Reduced Expression of Metastasis Suppressor RhoGDI2 Is Associated with Decreased Survival for Patients with Bladder Cancer

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ABSTRACT

Purpose: ***RhoGDI2*** **was recently shown to be a metastasis suppressor gene in models of bladder cancer. We sought to further understand its importance in human cancer by determining the level of its expression and the distribution of its encoded protein in normal human tissues and cell lines and to evaluate whether its protein expression is a determinant of human bladder cancer progression.**

Experimental Design: **RhoGDI2 mRNA and protein expression was evaluated in cell lines and human tissues using Affymetrix and tissue microarrays, respectively. Tissue microarrays represented most human normal adult tissues and material from 51 patients that had undergone radical cystectomy for bladder cancer. In these 51 patients, the χ^2 test was used to test for associations between RhoGDI2 and stage, grade of urothelial carcinoma, histological type, and disease-specific survival status. Cox proportional hazards regression analyses were used to estimate the effect of RhoGDI2 expression level on time to development of metastatic disease and disease-specific survival time, adjusting for grade, stage, and histological type.**

Results: **In normal tissues, there was strong RhoGDI2 protein expression in WBCs, endothelial cells, and transitional epithelium. RhoGDI2 mRNA expression was inversely related to the invasive and metastatic phenotype in human bladder cancer cell lines. In patients with bladder cancer, univariate analysis indicated that reduced tumor RhoGDI2 protein expression was associated with a lower actuarial 5-year disease-free and disease-specific survival ($P = 0.01$). In addition, patients with tumors that had low or absent RhoGDI2 had a shorter time to disease-specific death ($P \leq 0.01$). When tumor grade, stage, histological type, and RhoGDI2 staining level were examined using multivariate analysis, RhoGDI2 expression was found to be an independent predictive factor for disease-specific death ($P = 0.03$).**

Conclusions: **These results suggest that RhoGDI2 is an independent predictor of prognosis for patients with bladder cancer and provide clinical evidence in support of its involvement in cancer metastasis.**

INTRODUCTION

Rho GTPases form a distinct branch of the Ras-like low molecular weight GTP-binding protein superfamily. They are involved in actin cytoskeleton organization (1) and have been associated with invasion and metastasis (2). Rho GTPases switch between an inactive GDP-bound state and an active GTP-bound state. Regulators of this GDP/GTP cycle include GDP dissociation inhibitors (GDIs), which bind to the inactive form, thereby blocking its activation.

RhoGDI1 was first identified on the basis of its ability to inhibit GDP dissociation from RhoA (3), CDC42Hs (4) and Rac1 (5). RhoGDI2 (also known as D4-GDI or Ly-GDI) shares 67% amino acid identity with RhoGDI1 (6–8), but, in contrast to RhoGDI1, which is ubiquitous, this protein was believed to be exclusively expressed in cells of hematopoietic lineage (6, 7). However, our recent studies have suggested that the *RhoGDI2* gene is also expressed in nonhematopoietic neoplasms (9, 10). Also, in contrast to RhoGDI1, the function, effector targets, and biological role(s) of RhoGDI2 in health and disease are incompletely understood. Using an animal model of human bladder cancer metastasis (11) and DNA microarray technology (10), we have recently shown that RhoGDI2 is a putative metastasis suppressor gene in human cancer. In this model, re-expression of the *RhoGDI2* gene in cells with metastatic ability suppressed lung metastasis, whereas s.c. tumor growth was unaffected.

To gain further insight into the biological and clinical importance of RhoGDI2, we investigated the mRNA and protein expression of RhoGDI2 in normal human adult tissues and organs, and the potential prognostic relevance of its protein level on the survival of patients with bladder cancer. Taken together, our data suggest that RhoGDI2 expression in normal human tissues is not limited to the hematopoietic compartment. In addition, the loss or reduction of RhoGDI2 protein expression in bladder tumors is an independent predictor for the development of metastatic disease and disease-specific death from bladder cancer, supporting its role as a metastasis suppressor gene and its potential utility as a clinical prognostic marker.

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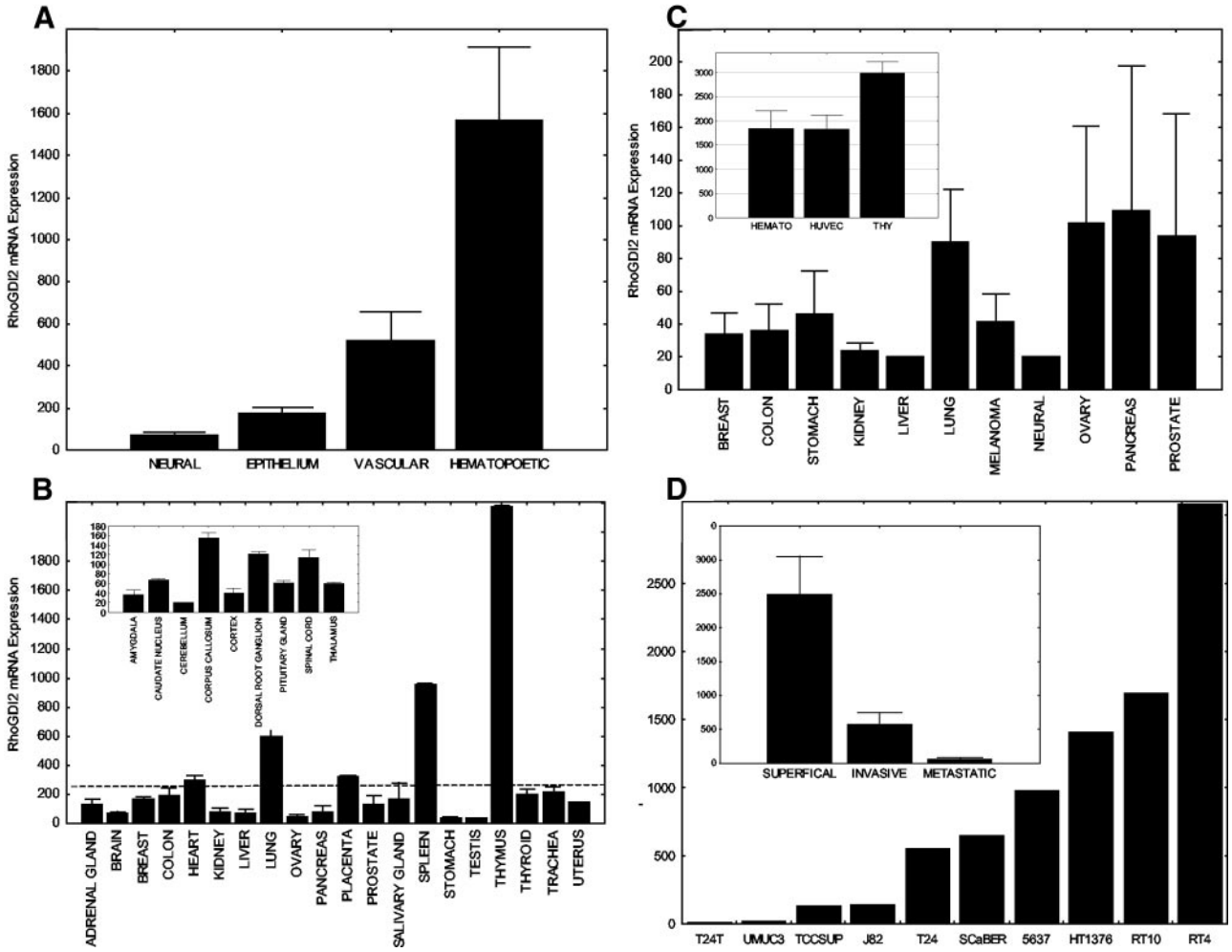

Fig. 1 RhoGDI2 mRNA expression in human tissues and cell lines; two to eight tissue samples from each site were evaluated for RNA expression. Average mRNA expression and SE are displayed. Values <20 are considered negative. *A*, average expression as a function of normal tissue lineage. *B*, expression as a function of individual normal tissues; *dotted line*, average tissue expression [214 ± 386 (SD)] for entire cohort; *inset*, detailed expression profile of neural tissue types. *C*, expression in nonbladder cells; one to five tumor cell lines were evaluated for each histological type; *inset*, malignant [hematopoietic (*HEMATO*)] and normal [human umbilical vein endothelial cells (*HUVEC*) and thymocytes (*THY*)] cells with markedly higher expression levels. *D*, expression in bladder tumor cell lines; expression in common bladder cell lines arranged as a function of expression level; *inset*, expression levels in cells grouped as a function of their original or xenograft properties as they relate to their invasive or metastatic behavior as described in "Materials and Methods."

MATERIALS AND METHODS

Normal Tissues, Tumor Cell Lines, and Chip Hybridization. Normal adult human tissue samples were obtained from commercial sources, as described previously (12). Detailed sample descriptions can be obtained on the Genomics Institute of the Novartis Research Foundation (GNF) web site.[5] Other normal tissues were counterparts to tumor samples surgically removed at the University of Virginia (described in Ref. 12). The University of Virginia Human Investigation Committee approved the use of the tissue samples obtained at the University of Virginia. The 85 human tumor-derived cell lines used here included the National Cancer Institute Developmental Therapeutic Program's NCI-60, as well as other commonly used cell lines.[6] Samples were labeled and hybridized to human (U95A) high-density oligonucleotide arrays (10) as described previously (13). Primary analysis of the arrays was performed using GENECHIP 3.2 (Affymetrix, Santa Clara, CA), and data were scaled to an average hybridization intensity of 200 (corresponding to ~3–5 copies of transcript/cell) for interchip comparison.

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Human Tissue Microarray. A tissue microarray that contained 160 0.6-mm cores representing the majority of normal human adult tissues was constructed from zinc formalin-fixed, paraffin-embedded blocks (Beecher Instruments, Silver Spring, MD). Another tissue microarray that was constructed contained four cores from each of 51 zinc formalin-fixed, paraffin-embedded blocks from carcinomas of the bladder harvested as described below.

RhoGDI2 Immunohistochemistry. For immunohistochemistry using the tissue microarrays, the avidin-biotin immunoperoxidase method was performed. After slides had been placed in citrate buffer and treated with microwave heat for 20 min, a rabbit polyclonal antibody (1:400 dilution; Spring Bioscience, Fremont, CA) to RhoGDI2 was applied for 1 h at room temperature. This antibody was verified for specificity for RhoGDI2 (*versus* RhoGDI1) in a Western blot of cells with and without RhoGDI2 and RhoGDI1 expression (data not shown). Immunohistochemical staining was scored by a board-certified genitourinary pathologist (H. F. F.) as negative, reduced (defined as staining weaker than that for normal adjacent urothelium in the same section), and positive (defined as staining, either uniformly or focally, stronger than, or as strong as, for normal adjacent urothelium in the same section). This scoring was carried out in a blinded fashion, without knowledge of the patient followup information.

Patient Population, Surgical Procedures, and Clinical Follow Up. For immunohistochemical (tissue microarray construction) and long-term prognostic evaluation studies, we used paraffin tissue blocks from 51 patients with clinical stage T_a–$T_3N_0M_0$ bladder cancer who underwent radical cystectomy. Stage was assigned using the 2002 Union International Contre Cancer (UICC) Tumor-Node-Metastasis System (14). All of the patients had computed tomography of the abdomen and/or i.v. pyelography before undergoing radical cystectomy with pelvic lymphadenectomy. Pathological review of tumor tissue was carried out by a single board-certified urological pathologist (H. F. F.). This retrospective study was approved by the University of Virginia Human Investigation Committee.

Statistical Analysis. The χ^2 test was used to test for associations between RhoGDI2 and stage, grade of urothelial carcinoma, histological type and disease-free survival (metastatic recurrence rate), and disease-specific survival (death from bladder cancer) status. Kaplan-Meier curves were used to estimate, and the log-rank test was used to compare, the survival time distributions for patients with RhoGDI2-positive tumors to those whose cancers had no or reduced staining for RhoGDI2. Cox proportional hazards regression analyses were used to estimate the effect of RhoGDI2 on survival time, adjusting for grade, stage, and histological type. Data graphs display mean ± SE.

RESULTS AND DISCUSSION

Evaluation of RhoDGI2 mRNA expression as a function of tissue type is shown in Fig. 1, *A* and *B*. The average expression level among all of the tissue samples was 214 ± 386 (SD). A striking difference in expression was noted between tissue categories with neural tissues expressing the lowest levels [73 ± 44 (SD)]. A detailed analysis of different central nervous system tissue types was undertaken and is shown in Fig. 1*B* (*inset*) and indicates that there were no obvious extremes in expression in this tissue. As previously noted (6, 7), tissues with significant hematopoietic cell components such as spleen and thymus had higher than average [1566 ± 703 (SD)] levels of RhoDGI2 mRNA expression (Fig. 1*A*). Interestingly, tissues with significant vasculature such as heart, lung, and placenta also had high levels of mRNA expression (Fig. 1, *A* and *B*), whereas pancreas, testis, and liver had very low levels of expression of this gene. In contrast to RhoDGI2 expression in normal tissues, an extensive survey of tumor-derived cell lines revealed its overall decreased expression (Fig. 1*C*). Interestingly, tumorigenic, immortalized, or normal cells of hematopoietic or endothelial lineage had high levels of expression (Fig. 1*C*, *inset*).

Using tissue microarrays of normal human adult specimens, we found that positive immunohistochemical staining for RhoGDI2 was most often cytoplasmic with occasional nuclear immunoreactivity. The highest intensity of staining was observed in cells of hematopoietic origin including lymphocytes, neutrophils, and histiocytes, confirming the microarray experiments. Immunoreactivity was also present in megakaryocytes and endothelial cells. Amnionic and chorionic cells also labeled for RhoGDI2. Notably, a variety of types of epithelial cells showed immunopositivity including: transitional epithelium; ba-

Table 1 Relationship of RhoGDI2 immunohistochemical expression with pathological and clinical variables: Association of RhoGDI2 immunohistochemical expression with tumor grade, stage, and histological type

Variable	Category	All $n = 51$	GDI2 = 0[a] $n = 14$ (27%)	GDI2 = 1[b] $n = 37$ (73%)	P [c]
Grade	1–2	14	2 (14%)	12 (86%)	0.08
	3–4	20	4 (20%)	16 (80%)	
	Nonurothelial	17	10 (59%)	7 (41%)	
Stage[d]	T_a	10	2 (20%)	8 (80%)	0.08
	T_1–T_4	24	4 (17%)	20 (81%)	
	Nonurothelial[e]	17	10 (59%)	7 (41%)	
Histology	Urothelial	34	6 (18%)	28 (82%)	0.03
	Nonurothelial	17	10 (59%)	7 (41%)	

[a] GDI2 = 0, absent or reduced staining for RhoGDI2.
[b] GDI2 = 1, staining for RhoGDI2 similar to normal urothelium.
[c] *P*s are based on the Pearson χ^2 test.
[d] Union International Contre Cancer (UICC)/2002 (14).
[e] All were stage ≥ T_2.

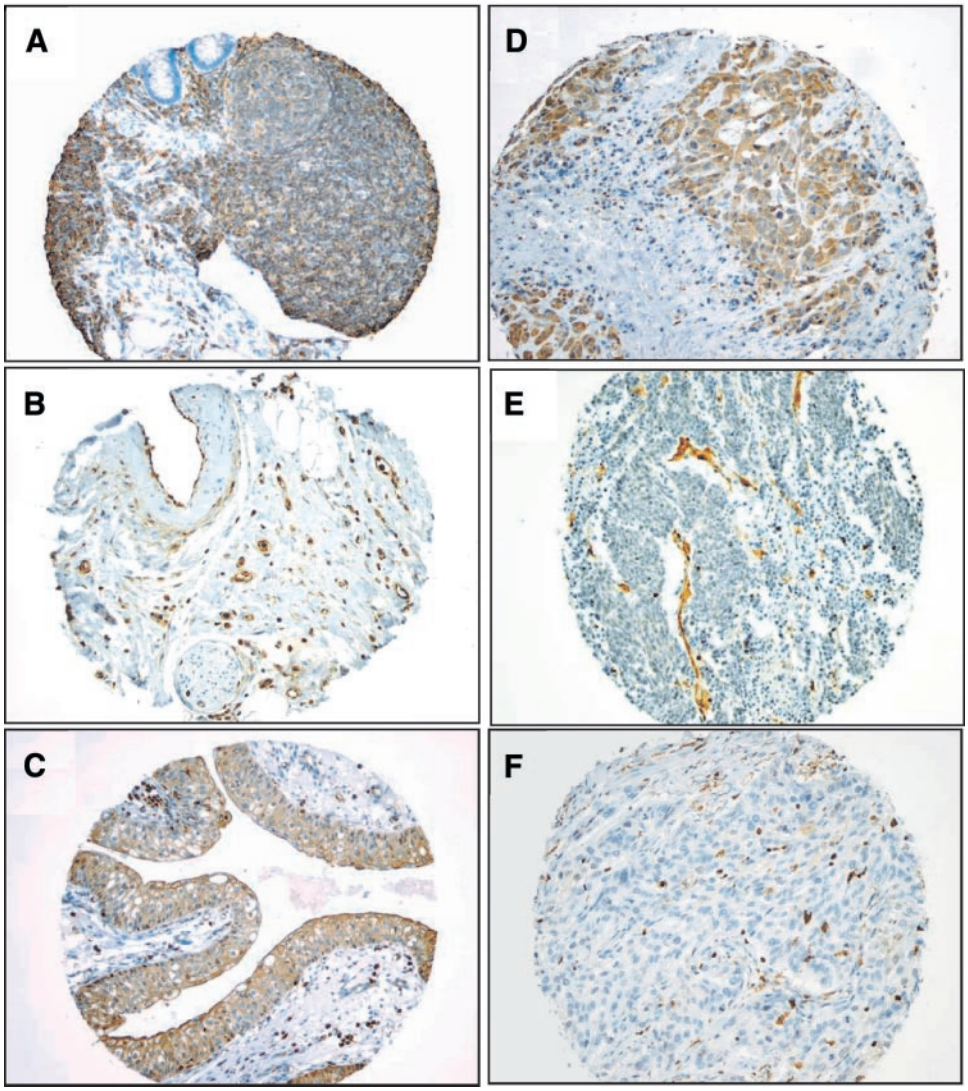

Fig. 2 *A*, *B*, and *C*, RhoGDI2 immunohistochemistry in normal tissues: normal immune cells (shown here in the appendix; *A*), endothelial cells (*B*), and urothelium (*C*) are strongly positive for RhoGDI2. *C*, *D*, and *E*, RhoGDI2 immunohistochemistry in bladder cancer: **(***D***)** most urothelial carcinomas of the bladder were immunoreactive for RhoGDI2 with staining similar to normal urothelium; *E*, three small-cell undifferentiated carcinomas lacked staining, and one had reduced intensity; *F*, 2 of 10 squamous cell carcinomas lacked immunopositivity. ×100.

sal cells of vas, seminal vesicle, and epididymis; fallopian tube; skin (basal layer); renal collecting ducts; and ducts and some acini of breast, sweat glands, Bartholin's gland, lacrimal gland, and salivary glands.

Taken together, these data lead to several interesting hypotheses. First, it would appear that RhoGDI2 expression is uniformly lower in tumor/immortal cell lines when compared with their normal tissue counterparts, with the exception of hematopoietic and endothelial lineages, which display typically high levels in tumor/immortal cells. This striking difference may suggest different roles for RhoGDI2 in transformation or tumor progression in hematopoietic, compared with epithelial, cancers. Further work is required to confirm these preliminary hypotheses. Second, these data indicate that RhoGDI2 expression is not restricted to cells of hematopoietic lineages but instead has a more extensive distribution in normal tissues, suggesting that it may play a role in nonhematopoietic cell biology.

Another interesting finding is the presence of RhoGDI2 in endothelial cells in both tumor and normal vasculature (arteries, veins, and capillaries). These data may account in part for the high expression values observed in heart and lung. Interestingly, most of the supposedly specific endothelial markers are present on both endothelial cells and immature and mature hematopoietic cells (15), which form the concept of a common embryonic precursor. This concept is further supported by recent data suggesting mechanistic links between hematopoietic progenitors and endothelial cells. Using a chronic myelogenous leukemia model, we have provided evidence for the existence of a hemangioblastic progenitor cell in the bone marrow of adult humans. Data suggest that chronic myelogenous leukemia arises from a hemangioblastic progenitor cell, the progeny of which are malignant blood cells and genotypically clonal endothelial cells (16). Similarly, Schmeisser *et al.* (17) have recently shown a phenotypic overlap between monocytes and vascular endothelial cells. Finding RhoGDI2 expression in both endothelial and hematopoietic cells further supports these ideas.

To gain further understanding of the relationship between RhoGDI2 mRNA expression and tumor progression in human

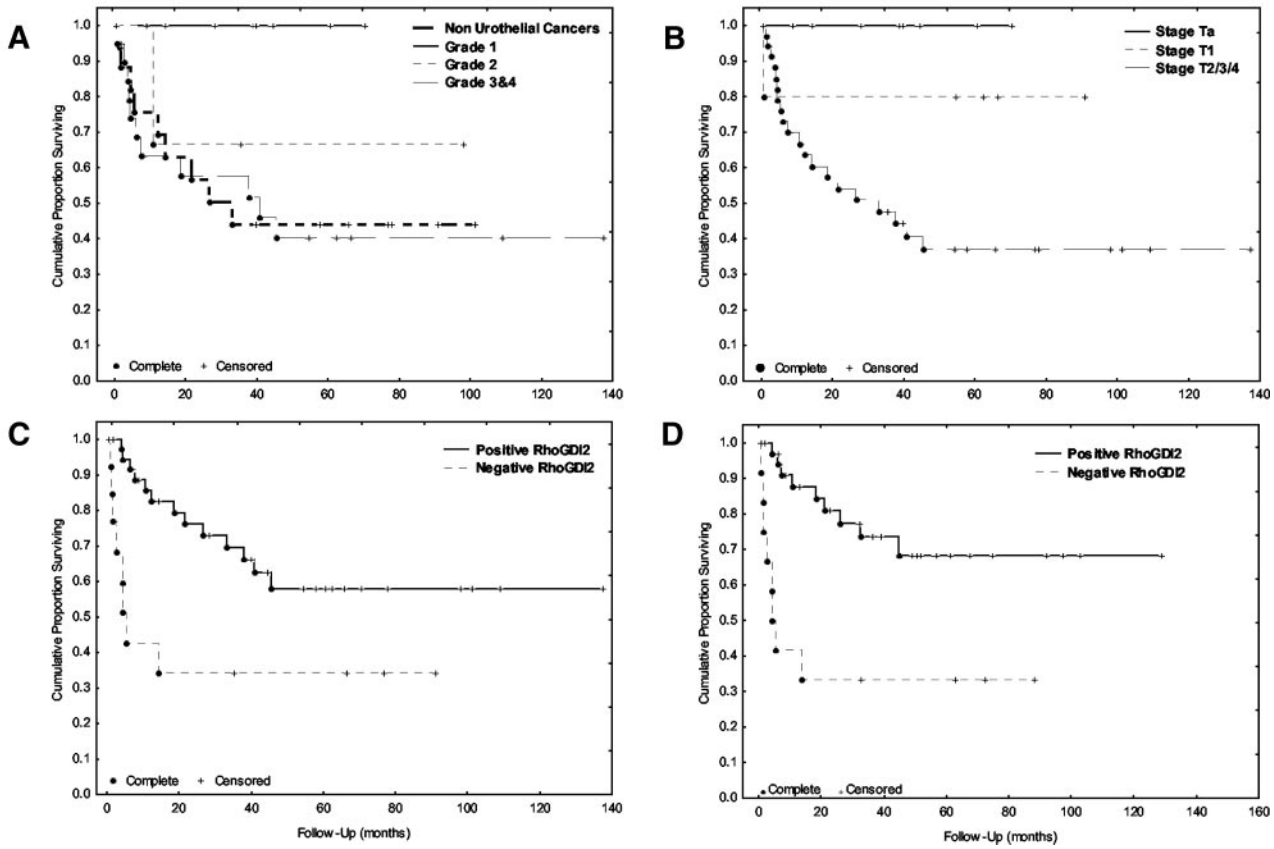

Fig. 3 Association between RhoGDI2 status and disease-free or disease-specific survival time: Kaplan-Meier curve estimates of the survival time distributions for patients as a function of *A*, disease-specific survival time as a function of tumor grade including nonurothelial cancers; *B*, disease-specific survival time as a function of pathological stage; *C*, disease-free survival time as a function of RhoGDI2 immunohistochemistry (positive compared with those with reduced intensity or negative by immunohistochemistry); *D*, disease-specific survival time as a function of RhoGDI2 immunohistochemistry (positive compared with those with reduced intensity or negative by immunohistochemistry).

bladder cancer, we carried out a survey of common human bladder tumor cell lines. We categorized these lines in terms of their invasive or metastatic abilities based on their initial description or subsequent biological behavior as observed in xenograft models of bladder cancer. As can be seen in Fig. 1*D*, there is a wide spectrum of expression of the RhoGDI2 mRNA in the cell lines. We used both published information on the original biological behavior of the tumors from which the cell lines were derived (18) as well as xenograft data (10, 19) to assign the cell lines to either superficial (not invading muscle); invasive (invading muscle of bladder wall), or metastatic to lung. Using this classification system, we observed a marked decrease in its expression as a function of invasive and metastatic tumor cell phenotypes (Fig. 1*D*, *inset*), strongly supporting the notion that this gene is related to tumor progression and metastasis.

Table 2 Relationship of RhoGDI2 immunohistochemical expression with pathological and clinical variables: Effect of RhoGDI2 immunohistochemical expression on disease-specific survival adjusting for grade, stage, and histological type

Variable	Hazard ratio	95% confidence interval	P^a
Grade	8.89	1.13–69.7	0.04
Stage	1.12	0.76–1.65	0.57
Histology[b]	0.15	0.02–1.27	0.08
RhoGDI2[c]	0.32	0.12–0.87	0.03

[a] *P*s based on Wald tests in the Cox proportional hazards model.
[b] Histology: urothelial or other tumor histology.
[c] RhoGDI2: immunohistochemical staining for RhoGDI2.

Our previous results (9, 10) and the data presented above on human bladder cancer cell lines suggests that RhoGDI2 is an important determinant of bladder cancer metastasis. Therefore, one would expect that the presence or absence of this protein in bladder tumors would potentially offer clinically useful prognostic information. To address this issue, we undertook an immunohistochemical study of 51 tumors from patients with bladder cancer to determine whether RhoGDI2 correlated with pathological parameters and clinical outcome. Patient age at the time of surgery ranged from 42 to 72 years with a median of 62. Median follow-up for patients still alive ($n = 30$) was 4.5 years (range, 0.03–11.4 years). Pathological and immunohistochemical features of the neoplasms are listed in Table 1. Twenty-eight urothelial carcinomas showed immunopositivity for RhoGDI2

similar to that for normal urothelium, whereas 2 had reduced staining intensity, and 4 were negative. Five squamous cell carcinomas showed a normal level of immunoreactivity, whereas five had reduced levels of staining, and two lacked immunoreactivity. Two adenocarcinomas of the bladder were positive for RhoGDI2, and one was negative. Three small-cell undifferentiated carcinomas lacked RhoGDI2, whereas one showed reduced positivity. Inflammatory cells and endothelial cells within these carcinomas showed immunopositivity.

Univariate analysis was carried out to determine whether there were any associations between conventional pathological prognostic markers and RhoGDI2 protein expression (Table 1). In these analyses, stage and grade categories with low frequencies were combined with adjacent categories of stage or grade. Four observations with grade-2 tumors were combined with 10 grade-1 tumors, and 8 grade-4 tumors were combined with 12 grade-3 tumors. Because bladder tumors of nonurothelial histology are not routinely graded, these were placed in a separate category. Superficial (T_a) tumors were evaluated separately from invasive (T_1–T_4) tumors and nonurothelial tumors. RhoGDI2 was not associated with stage ($P = 0.08$) or grade ($P = 0.08$). This is probably because of the small number of tumors in this early study. Interestingly, as a group, tumors of nonurothelial histological type generally appeared to express less protein than did their urothelial counterparts (Table 1; Fig. 2), and statistical analysis indicated that histological type was associated with RhoGDI2 expression ($P = 0.03$).

A comparison of Kaplan Meier survival curves as a function of grade (Fig. 3*A*) and stage (Fig. 3*B*) indicate that our 51-patient cohort was typical of that in the published literature, namely that stage, grade, and histology influence disease-specific survival. In addition, because tumors of nonurothelial histology were all stage $\geq T_2$ and are not routinely graded, we evaluated them as a separate group and found that their prognosis was similar to that of high-grade urothelial cancers. Importantly, there was a significant difference in disease-free ($P < 0.001$; Fig. 3*C*) and disease-specific ($P < 0.01$; Fig. 3*D*) survival times between patients with RhoGDI2 positive tumors and those with reduced or absent protein expression. In addition to the difference in recurrence and in long-term survival between the two groups, patients whose tumors lacked, or had reduced, RhoGDI2 had a steeper failure rate compared with those who had RhoGDI2-positive tumors ($P \leq 0.01$). This rapid appearance of metastatic disease is an interesting observation and is consistent with the notion that reduced protein expression of this gene is associated with the metastatic phenotype. In this model, patients harboring tumors with a high degree of metastatic potential would be expected to develop clinical metastasis and would succumb rapidly after treatment of the primary tumor because a significant number of cells would have already been present in distant organs at the time of treatment.

To understand the independent contribution of RhoGDI2 reduction to bladder tumor progression in comparison with other variables, we constructed a proportional hazard model involving all of the prognostic variables (Table 2). These data indicated that grade and RhoGDI2 immunohistochemical level provide independent predictive ability for disease-specific survival when used together in the model, even in this small cohort of patients. The independent prognostic ability of metastasis suppressor genes in human cancer is relatively uncommon thus far (20–22). Hence, our findings suggest that RhoGDI2 has fundamentally important biological functions and correlates with patient prognosis as well.

In summary, our results suggest that RhoGDI2 expression is a predictor of prognosis in patients with bladder cancer, which supports previous experimental data indicating a role for this gene in the suppression of metastasis. If confirmed in larger independent prospective studies, the evaluation of RhoGDI2 expression may offer additional predictive ability beyond conventional pathological markers and, in this way, may serve as a useful indicator of disease aggressiveness, perhaps leading to molecularly individualized treatment of patients with bladder cancer.

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