RhoGDI2 Is an Invasion and Metastasis Suppressor Gene in Human Cancer

John J. Gildea, M. Jabeed Seraj, Gary Oxford, Michael A. Harding, Garret M. Hampton, Christopher A. Moskaluk, Henry F. Frierson, Mark R. Conaway, and Dan Theodorescu

Abstract

To discover novel metastasis suppressor genes that are clinically relevant in common human cancers, we used isogenic human bladder cancer cell lines and used DNA microarray technology to identify genes whose expression diminishes as a function of invasive and metastatic competence. We then evaluated the expression profile of such genes in 105 pathologically characterized tumors from seven common organ sites, and we identified one gene, RhoGDI2, whose expression was diminished as a function of primary tumor stage and grade. When RhoGDI2 was transferred back into cells with metastatic ability that lacked its expression, it suppressed experimental lung metastasis but did not affect in vitro growth, colony formation, or in vivo tumorigenicity. In addition, RhoGDI2 reconstitution in these cells blocked invasion in an organotypic assay and led to a reduction of in vitro motility. These results indicate that RhoGDI2 is a metastasis suppressor gene, a marker of aggressive human cancer, and a promising target for therapy.

Introduction

Tumor cell growth at the site of metastasis is an important clinical target. Because cells must survive and proliferate to become overt metastases, there is ample opportunity for intervention. Understanding the genes responsible for either enhancing or suppressing this process would allow novel therapeutic and diagnostic applications to evolve and thus improve clinical care of cancer patients. However, although it takes the coordinated expression of many genes to allow the development of invasion and metastasis (1, 2), it may only take one gene to block this process because inability to complete any step of the metastatic cascade renders a cell nonmetastatic (3–6). Therefore, the discovery of metastasis suppressor genes (3–6), genes that inhibit the formation of spontaneous, macroscopic metastases without affecting the growth rate of the primary tumor, is critically important. In addition, at the time of their identification, strategies to define the relevance of such genes in human cancer progression would allow the prioritization of those whose expression is closely linked with this phenotype, thus accelerating the translational benefits to patient care afforded by such a discovery. In this study, we have used such a strategy to identify a novel metastasis suppressor gene in common human cancers.

Materials and Methods

Cell Lines. T24T is a highly metastatic, spontaneously occurring variant cell line of the human bladder cancer cell line T24. In contrast to this variant, T24 does not produce significant metastasis when injected i.v (7).

Microarray Hybridization. RNA extraction from cell lines and tissues was carried out as described previously (8). For cell line analysis of gene expression, the HG-U133A Array (Affymetrix Inc., Santa Clara, CA) was used. RNA extraction from human tissues and hybridization on the U95Av2 GeneChip (Affymetrix) that represents ~12,000 sequences, many of which have previously been characterized in terms of function or disease association, were performed as described previously (8). We included only those probe sets whose maximum hybridization intensity (AD) in at least one sample was >200; the other probe sets were excluded (the quantification of gene transcripts with AD values uniformly <200 is typically unreliable).

Primary Tumor Analysis. A set of 105 primary human carcinomas whose transcripts were profiled had known tumor stage and grade. This set of tumors comprised 24 prostate adenocarcinomas, 8 bladder carcinomas, 21 breast adenocarcinomas, 14 colorectal adenocarcinomas, 10 clear cell carcinomas of the kidney, 4 hepatocellular carcinomas, and 24 lung carcinomas.

Plasmid Constructs and Transfection and Derivation of Stable Cell Lines. A full-length RhoGDI2 was cloned into the pCDNA 3.1-Zeo vector containing a zeocin-selectable marker driven by the cytomegalovirus promoter. Transfections of pCDNA 3.1-RhoGDI2 and pCDNA 3.1 were performed by Lipofectin (Life Technologies, Inc.) using the manufacturer’s instructions. One day after transfection, 25 µg/ml zeocin (Invitrogen) was added to the cultures and maintained until no further death (~1 month) was observed in the cultures. Western Blot Analysis. Protein extracts were prepared from T24T/vector and T24T/RhoGDI2 cells grown to 80–90% confluence in 100-mm dishes. Western blotting was carried out as described previously (9) using commercial enhanced chemiluminescence reagents (SuperSignal West Femto; Pierce) and photographic film. Antibodies for cleaved RhoGDI2 were from Imgenex. Antibodies for intact RhoGDI2 were from Biosource, and antibodies for cleaved RhoGDI2 were from Imgenex. Positive control lysates of Jurkat cells treated with staurosporine from BD Transduction.

In Vitro Growth and Colony Formation. Single cell suspensions were obtained with cell counts performed using a model ZM Coulter Counter. Trypan blue staining was used to assess viability before plating of the cells. After incubation of 375, 750, 1500, and 3000 cells/well in 96-well plates for various times, cell numbers were estimated using the CyQUANT assay (Molecular Probes, Eugene, OR). Colony formation in agar by 1 × 104 cells inoculated into each well of 24-well culture plate was carried out using routine methods (10).

s.c. Tumorigenicity, Experimental Metastasis Assay, and Tissue Processing. Six-week-old nude mice were obtained from the University of Virginia breeding facility and maintained strictly according to the NIH and institutional guidelines. Subcutaneous tumorigenicity was evaluated as described previously (11). For experimental metastasis, mice were given an i.v. lateral tail vein injection with 106 tumor cells suspended in 0.1 ml of SFM.3 In both assays, mice were examined and weighed every week. Tumors were measured weekly, and mice were euthanized 8 weeks after injection. At the time of euthanasia, the lungs and liver were removed by dissection away from adjacent organs and examined grossly and microscopically. Visual counting of lung metastases was carried out as described previously (11).

3 The abbreviations used are: SFM, serum-free medium; EGF, epidermal growth factor.
Chemotaxis and Invasion Assays. Baseline and EGF-induced migration were assessed by measuring the movement of cells into a scraped, acellular area or "wound assay" as described previously (9). Briefly, cells were plated in DMEM-F12 media plus 5% serum and grown to ~90% confluence, serum-starved for 24 h in HyQ-CCM1 media (Hyclone, Houston, TX), scraped with a pipette tip, and assayed over a 12-h time period with and without EGF. A time 0 control was taken by fixing wells immediately after scraping. The assay was terminated when the EGF-stimulated control wound was completely closed, which occurred at approximately 12 h. The cells were then fixed in 100% methanol and stained with crystal violet. Analysis of the wound was performed by digitally capturing the wounded area using an Olympus Stereo dissecting scope as described previously (9).

Bladder Organ Culture Invasion Assay. We modified and optimized the assay described by Crook et al. (12). Rats were anesthetized, and bladders harvested under sterile conditions. The bladder was detubularized and glued in 1 well of a 6-well plate, urothelium side up, with cyanoacetate glue. Fifty μl of trypsin EDTA in HBSS (Life Technologies, Inc. catalogue number 25200-080) were added to the bladder and incubated for 15 min at room temperature. The trypsin was gently aspirated from the edge of the bladder, and any residual trypsin inactivated by 100 μl of 100% fetal bovine serum. The fetal bovine serum was removed, and 5

The trypsin was gently aspirated from the edge of the bladder, and any residual trypsin inactivated by 100 μl of 100% fetal bovine serum. The fetal bovine serum was removed, and 5 × 105 cells in 100 μl of complete Waymouth media (Gibco) with 10% FCS containing antibiotic/antimycotic were placed on the bladder surface and incubated for 4 h. After 4 h, 4 ml of complete media were added to each well. The media were changed twice a week for the duration of the experiment. After 3 weeks, the bladders were washed twice with PBS and fixed overnight in 10% buffered formalin. Routine histological sections were carried out as described previously (10) and examined by a board-certified anatomical pathologist (C. A. M.).

Results and Discussion

We have recently characterized a human bladder cancer cell line T24 and its more aggressive isogenic variant, T24T (7). When injected i.v., T24 forms metastases in approximately one-third of mice with 1–2 lesions/mouse, whereas T24T produces metastases in all mice with a mean of 24–28 lesions/mouse. In addition, T24 cells are noninvasive in organ culture and orthotopic models, whereas T24T are invasive in both (data not shown). To identify putative invasion and metastasis suppressor genes, we evaluated the gene expression profiles of these cell lines with low and high metastatic ability, focusing on genes whose expression is significantly diminished in the more metastatic and invasive T24T variant.

RNA was extracted from these two cell lines and hybridized to HG-U133A Affymetrix oligonucleotide arrays consisting of two GeneChip arrays. These arrays contain almost 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 human genes.4 A total of 2,368 genes were differentially diminished in the metastatic cell line. Table 1 shows a summary list of the top 30 genes whose expression was most diminished. Interestingly, several of these genes are known to be IFN inductive, but their relationship to tumor invasion and metastasis is unknown. Others, such as the genes for the matrix components fibronectin, collagen (13), and tissue inhibitor of metalloproteinase (14), have known associations with tumor invasion and metastasis. Expression of fibronectin has been linked with metastasis in a melanoma model (15), whereas other work has shown that fibronectin fragments can inhibit tumor growth, angiogenesis, and metastasis (16).

Two of the most diminished genes are related to the Rho family of GTPases and their regulators, Rac2 and RhogDI2 (an inhibitor of guanine nucleotide dissociation for Rho proteins). This is of particular interest because regulation of the actin cytoskeleton by Rho GTPases has been implicated in promoting a variety of cellular processes such as changes in morphology, motility, and adhesion (17) that may contribute to invasion and metastasis of cancer cells (18). Recently, overexpression of Rho C has been found in melanoma cells with increasing metastatic activity (15) as well as in inflammatory breast cancer (19). Also, overexpression of wild-type or constitutively active forms of RhoA has been shown to induce invasive behavior in noninvasive rat hepatoma cells in vitro (20) and to confer the metastatic phenotype to both NIH 3T3 fibroblasts and poorly metastatic melanoma cells in vivo. In addition, Tiam1, a gene shown to play a role in T-cell lymphoma invasion, encodes a guanine nucleotide exchange factor for Rho GTPases (21). Together, these results and others indicate that both overexpression and misregulation of Rho GTPases contribute to cancer cell invasion and metastasis.

Having uncovered 2368 genes whose expression patterns correlate with diminished invasion and metastasis in our cell line model, we used a secondary selection criteria to determine which genes warranted further mechanistic study, based on their expression levels in human tumors as a function of pathological stage and grade. We used the HG-U95Av2 GeneChip (Affymetrix) to determine the gene expression profiles of 105 primary human tumors whose stage and grade status were known. This set of tumors comprised the most common single histological type of carcinoma.

We then proceeded in descending order to evaluate the genes shown in Table 1, and we found that RhoGDI2 was the first gene that

Table 1 Top 30 genes differentially expressed in metastatic (T24T) and nonmetastatic (T24) human bladder cancer cell lines

<table>
<thead>
<tr>
<th>Probe set†</th>
<th>Genes</th>
<th>Signal log ratioβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>201859_at</td>
<td>Proteoglycan 1, secretory granule (PLG1)</td>
<td>9.7</td>
</tr>
<tr>
<td>208794_s_at</td>
<td>SW5/SNF related, transcriptional activator 4 (SMARCA4)</td>
<td>-8.1</td>
</tr>
<tr>
<td>204058_s_at</td>
<td>Keratin 19 (KRT19)</td>
<td>-7.9</td>
</tr>
<tr>
<td>204211_at</td>
<td>IFN, α-inducible protein 27 (IFI27)</td>
<td>-7.6</td>
</tr>
<tr>
<td>206655_s_at</td>
<td>Osteonectin (ON)</td>
<td>-7.2</td>
</tr>
<tr>
<td>202269_s_at</td>
<td>IFN-inducible guanylate-binding protein 1 (GBP-1)</td>
<td>-7.2</td>
</tr>
<tr>
<td>203571_s_at</td>
<td>Adipose-specific 2 (APM2)</td>
<td>-7.1</td>
</tr>
<tr>
<td>209865_s_at</td>
<td>IFN γ-inducible protein 16 (IFI16)</td>
<td>-7.0</td>
</tr>
<tr>
<td>201288_at</td>
<td>Rho GDP dissociation inhibitor (GDI) β (GDI2)</td>
<td>-6.8</td>
</tr>
<tr>
<td>211959_at</td>
<td>Insulin-like growth factor-binding protein 3 (IGFBP3)</td>
<td>-6.6</td>
</tr>
<tr>
<td>209160_at</td>
<td>3α-hydroxysteroid dehydrogenase, type II</td>
<td>-6.4</td>
</tr>
<tr>
<td>202286_s_at</td>
<td>Gastrointestinal tumor-associated antigen GA733-1</td>
<td>-6.4</td>
</tr>
</tbody>
</table>

† Probe set and gene description refers to the assignment on the HG-U133a chip (www.affymetrix.com).

β Signal log ratio indicates the log (base 2) of the gene expression ratio of T24T/T24.

4 Not present on HG-U95Av2 chip (www.affymetrix.com) and therefore not included in stage/grade analysis (Fig. 1).
correlated with both tumor stage and grade (Fig. 1A). In addition, we have previously shown that expression of RhoGDI2 is inversely correlated with the invasive phenotype in human bladder cell lines (11). Hence, the eight genes whose expression was found to be more diminished than RhoGDI2 in T24T cells were not uniformly related to both tumor grade and stage (Fig. 1A) and thus were not further investigated. Furthermore, in view of published evidence that overexpression and/or misregulation of Rho family members are associated with both tumor stage and grade, we have previously shown that expression of RhoGDI2 is inversely correlated with the invasive phenotype in human bladder cell lines (11). Hence, the eight genes whose expression was found to be more diminished than RhoGDI2 in T24T cells were not uniformly related to both tumor grade and stage (Fig. 1A) and thus were not further investigated. Furthermore, in view of published evidence that overexpression and/or misregulation of Rho family members are associated with both tumor grade and stage. A set of 105 primary carcinomas was expression profiled, and their clinicopathological data, including stage and grade, were assigned by a board-certified anatomical pathologist (H. F. F). The expression level of the eight most diminished genes in the metastatic T24T cells is shown as a function of stage and grade. B, expression of Rho family GTPases in human cancer. Expression analysis as in A for genes encoding Rho family GTPases and their regulators found on the HG-U95Av2 array. C, RhoGDI2 expression in bladder cell lines. Western blot analysis of RhoGDI2 protein levels in whole cell lysates (10 μg protein/lane) for T24, T24T, and T24T vector- and RhoGDI2-transfected cells. Lanes are as indicated. Jurkat cell lysates are positive controls for cleaved RhoGDI2-specific antibody.
lated with metastatic disease, we sought to evaluate such expression levels in our cohort of human tumors. As can be seen in Fig. 1B, among the genes present on the HG-U95Av2 array, only RhoGDI2 expression levels are associated with both tumor grade and stage.

Having established a strong correlation between loss of RhoGDI2 expression and higher stage and grade in human cancer, we set out to investigate whether RhoGDI2 expression levels are causally related to invasion and metastasis or are merely markers of this phenotype. A full-length human RhoGDI2 gene was cloned into the pCDNA 3.1 expression vector and transfected into the metastatic T24T bladder cell lines. Vector-only transfectants were also generated in parallel. Using zeocin selection, resistant cells were isolated in both transfection groups. RhoGDI2 expression in the transfected cell lines was evaluated at the protein level (Fig. 1C), using antibodies specific for intact RhoGDI2, indicating that the level of this expression in transfectants is in the same range as that found in the noninvasive and nonmetastatic T24 cells. This point is particularly important because supraphysiological expression of exogenous transgenes may lead to aberrant functional consequences and hence misinterpretations of the true function of a given gene. Because RhoGDI2 is cleaved by caspases during many forms of apoptosis, we also used an antibody specific for the cleaved form of RhoGDI2 (as shown in Jurkat cells induced to undergo apoptosis by staurosporine treatment) to determine that there is no detectable RhoGDI2 cleavage in both the T24 cells and the T24T transfectants.

By definition, a metastasis suppressor gene does not affect growth or tumorigenicity; we evaluated these features in the vector control and RhoGDI2-transfected T24T cell lines. As can be seen in Fig. 2A, in vitro growth was not significantly different between the two cell lines. When anchorage-independent growth (clonogenicity in soft agar) was evaluated, no difference was seen (Fig. 2B). Finally, tumorigenicity at s.c. sites in immunocompromised mice (Fig. 2C) was similar for both cell lines.

Because the growth and tumorigenicity of T24T were not affected by RhoGDI2 expression, we tested whether RhoGDI2 reexpression in T24T cells affected the metastatic phenotype of these cells. Using an experimental metastasis assay in immunocompromised mice, vector-and RhoGDI2-transfected cells were evaluated for metastasis formation 8 weeks after injection. All seven vector control-injected mice had lung metastases with an average of 32 metastases/mouse, consistent with previous results on T24T (11). In contrast, only four of eight mice injected with RhoGDI2-transfected cells exhibited lung metastases, and of these four, each mouse had an average of only five metastatic nodules. The differences in these results were statistically significant in both the number of mice with tumors ($P = 0.027$) and the number of tumors/mouse ($P = 0.007$). A representative picture of the two groups is shown in Fig. 3A.

Having established a causal role for RhoGDI2 in metastatic disease, we next asked how RhoGDI2 might regulate the ability of tumor cells to metastasize. Because Rho family members and related regulators affect cytoskeletal organization (22) with resultant effects on tumor invasion and motility, we hypothesized that RhoGDI2 functions as an invasion suppressor in human cancer. Thus, tumor cells expressing RhoGDI2 and reaching the vascular bed of the lung would lack the ability to effectively invade and colonize this organ, leading to reduced metastatic ability.

Using a novel organotypic bladder invasion assay, we evaluated the invasive ability of vector control- and RhoGDI2-transfected cell lines. As can be seen in Fig. 3B, the vector-transfected cell lines are invasive, as would be expected in view of their metastatic phenotype, whereas cells expressing the RhoGDI2 transgene remain superficial to the basement membrane, which is similar in behavior to their lineage-related noninvasive counterpart, T24 (data not shown).

Furthermore, because invasion is composed of several distinct steps (1), a critical one being cell motility, we sought to refine our understanding of the mechanisms by which RhoGDI2 blocks invasion by evaluating the impact of RhoGDI2 reconstitution on the motile phenotype. Because EGF induces in vitro cell migration in T24T cells (9) and is a significant factor in the biology of invasive bladder cancer (23), we examined the role of RhoGDI2 in EGF-induced cell migration. In the standard wound assay, cells expressing the RhoGDI2 transgene had poor baseline migration and a minimal response to EGF addition (Fig. 3C). Because phosphatidylinositol 3'-kinase is a known mediator of EGF-mediated motility in these cells (9), LY294002 was used as an additional specificity control for motility inhibition.

In summary, we have identified RhoGDI2 as a novel metastasis suppressor gene in human cancer. This gene is mechanistically in-
involved in suppressing tumor invasion and metastasis in model systems of human bladder cancer, and its expression is diminished as a function of tumor stage and grade in a variety of common human carcinomas, suggesting an important and fundamental role in the pathogenesis of tumor progression in human cancer irrespective of tumor type. As mentioned above, the role of Rho proteins in cancer metastasis is growing (15). The GDP-bound form of Rho family members is complexed with RhoGDIs and remains in the cytosol as an inactive form until their release from RhoGDI (24). RhoGDI is a soluble protein, and it inhibits the association of the GDP-bound form of prenylated Rho with the membrane by forming a ternary complex (25) and thus limits the availability of Rho molecules to be converted from an inactive to an active (GDP→GTP) form. Because Rho GTPases are strongly implicated in experimental metastasis of breast (26) and melanoma (15) cancer model as well as in human inflammatory breast cancer (19), the inferred effect of decreased expression of a RhoGDI would be to provide increased access of Rho guanine nucleotide exchange factors to the Rho GTPases, thus allowing the cell to become more invasive and metastatic. In addition, our findings challenge the notion that expression of RhoGDI2 is specific to hematogenous cell lineages (27).

This work also highlights the power and utility of combining gene profiling of well-defined experimental model systems with human data to pinpoint functionally relevant gene products in human disease. Although the in vivo specificity of RhoGDI2 for Rho protein targets remains to be determined, preliminary results indicate that it may
regulate the activity of RhoB.5 These observations suggest important functional differences between Rho family members with respect to their role in cell motility, invasion, and metastasis. In addition, interpretation of the expression data from human tumors suggests that Rho family members may be actively involved in the metastatic cascade via deregulated activity from loss of GDI function, in addition to possible overexpression or activating mutations. One of the most important ramifications of this work concerns the therapeutic and diagnostic possibilities offered by these observations. There is a critical need for markers that will accurately distinguish those histological lesions with a high probability of causing clinically important metastatic disease from those that will remain indolent. Finally, because guanine nucleotide dissociation inhibitors such as RhoGDI2 function to block specific enzymatic reactions, high-throughput systems can be readily designed for discovery of mimetic agents that target such reactions.

References


5 G. Oxford and D. Theodorescu, unpublished observations.

RhoGDI2 Is an Invasion and Metastasis Suppressor Gene in Human Cancer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/22/6418

Cited articles
This article cites 26 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/22/6418.full#ref-list-1

Citing articles
This article has been cited by 34 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/22/6418.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/62/22/6418.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.