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) has been shown to be inactivated in a wide variety of cancers, including prostate, breast, lung, and head and neck cancer. The role of PTEN as a tumor suppressor has been well established. 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. 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. 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 prostate 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 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
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 ALVA 41 was kindly provided by Dr. V. Rosner (Columbia University, New York). All cell lines were cultured in RPMI 1640 supplemented with 10% FCS, 100 μg/mL streptomycin, 100 units/mL penicillin, and 250 mmol/L dexamethasone, at 37°C in a 5% CO₂ 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. 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/TTO to obtain an inducible PTEN expression plasmid designated as Tet/PTEN. For construction of Drg-1-chromophenicol 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 (Minus Corp., Madison, WI). In each case, green fluorescent 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 (1:500), tubulin (1:1,000, Upstate Biotechnology), phospho-Akt (1:500, Cell Signaling Technology), or total Act (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'–ATGCAAGGTGTCACCTCCCG and 5'–ATGTAAGGATGAGTACAGC) 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.

Chromophenicol 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 [14C]chromophenicol 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., Carparrillo, 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 χ² 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. 1A, 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. 1A, 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. 1B, 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
cancer cell line DU-145, by siRNA would lead to attenuation of Drg-1 expression. As shown in Fig. 1C, 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. 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. 1D). 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. 1E, 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 Drg-1-CAT–reporter plasmid and inducible PTEN expression vector (Tet/PTEN) as indicated. Twenty-four hours after transfection, cells were collected 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 LY294002 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.
3'-kinase inhibitor, Ly294002 (Sigma Chemical Co.). As shown in Fig. 1G, 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. 2A, 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. 2B). 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 (61.1%) 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. 2B). Furthermore, consistent with our previous observations (14, 17), the Drg-1 expression also correlated significantly with metastasis in both prostate and breast cancer ($P = 0.004$, $P = 0.01$, respectively, Fig. 2B). 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, $\chi^2$ test was done to test the significance of association between Drg-1 and each of these parameters. * indicates statistically significant correlation ($P < 0.05$).

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<th>All (81)</th>
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<th>reduced (30)</th>
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<td></td>
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<tr>
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<td>63</td>
<td>44 (69.8%)</td>
<td>19 (30.16%)</td>
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<td>18</td>
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<td>11 (61.11%)</td>
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<tr>
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<td>0 (0%)</td>
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<td>48 (61.5%)</td>
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<td>40 (66.7%)</td>
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<tr>
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<td>10 (47.6%)</td>
<td>11 (52.4%)</td>
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<td>$T_{N_0}M_{0}$</td>
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<td>38 (74.5%)</td>
<td>13 (25.5%)</td>
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<td>12(60.0%)</td>
<td>8 (40.0%)</td>
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<th>reduced (21)</th>
<th>$P$ value</th>
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<tr>
<td>Wild type</td>
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<td>40 (70.2%)</td>
<td>17 (29.8%)</td>
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<td>12 (26.7%)</td>
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<td>54 (96.4%)</td>
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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. 2B, 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. 3A 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. 3C) revealed that the combination of PTEN and Drg-1 expression was an independent prognostic marker in both prostate and breast cancer. As shown in Fig. 3C, 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
PTEN UP-REGULATES Drg-1

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 gene 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.

References


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