Overexpression of RhoA, Rac1, and Cdc42 GTPases Is Associated with Progression in Testicular Cancer

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ABSTRACT
The Rho family of GTPases are involved in actin cytoskeleton organization and associated with carcinogenesis and progression of human cancers. We investigated the roles of Rho family GTPases, prototypes RhoA, Rac1, and Cdc42, and the major downstream targets of RhoA, ROCK-I, and ROCK-II in testicular cancer. We quantified protein expression in paired tumor and nontumor samples from surgical specimens from 57 consecutive patients with testicular germ cell tumors using Western blotting. Protein expression of RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 was significantly higher in tumor tissue than in nontumor tissue (P < 0.0001). Expression of protein for RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 was greater in tumors of higher stages than lower stages (P < 0.0001, P < 0.001, P < 0.001, P < 0.0001, P < 0.0001, respectively). Within stage II nonseminoma (31 patients), protein levels of RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 in the primary tumor were lower in the group of 24 patients with no evidence of disease after therapy compared with 7 patients with disease that was recurrent (P < 0.05). Rho family GTPases may be involved in the progression of testicular germ cell tumors.

INTRODUCTION
Because of the availability of highly effective combination modalities of surgery, radiotherapy, and cisplatin-containing chemotherapy, disease-specific survival in testicular germ cell tumors (GCTs) is >90% (1). However, cure rates of patients with a higher stage at presentation and/or recurrent tumor, in particular those refractory to high-dose chemotherapy with stem-cell rescue, are low (5-year survival rate < 30%: Refs. 2–4). Cancer cell migration is central to the process of metastasis (5). Therefore, new treatment modalities, e.g., inhibitors of metastasis, for high-risk patients should be sought.

Members of the Rho GTPases family, prototypes RhoA, Rac1, and Cdc42, are involved in the regulation of a variety of cellular processes, including organization of the microfilament network and cell-cell contact, and they perform essential and specialized functions in actin cytoskeleton organization (6). RhoA regulates formation of stress fibers and focal adhesion of cells. Rac1 regulates formation of lamellipodia and membrane ruffling, whereas Cdc42 regulates formation of filopodia (6, 7).

There is increasing evidence that Rho-family GTPases influence a variety of processes in cancer, including cell transformation, survival, invasion, metastasis, and angiogenesis (8). It has been reported that overexpression of RhoA (9–12), Rac1 (9), and Cdc42 (9) is associated with carcinogenesis and progression of several human tumors (8).

ROCK (Rho-associated serine-threonine protein kinase; Refs. 13, 14), one of the best characterized downstream effectors of RhoA, is activated when it binds selectively to the active GTP-bound form of RhoA. ROCK mediates RhoA action on the actin cytoskeleton through stress-fiber formation and assembly of focal contacts (15). Furthermore, the Rho/ROCK pathway is involved in cancer progression, and a specific ROCK inhibitor suppresses tumor growth and metastasis (16, 17).

For patients with solid human tumors, the biggest threat to survival is metastasis, and cell migration is a pivotal step in metastasis (5). In moving cells, lamellipodia and filopodia can be observed at the advancing aspect of the cell, whereas retraction can be seen on the opposite side (18). These findings are accompanied by reorganization of the actin cytoskeleton within the cell.

To clarify the roles of Rho family GTPases in testicular GCTs, we examined protein expression of RhoA, Rac1, and Cdc42 and major downstream effectors of RhoA, ROCK-I, and ROCK-II using Western blotting. We compared protein expression in testicular GCT tissue with the nonneoplastic portions of the same resected specimen. The relationship between protein expression and pathological features of the tumors was examined.

MATERIALS AND METHODS
Patients and Tissue Preparation. Specimens from surgery for newly diagnosed testicular GCTs between 1995 and 2001 from 57 consecutive Japanese patients were studied (age range, 16–59 years; mean, 37.8 years). Eighteen patients had a histological diagnosis of pure seminoma, whereas 39 patients had a nonseminoma component. All patients underwent imaging studies (computed tomography and/or magnetic resonance imaging) before surgery for staging. Postoperative follow-up ranged from 3 to 60 months (median follow-up, 29 months). In
all cases, three sites of tumor and varying portions of adjacent nonneoplastic testis were examined for the study (10, 11). Clinical staging was carried out according to the criteria of Tumor-Node-Metastasis classification (19). The study was conducted in accord with the Helsinki Declaration. Institutional Review Board approval was obtained for this investigation. Each patient signed a consent form approved by the Committee on Human Rights in Research at our institution.

After radical inguinal orchiectomy, patients with stage I seminoma were treated with radiotherapy at the lymphatic drainage area, and patients with stage I nonseminoma were treated with chemotherapy. Stage II disease was treated with radical inguinal orchiectomy and postchemotherapy retroperitoneal lymph node dissection. For patients with recurrent and/or refractory stage II nonseminoma or those with stage III, cisplatin-based high-dose chemotherapy with stem-cell rescue was performed.

Western Blotting. Tumors and normal testis were dissected by omitting stromal tissue. Western blotting was carried out as described previously (9–11). Briefly, 50 μg of cytosolic proteins were separated by SDS-PAGE (12.5% gel) for immunological detection of proteins. After blotting to nitrocellulose, proteins bound to the membrane were stained by Ponceau S to confirm that identical amounts of protein had been transferred. Rho family protein expression was analyzed using specific antibodies (sc-179, RhoA; sc-6055, ROCK-I; sc-1851, ROCK-II; sc-95, Rac1; sc-87, Cdc42; each diluted 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with peroxidase-conjugated antirabbit IgG, these proteins were visualized by chemiluminescence. The blotted membrane was scanned densitometrically with a PDI imaging scanner (Agfa Japan, Tokyo, Japan) and analyzed with NIH image software. For quantitation of proteins, relative amounts of Rho GTPases in tumors were expressed as a ratio of absorbance of bands from the tumor specimen to those from the corresponding normal tissue; the latter was set at 1.0 by densitometric analysis as described previously (9–11). Mean values from three experiments were obtained for tumor and nontumor tissues (10, 11).

Immunohistochemistry. Immunohistochemistry, using the same specific antibodies as antibodies for Western blotting, was performed to support the data obtained by Western blotting as described previously (9).

Statistical Analysis. Results of Western blotting were analyzed using the Mann-Whitney U test as described previously (10, 11). Spearman rank correlation coefficient was used to determine the relationships between proteins (10). P values < 0.05 were considered significant. Data were analyzed using commercially available software.

RESULTS

Protein expression of RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 was observed in both tumor and nontumor tissues (Fig. 1). Two isoforms of ROCK-I and ROCK-II have high homology (20, 21) and thus show a cross-reaction (Fig. 1).

Expression levels of RhoA protein correlated positively with ROCK-I and ROCK-II in tumor tissues (correlation coefficient $r^2 = 0.586$, $P < 0.0001$, and $r^2 = 0.459$, $P < 0.0001$, respectively; Fig. 2). The expression of RhoA also showed a positive correlation for Rac1 ($r^2 = 0.473$, $P < 0.0001$) and Cdc42 ($r^2 = 0.322$, $P < 0.0001$). There was a positive correlation between Rac1 and Cdc42 expression ($r^2 = 0.554$, $P < 0.0001$).

The amounts of protein for RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 was significantly greater in cancerous (mean ± SD, 3.52 ± 1.31, 3.90 ± 1.43, 2.05 ± 0.51, 4.48 ± 1.48, 3.22 ± 0.88, $P < 0.0001$ for all; Fig. 3).

Although tumor cells showed moderate (for anti-RhoA, anti-ROCK-I, anti-ROCK-II, and anti-Cdc42 antibodies) to strong (for anti-Rac1 antibody) staining, nontumor cells showed very weak reaction to these antibodies in immunohistochemistry (Fig. 4).

In stage I tumors, there was a difference of expression level of RhoA protein between nonseminomas and seminomas.
(mean ± SD, 2.92 ± 0.29 versus 2.62 ± 0.46; Fig. 5), and this was similar for ROCK-I (2.88 ± 0.61 versus 2.43 ± 0.73), ROCK-II (2.21 ± 0.31 versus 1.81 ± 0.50), Rac1 (4.09 ± 0.47 versus 3.15 ± 0.73), and Cdc42 (3.12 ± 0.48 versus 2.40 ± 0.50).

In nonseminomas, expression of RhoA protein in stage II/III tumors (mean ± SD, 4.70 ± 1.38) was increased in comparison with stage I tumors (Fig. 5), and the same was noted for ROCK-I (5.03 ± 1.37), ROCK-II (3.05 ± 0.78), Rac1 (5.64 ± 1.22), and Cdc42 (4.02 ± 0.65).

All stage I patients (17 seminoma and 5 nonseminoma) and patients with stage II seminoma (1 patient) were alive with no evidence of disease (NED) after systemic therapy. Of the patients with stage II nonseminoma (31 patients), 24 patients were alive with NED, whereas 7 patients showed refractory or recurrent disease (median; 17 months). Stage III patients (3 nonseminoma) were unresponsive to therapy, and all patients died from disease (median; 7 months).

We classified stage II nonseminoma into two groups: refractory/recurrence (7 patients) and NED (24 patients). We then compared expression levels of Rho GTPases of the primary tumor between the groups to evaluate the relationship between expression status and the effect of chemotherapy (Fig. 6). Although the differences in the expression levels were small, RhoA protein levels in primary tumors were higher in the refractory/recurrence group (mean ± SD, 5.34 ± 1.59) than those with NED (4.12 ± 0.86). Similar results were noted for ROCK-I: 5.83 ± 1.42 versus 4.29 ± 0.84; ROCK-II, 3.70 ± 0.49 versus 2.46 ± 0.46; Rac1, 6.19 ± 1.42 versus 5.14 ± 0.78; and Cdc42, 4.33 ± 0.69 versus 3.74 ± 0.48, (Fig. 6).

The existence of viable cells in resected lymph nodes obtained by postchemotherapy retroperitoneal lymph node dissection was examined microscopically. In resected involved lymph nodes, no viable cells were found in 24 patients with NED and in 2 of 7 refractory/recurrence patients. However, RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 proteins were weakly detected in resected involved lymph nodes from all 7 refractory/recurrence patients but only 2 of 24 patients with NED (data not shown). This occurred despite normalization of serum values of α-fetoprotein, β-human chorionic gonadotropin, and lactate dehydrogenase.
DISCUSSION

In the present study, RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 protein expression was greater in tumors than in paired nontumor testis. In our previous article (22) and the preliminary study, expression levels of mRNA for RhoA, ROCK, Rac1, and Cdc42 were increased in tumor than in nontumor tissues using PCR after reverse transcription in testicular GCTs. In these studies, gene expression of RhoA, ROCK, Rac1, and Cdc42 was presented by the relative yield of the PCR product from target sequences to that from the \( \beta \)-2-microglobulin gene used as an internal control, which did not change between tumor and normal. Furthermore, expression levels of mRNA for Rho correlated positively with its protein levels (11). These findings suggest that all of the proteins under study are increased in the tumor samples. Overexpression of RhoA protein has been reported in breast, lung, colon (9), and uroepithelial cancers (10, 11), as have Rac1 and Cdc42 in breast cancer (9). The Rho/ROCK pathway is involved in cancer progression (10, 16, 17), and overexpression of Rac1 and/or Cdc42 in breast (9) and head and neck cancer (12) has been shown. Several lines of evidence directly link Rho family GTPases to acquisition of a migratory, invasive, and metastatic phenotype (23). Higher expression of Rho family GTPases has been linked to higher stages in human cancers (9–11). In bladder and upper urinary tract cancer, RhoA and ROCK were abundantly expressed not only in primary lesions but also in metastatic lesions (10, 11). In the present study, higher expression of RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 protein in the primary lesion was associated with higher stages of disease. These observations suggest that Rho family GTPases are likely to be involved in carcinogenesis and the migration of tumor cells in testicular GCTs.

Testicular GCTs are classified as seminoma or nonseminoma, reflecting their origin in primordial germ cells and their remarkable ability to differentiate in vivo (1). Histologically, five different forms can be identified, and mixed tumors are frequently found: (a) seminoma; (b) embryonal carcinoma; (c) teratoma; (d) choriocarcinoma; and (e) yolk-sac tumor. Most nonseminomas include multiple cell types, and seminoma may...
be a component. Seminoma has the best prognosis of these cell types. Nonseminomas are clinically more aggressive than seminomas (1). Metastatic spread occurs along the lymphatic vessels of the funiculus through the inguinal canal to the renal and para-aortic lymph nodes or by a hematogenous route. In the current study, nonseminoma correlated with a higher stage than seminoma (P = 0.0018, data not shown). Because neoplasms are heterogeneous and contain subpopulations of cells with different angiogenic, invasive, and metastatic properties, their response to therapeutic agents is likewise heterogeneous (24). Although we could not examine in the present study, the protein levels of Rho family GTPases in each tumor component, seminoma, embryonal carcinoma, teratoma, choriocarcinoma, and yolk-sac tumor, should be examined to elucidate the biological differences and metastatic characteristics of each cell type in the future.

Therapy is based largely on stage, the histological differentiation, and serum values of α-fetoprotein, β-human chorionic gonadotropin, and lactate dehydrogenase (1). Cisplatin-containing chemotherapy is the optimal regimen for testicular GCTs because of its improved effectiveness (1). Despite extensive evaluation of many different treatment modalities, however, some metastatic or recurrent tumors are resistant to high-dose chemotherapy with stem-cell rescue (2–4).

A specific ROCK inhibitor, Y-27632 (25, 26), blocks both RhoA-mediated activation of actomyosin and invasive activity of cultured rat MM1 hepatoma cells (17). Continuous treatment with this inhibitor reduced dissemination of MM1 cells im-
planted into the peritoneal cavity of syngeneic rats (16). These studies suggest that ROCK inhibition may represent a potential approach to prevention of cancer invasion and metastasis by inhibiting cell migration and morphological alterations. In the present study, higher expression of RhoA/ROCK proteins were involved in tumor progression and associated with poor response to therapy. Therefore, we should elucidate the effects of this inhibitor, Y-27632, on testicular GCTs in vivo and in vitro.

Within stage II nonseminomas, the comparison between the refractory/recurrence (7 patients) and NED (24 patients) groups showed that protein expression of RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 in primary lesions was higher in the refractory/recurrence subset. The existence of viable cells in involved lymph nodes obtained by postchemotherapy retroperitoneal lymph node dissection is considered to be good index of the need for more aggressive therapy. Indeed, some Rho GTPases were weakly expressed in involved lymph nodes in which viable cells were not found; these cases showed refractory/recurrent disease. These results suggest that higher expression levels of Rho GTPases are associated with higher rates of refractory/recurrent disease. Examining Rho expression status in resected nodes after postchemotherapy retroperitoneal lymph node dissection may predict prognosis. Thus, 2 of the 24 patients with NED in whom GTPases were weakly expressed within resected nodes should be strictly followed for recurrence regardless of normal serum values of α-fetoprotein, β-human chorionic gonadotropin, and lactate dehydrogenase. Although the follow-up period in the current study was too short to draw definitive conclusions regarding a possible relationship between Rho protein levels and prognosis, this relationship will be the subject of our forthcoming studies with larger numbers of testicular GCT patients.

It is likely that Rho family GTPases are involved at different stages of tumor progression (8). Cell migration and invasion are differentially modulated by the Rho family GTPases (27, 28), and the mutational activation or overexpression of these proteins leads to metastasis in animal models (23, 29). Although cross-talk among the members of Rho-family GTPases occurs,

Fig. 6 RhoA, ROCK-I, ROCK-II, Rac1, and Cdc42 expression in stage II nonseminomas with NED and poor prognosis. NED, the patients with no evidence of disease. Refrac/Recurr, the patients with recurred tumor/refractory to therapy. The data show the 95% confidential interval.
each is activated in response to specific environmental signals and each induces specific changes in the actin cytoskeleton (30). In the present study, there was a positive relationship between expression levels of Rho family GTPases within tumor cells (Fig. 2). Cross-talk and differences in expression patterns between members of the Rho family may result in differing behavior in various cancers. There is also evidence for sequential activation of Cdc42→Rac1→RhoA (18). However, it still remains unclear how expression of Rho family GTPases is regulated. In the present study, the correlation coefficients (r²) of Rac1/Cdc42 (r² = 0.554) was higher than that of RhoA/Rac1 (r² = 0.473) and RhoA/Cdc42 (r² = 0.322). Recent studies have shown that overexpression of p120 catenin leads to morphological change and increased cell migration by modulating Rho GTPases, e.g., Rac1/Cdc42 activation and RhoA inactivation (31–33), suggesting that p120 catenin is a key regulator of Rho-family GTPases in both cell-cell adhesion and migration. Furthermore, tyrosine phosphorylation of p120 catenin is associated with tumor relapse, and p120 catenin is available as a clinical marker in human lung cancer (34). It should be possible to elucidate the specific roles of each member of the Rho family GTPases, the cross-talk between members, and the regulators of their function to understand the molecular mechanisms of carcinogenesis and spread of testicular GCT.

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