western blot (right panel), using the anti-Drg-1 rabbit polyclonal antibody (1 : 5000), as described before (Bandyopadhyay *et al.*, 2003)

(Figure 3). In the case of MDA-MB-231 and MCF-7 cells, significant enhancement of Drg-1 protein was observed even by 0.05 μM 5-Azacytidine treatment, while in MDA-MB-435 and MDA-MB-468 cells, the significant effect was observed at 0.2 μM dose. In all cases, the expression of β-actin or tubulin remained unchanged (Figure 3) and the rate of proliferation remained unaffected (data not shown). These results suggest that Drg-1 expression at the transcriptional level is controlled at least in part by hypermethylation of CpG islands and that inhibition of the methylation is capable of restoring the Drg-1 expression, which is in good agreement with the previous finding in a colon cancer cell line (Guan *et al.*, 2000).

Since the expression of the Drg-1 gene is found to be inversely correlated with metastasis in the clinical samples, we examined the effect of Drg-1 on cell motility and invasiveness to obtain further insight into the functional role of Drg-1 in breast tumor progression. As shown in Figure 4a, overexpression of Drg-1 significantly reduced invasiveness of the MDA-MB-468 cells in Matrigel-coated invasion chamber ($P=0.01$), as compared to cells transfected with empty vector. However, the motile abilities of both the parental and transfected cells were virtually unaffected (Figure 4b). These data suggest that Drg-1 suppresses the invasive ability of breast cancer cells *in vitro*, which is consistent with the marked reduction of Drg-1 expression in patients with metastasis, as evident by the immunohistochemical analysis. This result is also in good agreement with the results previously reported by us and others for prostate and colon cancer cells, respectively (Guan *et al.*, 2000; Bandyopadhyay *et al.*, 2003). Since Drg-1 expression was found to be enhanced by demethylation of CpG islands, we tested whether the same demethylating agent could reduce the invasiveness of the MDA-MB-468 cells by restoring the Drg-1 expression. We found that 5-Azacytidine treatment reduced the invasive capability of MDA-MB-468 cells by approximately threefold ($P<0.05$), which was associated with enhancement of Drg-1 expression (Figure 4c and inset). These results suggest that the effect of 5-Azacytidine on invasiveness of mammary

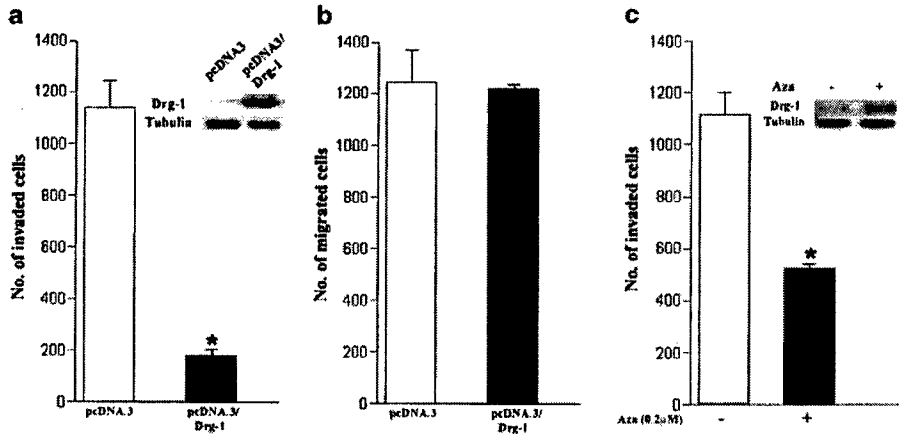


Figure 4 Drg-1 suppresses invasiveness of breast cancer cells *in vitro*. Drg-1 expression plasmid, pcDNA.3/Drg-1 (Bandyopadhyay *et al.*, 2003), or the vector, pcDNA.3, was transiently transfected into the MDA-MB-468 cell line by TransIT-TKO transfection reagent (Mirus Corporation, WI, USA). At 48 h post-transfection, the cells were collected and subjected to an *in vitro* invasion assay (**a**) and motility assay (**b**) as described before (Bandyopadhyay *et al.*, 2003). The expression of Drg-1 was tested by Western blot for Drg-1, as described in Figure 3 legend. (**c**) MDA-MB-468 cells were treated with (+) or without (−) 0.2 μM 5-Azacytidine for 4 days. The cells were then trypsinized and subjected to *in vitro* invasion assay. The same cells were also examined for Drg-1 and tubulin expression by Western blot (inset). All assays were performed in triplicate. *Statistically significant difference ($P<0.05$) as calculated by one-way ANOVA using SPSS software

tumor cells is at least partially mediated through Drg-1 and further support our notion that Drg-1 could act as a metastasis suppressor for breast cancer by affecting the step of invasion through the extracellular matrix.

The role of the Drg-1 gene in the context of human cancer has just begun to be explored. In this report, we have shown for the first time by statistical clinical evaluation that Drg-1 downregulation predicts breast cancer metastasis and poor survival. In addition to the diagnostic and prognostic value, understanding the mechanism of action of this gene is of considerable interest. Our results indicate that Drg-1 suppresses invasion of MDA-MB-468 breast tumor cells through the resconstituted extracellular matrix *in vitro*. Previously, it was shown that induction of MAPK activity by TGF alpha correlates with *in vitro* invasion in MDA-MB-468 cells, which can be inhibited by anti-sense MEK (Krueger *et al.*, 2001). Therefore, it is tempting to speculate that Drg-1 may cross-talk with the MAPK pathway components leading to suppression of invasion. It is noteworthy that another downstream component of the MAPK pathway, MKK4, has been shown to suppress metastasis of prostate and ovarian cancer *in vivo* (Kim *et al.*, 2001; Yamada *et al.*, 2002).

The organ-specific function of Drg-1 is also an intriguing observation. Drg-1 overexpression specifically suppressed metastases of prostate and colon carcinoma cells without affecting the primary tumor growth, while the same gene significantly reduced *in vivo* tumorigenicity of bladder cancer cells. This apparent contradiction could be at least partly explained by organ-specific microenvironment, since numerous studies have pointed toward the influence of microenvironment in tumor progression. However, the difference in microenvironments may not be sufficient to account for this discrepancy because Drg-1 overexpression *in vitro* was shown to cause cell growth arrest in EJ bladder cancer cell line, while the growth rates of the prostate and colon cancer cells were reported to be unaffected (Kurdistani *et al.*, 1998; Guan *et al.*, 2000; Bandyopadhyay *et al.*, 2003). Therefore, the distinctive property of the cell types is also considered to be an important factor leading to this phenomenon.

Our finding of the significant downregulation of Drg-1 with metastatic progression of breast cancer in clinical setting prompted us to explore the regulatory mechanism of this gene with the hope of restoring its expression in tumor cells. RNA analysis from clinical samples suggested that the downregulation of Drg-1 is at least partially at the RNA level. This is also consistent with a previous report in which Drg-1 mRNA was shown to be reduced in a small set of breast and prostate tumors by *in situ* hybridization (Kurdistani *et al.*, 1998). More importantly, we observed that reduced expression of Drg-1 could be restored by DNA methylation inhibition and that this was associated with restoration of the anti-invasive phenotype of the Drg-1 gene. Notably, metastasis suppressor gene nm23H1 has also been shown to be controlled by methylation of CpG islands, the inhibition of which restores the gene expression and its effect on motility suppression (Hartsough *et al.*, 2001). Alteration of the methylation pattern of genes that predict breast cancer metastasis, such as nm23H1 and Drg-1, can have a potential diagnostic value since sensitive methylation-specific PCR technique can detect the methylation pattern of the genes from tumor cells in ductal lavage fluid (Evron *et al.*, 2001).

In conclusion, the data presented in this paper clearly indicate the significant involvement of the Drg-1 gene in breast cancer progression and point toward a potential role of this gene in suppressing tumor metastasis, which remains the most crucial cause of mortality in breast cancer.

Acknowledgements

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Advances in Brief

PTEN Up-Regulates the Tumor Metastasis Suppressor Gene *Drg-1* in Prostate and Breast Cancer

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Abstract

***PTEN* (phosphatase and tensin homologue deleted on chromosome 10) has been shown to be inactivated in a wide variety of cancers, and the role of this gene as a tumor suppressor has been well established. On the other hand, results of recent animal studies as well as clinical evidence indicate that PTEN is also involved in tumor metastasis suppression. Although PTEN is known to play a key role in controlling cell growth and apoptosis, how PTEN exerts the metastasis suppressor function remains largely unknown. Recently, a microarray analysis identified the *Drg-1* gene (differentiation related gene 1) as one of the potential targets of PTEN. The *Drg-1* gene has been shown to suppress tumor metastasis in animal models of prostate and colon cancer, and the expression of this gene is significantly reduced with advancement of prostate and breast cancers in clinical setting. In this study, we explored the possibility that PTEN controls tumor metastasis by regulating the expression of the *Drg-1* gene. Our results indicate that overexpression of PTEN significantly augments the endogenous expression of Drg-1 protein, whereas inhibition of PTEN by small interfering RNA decreases Drg-1 in a dose- and time-dependent manner. We also found that the control of the *Drg-1* gene by PTEN seems to be at the transcriptional level, and that a phospho-Akt inhibitor restores the Drg-1 expression, indicating that PTEN controls Drg-1 by an Akt-dependent pathway. Consistent with these results, our immunohistochemical analysis revealed that PTEN expression correlates significantly with Drg-1 in both prostate and breast cancer cases. Furthermore, combination of the two markers, PTEN and Drg-1, emerged as a significantly better predictor of prostate and breast cancer patient survival than either marker alone.**

Introduction

PTEN (phosphatase and tensin homologue deleted on chromosome 10)/mutated in multiple advanced cancers was originally identified as a candidate tumor suppressor gene on chromosome 10q23.3 with dual specificity phosphatase function (1). Subsequently, it has been found that PTEN heterozygote mice show spontaneous tumor development in a variety of organs, including prostate and mammary glands, whereas homozygous deletion of the *PTEN* gene causes embryonic lethality (2, 3). Therefore, these observations confirmed the functional role of PTEN as a tumor suppressor. Consistent with these results, the *PTEN* gene has been shown to be frequently mutated in a wide variety of cancers, including glioblastoma, melanoma, endometrial, renal, prostate, breast, lung, and head and neck cancer (4). Notably, in most of these cases, PTEN inactivation was also found to have a significant correlation with invasiveness and metastasis, thus pointing toward a potential role of PTEN in metastatic advancement of these cancers (5–7). Indeed, recent studies with various mouse models have begun to reveal a functional involvement of PTEN in suppressing tumor metastasis. Using a series of hypomorphic *PTEN* mutant mice with decreasing PTEN activity, Trotman *et al.* (8) have shown that the extent of PTEN inactivation dictates metastatic progression of prostate cancer in a dose-dependent manner. In a separate study, Wang *et al.* (9) showed that mice with prostate-specific bi-allelic deletion of the *PTEN* gene spontaneously develop prostatic intraepithelial neoplasia lesions followed by invasive adenocarcinoma, and >50% of the animals develop pulmonary metastasis by 29 weeks of age. More direct link between PTEN and prostate cancer metastasis was shown by Davies *et al.* (10) who showed in an orthotopic mouse model that *ex vivo* treatment of PC3 prostate cancer cells with adenoviral PTEN completely inhibited lymph node metastases without inhibiting tumorigenicity. *In vivo* treatment of pre-established PC3 tumors with adenoviral PTEN also markedly diminished lymph node metastasis formation without causing significant regression of local tumor (10). These results agree well with the previous observation that reintroduction of the human 10q23–25 region into highly metastatic rat prostate cancer cells significantly suppressed metastasis without affecting their tumorigenic potential (11). The metastasis suppressor role of PTEN was also suggested in the case of a melanoma mouse model where overexpression of PTEN in B16F10 cells inhibited experimental pulmonary metastasis (12). Therefore, the results of these animal experiments strongly implicate the critical role of the *PTEN* gene in metastasis; however, the mechanism of metastasis suppression by PTEN remains an intriguing question.

Recently, Unoki *et al.* (13) did a microarray analysis and identified Drg-1 (differentiation related gene 1) as one of the several potential targets of PTEN. Notably, Drg-1 has been shown recently to play an important role in the context of human cancer progression (14–17). We have shown that expression of the *Drg-1* gene is inversely correlated with Gleason grades in prostate cancer, and importantly, this down-regulation is more significant in patients with metastasis to lymph nodes than in those with organ-confined disease (14). We have also observed similar inverse correlation of Drg-1 expression with metastasis in breast cancer patients (17). Consistent with these observations, the results of our animal experiment indicate that Drg-1 is capable of suppressing lung metastasis of prostate cancer cells without affecting the growth of primary tumor (14). Drg-1 has also been shown to exert similar metastasis-suppressive effect in colon cancer cells in a mouse model (15). These data strongly indicate a negative involvement of Drg-1 in the metastatic progression of cancer. Therefore, the results of the above studies raise a possibility that PTEN suppresses metastasis by regulating the expression of the *Drg-1* gene. Here, for the first time, we present evidence that PTEN augments Drg-1 expression via an Akt-dependent pathway *in vitro*, and this

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regulation occurs largely at the transcriptional level. Our results also indicate that expression of the *Drg-1* gene has significant positive correlation with PTEN expression status in prostate and breast cancer patients and that the combination of Drg-1 and PTEN has a better prognostic value than either marker alone.

Materials and Methods

Cell Lines. Human prostate cancer cell line PC3, DU-145, and breast cancer cell lines MDA-468 and BT-549 were obtained from American Type Culture Collection (Manassas, VA). Human prostate cancer cell line ALVA41 was kindly provided by Dr. W. Rosner (Columbia University, New York). All cell lines were cultured in RPMI 1640 supplemented with10% FCS, 100 μg/mL streptomycin, 100 units/mL penicillin, and 250 nmol/L dexamethasone, at 37°C in a 5% CO_2 atmosphere.

Expression Plasmids and Transfection. To create a mammalian expression plasmid of PTEN, total human placental RNA (Clontech, Palo Alto, CA) was reverse-transcribed, and the cDNA was PCR amplified. The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), and the resultant clone was designated as pcDNA3/PTEN. The protein and lipid phosphatase-deficient mutant form of PTEN, C124S, and its wild-type counterpart, each cloned into Flag-tagged vector, were kindly provided by Dr. M. L. Georgescu (M. D. Anderson Cancer Center, TX). To create a cell line with inducible PTEN expression, the tetracycline inducible system T-Rex (Invitrogen) was used. First, a derivative of human prostate cancer cell line PC3 (PC3MM) was transfected with the regulatory plasmid pcDNA6/TR encoding the Tet repressor, and a stable cell line (PC3MM/Tet) was generated by blasticidin selection (0.625 μg/mL). PTEN cDNA was cloned into the inducible expression vector pcDNA5/TO to obtain an inducible PTEN expression plasmid designated as Tet/PTEN. For construction of Drg-1-chloramphenicol acetyl transferase (Drg-1-CAT)–reporter plasmid, 1.5 kb long 5′upstream region of the Drg-1 gene was PCR-amplified from human genomic DNA and was cloned into the pBLCAT3 plasmid. For DNA transfection into ALVA and PC3 cells, Lipofectamine (Invitrogen) was used, whereas DU-145, MDA-468, and BT-549 were transfected by *trans*-TKO transfection reagent (Mirus Corp., Madison, WI). In each case, green florescent protein (GFP) expression plasmid was cotransfected, and the percentage of the GFP-positive cells was determined under fluorescent microscope to monitor the transfection efficiency.

Small interfering RNA Transfection. Four individual small interfering (si)RNAs against PTEN combined into one pool and one siRNA duplex targeting GFP were purchased from Dharmacon Inc. (Lafayette, CO). The *trans*-TKO transfection reagent was used to transfect the siRNA into the DU-145 cells.

Western Blot. Forty-eight hours after transfection, the cells were collected and subjected to Western blot with antibodies against PTEN (1:1,000, Upstate Biotechnology, Waltham, MA), Drg-1 (1:5000), tubulin (1;1,000, Upstate Biotechnology), phospho-Akt (1:500, Cell Signaling Technology), or total Akt (1:500, Cell Signaling Technology). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by ECL Plus system (Amersham Life Sciences, Piscataway, NJ).

Real-Time Reverse Transcription-PCR. Forty-eight hours after transfection of plasmid DNA, total RNA was isolated from the cells and reverse-transcribed. The cDNA was then amplified with a pair of forward and reverse primers for the Drg-1 gene (5′-ATGCAGGATGTAGACCTCGC and 5′-ATGGTAGGTGAGGATGACAGG) and for the human β-actin gene. PCR reactions were done with DNA engine opticon2 system (MJ Research, Waltham, MA) and the Dynamo SYBR Green qPCR kit (Finnzyme Corp., Oy, Finland). The thermal cycling conditions comprised an initial denaturation step at 95°C for 15 minutes followed by 30 cycles of PCR with the following profile: 94°C, 30 seconds; 57°C, 30 seconds; 72°C, 30 seconds.

Chloramphenicol Acetyl Transferase Reporter Assay. Twenty-four hours after transfection of plasmid DNAs, the expression of PTEN was induced by adding tetracycline (2 μg/mL). Cells were incubated for an additional 48 hours and then subjected to CAT assay as described previously (18). The reaction was done and acetylated [^{14}C]chloramphenicol was quantified with a PhosphorImager (Packard Instruments, Meriden, CT).

Tumor Specimens. Formaldehyde-fixed and paraffin-embedded tissue specimens from 81 prostate cancer and 85 breast cancer patients were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan) and Cooperative Human Tissue Network (Ohio State University, Columbus, OH), dating from 1988 to 2001. Each prostate cancer patient sample was assigned two separate Gleason grades, corresponding to the two predominant histologic patterns. Complete 5-year follow-up data were available for 43 prostate cancer and 85 breast cancer patients, and those who died of other causes were eliminated from the study.

Immunohistochemical Staining. Four micron-thick sections were cut from the paraffin blocks of prostate and breast tumors and mounted on charged glass slides. The sections were deparaffinized, rehydrated, and antigen retrieval was done by treatment with 25 mmol/L sodium citrate buffer (pH 9; for Drg-1, p53, and estrogen receptor) or 10 mmol/L sodium citrate buffer (pH 6; for PTEN and androgen receptor). The slides were incubated overnight at 4°C with the following antibodies: anti-Drg-1 rabbit polyclonal antibody (1:100), anti-PTEN rabbit polyclonal antibody (1:200, Upstate Biotechnology), anti-p53 mouse monoclonal antibody (1:100, Clone DO-7, Dako Corp, Carpentaria, CA), antiandrogen receptor (AR) rabbit polyclonal antibody (1:100, Zymed Corp., Camarillo, CA), and antiestrogen receptor (ER) mouse monoclonal antibody (1:70, clone 1D5, Dako Corp). The sections were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies, and DAB substrate chromogen solution (Envision-plus kit, DAKO Corp) was applied followed by counterstaining with hematoxylin. Results of the immunohistochemistry for Drg-1 and PTEN were judged based on the intensity of staining, and the grading of the Drg-1 and PTEN expression was done by two independent persons (S. B. and K. W.) without prior knowledge of the grade, stage, or patient survival. For p53, AR, and ER immunostaining, percentage of the tumor cells with positive nuclear staining was determined. In addition, 10 fields on each slide were chosen and the expression of each of Drg-1, PTEN, p53, AR, and ER was comparatively observed in the same field.

Statistical Analysis. For *in vitro* experiments, one-way ANOVA was used to calculate the *P* values. The association between Drg-1 and other prognostic markers was calculated by χ^2 analysis. The Kaplan-Meier method was used to calculate the survival rates, and prognostic significance was evaluated by the log-rank test. Cox proportional hazard regression model was used to evaluate the prognostic value of the different markers.

Results

PTEN Augments the Expression of the Drg-1 Gene *in Vitro*. To explore the possibility that PTEN controls the expression of the *Drg-1* gene in prostate and breast cancer, we examined the effect of PTEN on endogenous Drg-1 protein expression by using two sets of PTEN expression vectors. First, the empty pcDNA3 vector or the pcDNA3/PTEN expression plasmid was transiently transfected into the prostate (ALVA and PC3) and breast cancer (MDA-468 and BT-549) cell lines that were negative for PTEN expression, and the endogenous level of Drg-1 was examined by Western blot. We found, as shown in Fig. 1*A*, *b-e*, *left panel*, PTEN augmented the Drg-1 expression in a dose-dependent manner in all of the cell lines tested, whereas the empty vector did not have any notable effect. Next, to clarify whether the effect of PTEN on Drg-1 is dependent on the phosphatase activity of PTEN, we transfected the above cells with the second set of PTEN expression vectors (pFlag/PTEN), which includes the lipid and protein phosphatase-deficient mutant (C124S) and its wild-type counterpart. Our Western blot results showed that this mutant failed to up-regulate the Drg-1 expression (Fig. 1*A*, *b-e*, *right panel*), suggesting that the phosphatase activity of PTEN is essential for this function. To further validate the above observation, we used an inducible expression system of the *PTEN* gene. PC3MM/Tet cells were transiently transfected with an inducible PTEN expression vector, and endogenous level of the *Drg-1* gene was examined after induction of PTEN expression by tetracycline. As shown in Fig. 1*B*, we found that PTEN induction considerably elevated the expression of the *Drg-1* gene, thereby lending strong support to our notion that Drg-1 is up-regulated by PTEN.

As a complementary approach to confirm the above result, we sought whether inhibition of PTEN, in the PTEN-positive prostate

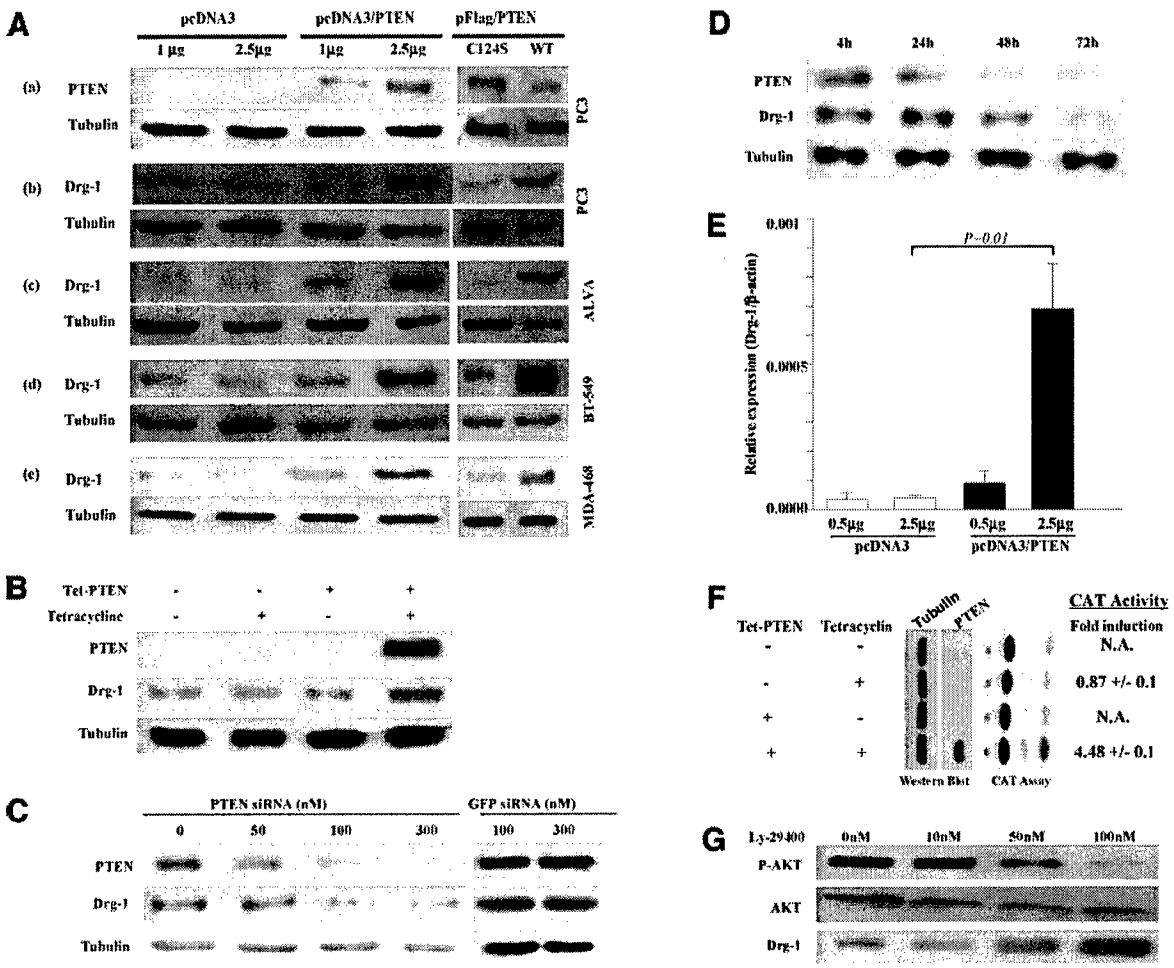


Fig. 1. Expression of the Drg-1 gene is up-regulated by PTEN. *A*, expression vector pcDNA3 or pcDNA3/PTEN, at indicated amounts, was transfected into the prostate cancer cell lines (PC3 and ALVA) and the breast cancer cell lines (BT-549 and MDA-468). Another set of PTEN expression vector (pFlag/PTEN), which includes mutant (C124S) and wild-type (WT) was also transfected into the above cell lines (*right panels*). Forty-eight hours after transfection, cells were lysed, and Western blot was done using antibodies against PTEN and Tubulin (*a*) or Drg-1 and Tubulin (*b-e*). *B*, PC3MM/Tet cells were transiently transfected with (+) or without (−) an inducible PTEN expression vector (Tet/PTEN). Twenty-four hours after transfection, cells were treated with (+) or without (−) tetracycline to induce PTEN expression. Forty-eight hours later, cells were lysed, and the levels of PTEN, Drg-1, and tubulin were examined by Western blot. *C*, siRNA for PTEN or GFP was synthesized as described in Materials and Methods. Various amounts of the siRNA, as indicated, were transfected into DU-145 cells (PTEN-positive). After 72 hours, cells were lysed, and lysates were examined by Western blot with antibodies for PTEN, Drg-1, and Tubulin. *D*, PTEN siRNA (300 nmol/L) was transfected into DU-145 cells, and cells were collected at different time points as indicated. The cell lysates were then examined for the expression of PTEN, Drg-1, and Tubulin by Western blot. *E*, various amounts of pcDNA3 or pcDNA3/PTEN were transfected into PC3 cells. Forty-eight hours after transfection, total RNA was isolated from the cells, and the expression of the Drg-1 and β-actin genes was examined by real-time-quantitative reverse transcription-PCR. *F*, PC3MM/Tet cells were cotransfected with Drg-1-CAT–reporter plasmid and inducible PTEN expression vector (Tet/PTEN) as indicated. Twenty-four hours after transfection, cells were treated with (+) or without (−) tetracycline to induce PTEN expression. Forty-eight hours later, cells were harvested and CAT assay was done. Induction of PTEN expression was also confirmed by Western blot. *G*, PC3 prostate cancer cells were incubated with various concentrations of the phosphatidylinositol 3′-kinase inhibitor LY29400 as indicated. After the treatment for 48 hours, cells were collected and expression of phospho-Akt (P-AKT), total Akt, and Drg-1, was examined by Western blot analysis.

cancer cell line DU-145, by siRNA would lead to attenuation of Drg-1 expression. As shown in Fig. 1*C*, we found that inhibition of PTEN expression by 100 and 300 nmol/L siRNA was followed by substantial decrease in the Drg-1 level. This inhibitory effect was mediated specifically by PTEN siRNA, because equivalent dose of GFP siRNA did not affect the expression of PTEN or Drg-1 (Fig. 1*C*, *right panel*). PTEN siRNA also attenuated the Drg-1 expression in a time-dependent manner, and at 72 hours the Drg-1 level was found to be diminished significantly (Fig. 1*D*). These results further corroborate our notion that PTEN controls the expression of the *Drg-1* gene and raise a possibility that the loss of PTEN expression in human cancers leads to down-regulation of the Drg-1 expression.

To examine whether the regulation of Drg-1 by PTEN is mediated at the RNA level, pcDNA3 empty vector or pcDNA3/PTEN was transfected transiently into PC3, and the level of Drg-1 mRNA was measured by a real-time quantitative reverse transcription-PCR. As shown in Fig. 1*E*, PTEN significantly enhanced Drg-1 expression in a dose-dependent manner whereas the empty vector did not affect Drg-1 level. To further clarify whether up-regulation of Drg-1 expression by PTEN is mediated at the transcriptional level, PC3MM/Tet cells were cotransfected with inducible PTEN expression vector and Drg-1-CAT–reporter plasmids. After induction of PTEN expression by tetracycline, CAT assay was done. As shown in Fig. 1*F*, we found that the resultant CAT activity was significantly augmented by PTEN, thereby strongly suggesting that the Drg-1 gene is positively controlled by PTEN at the transcriptional level.

PTEN is a dual specificity phosphatase that inhibits phosphatidylinositol 3′-kinase–dependent activation of Akt, and deletion or inactivation of PTEN results in constitutive Akt activation (19). Therefore, if Drg-1 expression is indeed controlled by PTEN through an Akt-mediated pathway, blocking Akt phosphorylation would restore expression of Drg-1. To test this possibility, PC3 prostate cancer cells, which exhibit a high level of phosphorylated Akt but lacks the PTEN gene, were treated with increasing dose of the phosphatidylinositol

3′-kinase inhibitor, Ly294002 (Sigma Chemical Co.). As shown in Fig. 1*G*, 50 and 100 nmol/L Ly-29400 specifically decreased the phospho-Akt level that was associated with a concomitant increase in Drg-1 expression. This result indicates that PTEN controls Drg-1 expression largely through an Akt-dependent pathway. Together, the results of our *in vitro* experiments implicate that PTEN transcriptionally up-regulates the expression of the *Drg-1* gene via an Akt-mediated pathway.

Expression of Drg-1 and PTEN Correlate in Clinical Setting. The result of our *in vitro* experiments prompted us to examine whether there is any correlation between the PTEN and Drg-1 expression levels in the clinical setting. We did an immunohistochemical analysis on an archive of 81 prostate and 85 breast cancer tissue samples. The results showed that Drg-1 expressed strongly in the epithelial cells of normal ducts and glands in both prostate and breast tissue sections, whereas the poorly differentiated tumor cells in the same specimen had significantly reduced level of Drg-1. Similarly, PTEN was also found to be highly expressed in the epithelial cells of normal ducts and glands, where the protein was detected mostly in the cytoplasm. Importantly, as shown in two representative fields in Fig. 2*A*, almost identical staining pattern was obtained when the same field was examined for PTEN and Drg-1 expression. Statistical evaluation revealed a strong correlation ($P = 0.03$) between Drg-1 and PTEN expression status in the case of prostate cancer (Fig. 2*B*). Of 63 patients who were positive for PTEN, 44 (69.8%) exhibited positive Drg-1 expression, and among 18 patients with reduced PTEN expression, 11(61.11%) also had reduced Drg-1 level. Even stronger correlation ($P < 0.001$) between the two genes was found in the case of breast cancer (Fig. 2*B*). Furthermore, consistent with our previous observations (14, 17), the Drg-1expression also correlated significantly with metastasis in both prostate and breast cancer ($P = 0.004$, $P = 0.01$, respectively, Fig. 2*B*). Therefore, results of this immunohistochemical analysis are consistent with our notion that PTEN controls the expression of Drg-1.

Recently, Drg-1 mRNA expression has been shown to increase in p53-dependent manner in certain bladder and breast cancer cell lines *in vitro* (16). Drg-1 expression has also been suggested to be modulated by androgen in prostate cancer cell lines, although there is some

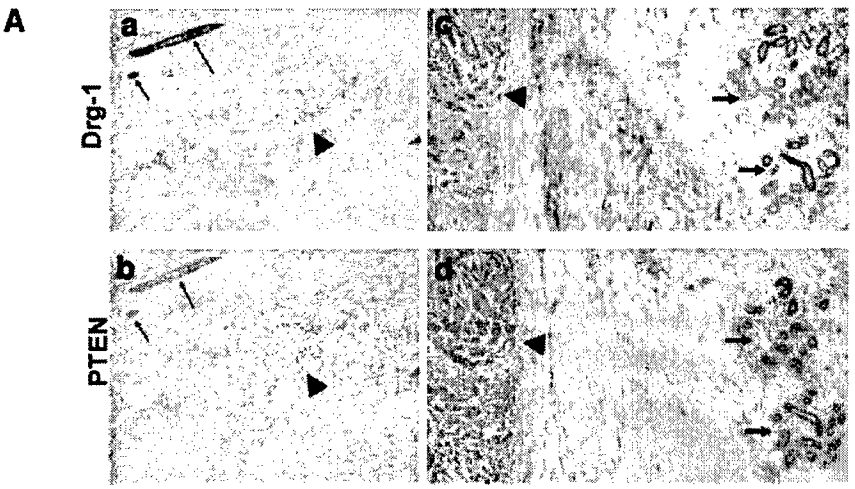


Fig. 2. Immunohistochemical analysis of Drg-1 with respect to PTEN and other clinicopathologic parameters in human prostate and breast cancer. *A*, immunohistochemistry for Drg-1 and PTEN was done on paraffin tissue sections from prostate (*a-b*) and breast (*c-d*) cancer patients. *a*, Drg-1 immunostaining in a representative field from a prostate cancer patient sample showing normal prostatic gland (*arrow*) and poorly differentiated prostate carcinoma cells (*arrowhead*). *b*, the same field is shown after immunostaining for PTEN. *c-d*, a representative field from a breast cancer specimen immunostained with Drg-1 (*c*) and PTEN (*d*) antibodies. *B*, association of Drg-1 with PTEN, p53, AR, and ER and degree of metastasis. Immunohistochemistry was done on prostate and breast tissue specimens as described in Materials and Methods. In each case, χ^2 test was done to test the significance of association between Drg-1 and each of these parameters. * indicates statistically significant correlation ($P < 0.05$).

B **Relationship between Drg-1 and other clinical parameters**

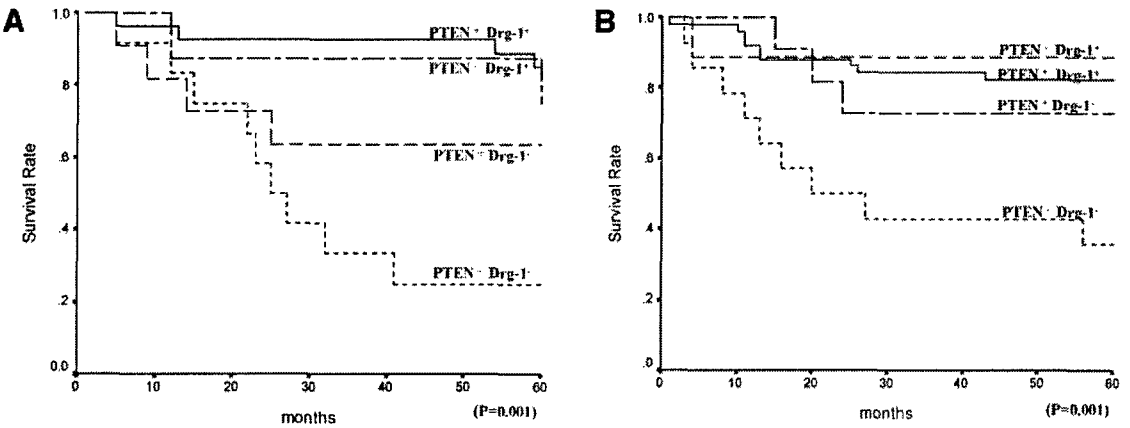
		Drg-1 expression		P value
Prostate Cancer	All (81)	positive (51)	reduced (30)	
PTEN status				
Positive	63	44 (69.8%)	19 (30.16%)	
Negative	18	7 (38.89%)	11 (61.11%)	0.03*
P53 status				
Wild type	3	3 (100%)	0 (0%)	
Mutant	78	30 (38.5%)	48 (61.5%)	0.46
AR				
Positive	60	40 (66.7%)	20 (33.3%)	
Negative	21	10 (47.6%)	11 (52.4%)	0.20
Metastasis status				
$T_xN_0M_0$	51	38 (74.5%)	13 (25.5%)	
$T_xN_1M_{0,1}$	30	12(40.0%)	18(60.0%)	0.004*
Breast Cancer	All (85)	positive (60)	reduced (25)	
PTEN status				
Positive	62	51 (82.3%)	11 (17.7%)	
Negative	23	9 (39.1%)	14 (60.9%)	<0.001*
P53 status				
Wild type	57	40 (70.2%)	17 (29.8%)	
Mutant	28	20 (71.4%)	8 (28.6%)	0.99
ER status				
Positive	40	27 (67.5%)	13 (32.5%)	
Reduced	45	33 (73.3%)	12 (26.7%)	0.73
Metastasis status				
$T_xN_0M_0$	29	26 (89.7%)	3 (10.3%)	
$T_xN_{1-3}M_{0,1}$	56	34 (60.7%)	22 (39.3%)	0.01*

controversy (20, 21). Therefore we examined the status of Drg-1 with respect to these markers in clinical samples by immunohistochemistry. However, as shown in Fig. 2*B*, no significant correlation of Drg-1 protein expression was observed with either p53, AR, or ER status in the case of either prostate or breast cancer.

Combination of PTEN and Drg-1 Predicts Clinical Outcome of Prostate and Breast Cancer. Because there was significant correlation between PTEN and Drg-1 expression in clinical samples of prostate and breast cancer, we next evaluated the prognostic importance of the combination of these two markers. Kaplan-Meier method was used to do univariate survival analysis in the prostate and breast cancer cases with 5-year follow-up. As shown in Fig. 3*A* and *B*, patients negative for both PTEN and Drg-1 had significantly worse prognosis than those with positive expression of either one or both markers (overall log-rank P value = 0.001 in both types of cancer). Importantly, Cox regression analysis (Fig. 3*C*) revealed that the combination of *PTEN* and *Drg-1* gene expression was an independent prognostic marker in both prostate and breast cancer. As shown in Fig. 3*C*, in the case of prostate cancer, the hazard ratio of Drg-1 and PTEN as an individual marker was 4.965 and 2.819, respectively. When both markers were combined, however, the hazard ratio was 8.537, meaning that the death risk of a patient with negative expression of both markers was 8.537 times compared with a patient positive for both or either PTEN and Drg-1. Similar trend was also observed in breast cancer. These data underscore the prognostic importance of the combination of PTEN and Drg-1 and also point toward the clinical relevance of the PTEN-Drg-1 pathway in advancement of the prostate and breast cancer.

Discussion

PTEN is a tumor suppressor gene that has been found to be one of the most common targets of mutation in human cancer, with a mutation frequency approaching that of p53 (4). In the case of human prostate cancer, deletion and/or mutations of the *PTEN* gene are reported in 30% of primary and 63% of metastatic tumors, placing *PTEN* among the most common genetic alterations in this type of cancer (5, 22). These results also strongly indicate the involvement of PTEN in late stage and metastasis of prostate cancer. Similar observations were reported in the case of breast cancer, in which loss of PTEN expression was found to be significantly correlated with lymph node metastasis (6). In fact, Davies *et al.* (10) have shown recently that PTEN significantly suppressed metastasis without affecting primary tumorigenesis in a prostate cancer animal model. However, the exact mechanism of metastasis suppression by PTEN remains unknown. In this report we have shown that PTEN up-regulates the



C **Cox regression analysis of disease free survival in prostate and breast cancer**

Variables	reference level	b	SE	Wald's x^2	Hazard ratio	95% CI	P
Prostate Cancer							
Drg-1 status	positive expression	1.602	0.498	10.363	4.965	1.872-13.173	0.001
PTEN status	positive expression	1.036	0.466	4.939	2.819	1.130-7.030	0.026
Combination of Drg-1 PTEN status	positive Drg-1and /or positive PTEN expression	2.144	0.615	12.143	8.537	2.556-28.520	<0.001
Breast Cancer							
Drg-1 status expression	positive	1.221	0.429	8.086	3.389	1.461-7.860	0.004
PTEN status expression	positive	0.984	0.429	5.267	2.674	1.154-6.195	0.022
Combination of Drg-1 PTEN status	positive Drg-1and/or positive PTEN expression	1.597	0.474	11.349	4.937	1.950-12.499	0.001

Fig. 3. Combination of PTEN and Drg-1 expression significantly correlate with survival. Overall survival rate over a period of 5 years was measured in 43 patients with prostate cancer (*A*) and 85 patients with breast cancer (*B*) in relation to expression of the *PTEN* and *Drg-1* genes. The patients were categorized into four groups ($PTEN^+$ $Drg\text{-}1^+$, $PTEN^+$ $Drg\text{-}1^-$, $PTEN^-$ $Drg\text{-}1^+$, and $PTEN^-$ $Drg\text{-}1^-$) as indicated in the figure. The P values were determined by log-rank test. *C*, Cox regression analysis was conducted to assess the contribution of the indicated variables to disease prognosis. b, the estimation for the regression coefficient β; *CI*, confidence interval.

putative tumor metastasis suppressor gene Drg-1 in both prostate and breast cancer cells *in vitro*. These results are corroborated by significant-positive correlation of the expressions of these two genes in the clinical setting. *Drg-1* was originally identified as a gene induced by differentiation in a colon carcinoma cell line (23). The *Drg-1* gene is almost ubiquitously expressed and encodes a 43,000 daltons protein, the biochemical function of which is yet to be understood. Previously, we have shown that overexpression of the *Drg-1* gene in a highly metastatic rat prostate cancer cell almost completely abrogated lung metastasis without affecting primary tumor formation (14). Drg-1 was also reported to suppress metastasis in an animal model of colon cancer (15). These results strongly suggest that PTEN exerts its tumor metastasis suppressor function by controlling the Drg-1 gene.

Previously we showed that expression of Drg-1 has a significant inverse correlation with degree of metastasis and patient survival in both prostate and breast cancers (14, 17). In this report, we further show that expression of Drg-1 is also significantly correlated with PTEN expression in patient samples of these malignancies. These results are in good agreement with our concept that PTEN up-regulates the Drg-1 gene, which in turn suppresses metastasis. Importantly, our results also suggest that the combination of PTEN and Drg-1 expression status has a better value in predicting patient outcome than either marker alone.

Understanding how PTEN suppresses metastasis through Drg-1 is of considerable interest. Results of several recent studies suggest that PTEN is able to suppress the invasiveness and motility of various types of tumor cells (24, 25). As a possible anti-invasive mechanism of PTEN, Koul *et al.* (24) showed that PTEN down-regulates matrix metalloproteinase-2 at the transcriptional level. Notably, others and we have shown that Drg-1 can suppress invasion of prostate, colon, and breast tumor cells through extracellular matrix *in vitro*. Therefore, it is plausible that PTEN blocks the invasion step resulting in metastasis suppression via a Drg-1–dependent pathway. PTEN is a dual-specificity phosphatase and is capable of inhibiting phosphatidylinositol 3′-kinase–dependent activation of Akt, a serine threonine kinase. Inactivation of Akt via dephosphorylation has been shown to result in reduced invasiveness of melanoma and bladder cancer cells (26, 27). Furthermore, Malik *et al.* (28) showed that overexpression of phospho-Akt significantly correlates with high Gleason grade of prostate cancer. It has also been shown that overexpression of phospho-Akt leads to enhanced invasiveness and metastasis of breast and ovarian cancer cells in animal models (29). In this report, we have shown that PTEN up-regulates expression of Drg-1 by an Akt-dependent pathway. These results are consistent with our notion that invasion and metastasis suppressor function of PTEN is mediated through inactivation of Akt, which leads to down-regulation of Drg-1. Identification of further downstream target and study of more detailed molecular mechanism of PTEN/Drg-1–mediated pathway of metastasis suppression is currently underway.

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