RECK Expression in Pancreatic Cancer: Its Correlation with Lower Invasiveness and Better Prognosis

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ABSTRACT

Background: The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) gene was initially isolated as a transformation suppressor gene. The RECK gene is expressed widely in normal organs but is undetectable in many tumor-derived cell lines. When artificially expressed in such cell lines, RECK negatively regulates at least matrix metalloprotease (MMP)-9, MMP-2, and MT1-MMP activation and suppresses the invasive and metastatic potentials of these cells. Clinical relevance of these observations, however, is yet to be established. The aim of this study was to examine RECK expression in pancreatic cancer, where intensive invasiveness and metastasis are frequently observed, and investigate its clinical significance. We also analyzed the correlation between RECK expression and MMP activation.

Methods: (a) RECK expression in surgically resected tissue samples of invasive ductal carcinomas of the pancreas (n = 50) was examined immunohistochemically, and its correlation with clinicopathological factors was analyzed; and (b) gelatin zymography was used for the detection of latent and activated forms of MMP-2 and MMP-9 in some of the tissue samples (n = 33). The gelatinase activity was quantified by densitometry, and the ratio of intensity of the active MMP-2 band to the total intensity of the pro- and active MMP-2 bands was evaluated as an indicator of MMP-2 activation. The MMP-9 activation was also studied.

Results: Among the 50 ductal carcinoma samples, 26 (52%) were stained positive for RECK. In the normal pancreas, both acinar and β cells were stained positive, but ductal cells did not. Tumors with positive RECK staining were significantly less invasive as compared with RECK-negative tumors (P = 0.0438). Importantly, patients who had tumors with high RECK expression showed significantly better prognosis than those who had RECK-negative tumors (P = 0.0463, by Log-rank test). Zymographic analysis indicated significant inverse correlation between the level of RECK expression and extent of MMP-2 activation (P = 0.0374).

Conclusions: Our findings support the hypothesis that the RECK protein has negative effects on the invasiveness of pancreatic cancer by inhibiting MMP-2 activation and suggest the potential value of RECK as a prognostic molecular marker for pancreatic cancer.

INTRODUCTION

The RECK1 gene was isolated through an expression-cloning approach designed to isolate genes inducing flat morphology when expressed in a v-Ki-ras-transformed NIH3T3 cell line (1–3). The RECK gene encodes a membrane-anchored glycoprotein of Mr ~110,000 with multiple epidermal growth factor-like repeats and serine protease inhibitor-like domains and is associated with the cell membrane through a COOH-terminal glycosylphosphatidylinositol modification (4). The RECK gene is widely expressed in various human tissues and non-neoplastic cell lines, whereas its expression is diminished in oncogene-transformed fibroblasts or tumor-derived cell lines (4, 5). Restored expression of RECK in malignant cells results in the suppression of their invasive and metastatic activities with concomitant suppression of proteolytic activity of MMP-9, MMP-2, and MT1-MMP, suggesting a role for RECK in the regulation of MMPs and tumor invasiveness (6). Although the earlier observation indicated that the RECK gene is widely expressed in human organs and down-regulated in tumor-derived cell lines, Furumoto et al. (7) found that 40% of hepatocellular carcinoma tissues expressed RECK mRNA at the levels even higher than that in the adjacent noncancerous liver tissues, and high RECK expression was significantly correlated with good prognosis, possibly attributable to the lower invasiveness of the tumors. In light of its wide tissue distribution, however, RECK expression in a wider variety of tumor types should be examined to fully understand the importance of RECK in tumor formation and progression. In addition, the

1 The abbreviations used are: RECK, reversion-inducing cysteine-rich protein with Kazal motifs; NGS, normal goat serum; TIMP, tissue inhibitors of metalloprotease; MMP, matrix metalloprotease.
relationship between RECK expression and MMP activation among clinical samples remains to be confirmed.

Pancreatic carcinomas are often associated with extensive invasion into adjacent tissues and metastasis to the liver at diagnosis; the disease, therefore, tends to show poor prognosis after surgical treatments. MMPs are believed to play important roles in the expression of such malignant phenotypes, and a disruption in the balance between MMPs and their inhibitors, such as TIMPs, has been implicated in the progression of this disease (8). In the present study, we analyzed the level of RECK protein expression and extent of MMP activation in pancreatic cancer tissues to address the question of whether this newly identified, membrane-associated MMP regulator plays any roles in defining the malignancy of this type of tumors. Our findings are consistent with the model that when RECK is expressed, MMP-2 activation is inhibited, and tumor invasion and metastasis are suppressed.

MATERIALS AND METHODS

Patients and Tumor Samples. Fifty patients with pancreatic ductal adenocarcinoma, who had undergone pancreatectomy at our department between January 1991 and June 1999, were included in this study. Patients with other pancreatic malignancies, such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma, and endocrine tumor, were excluded. The average age at surgery was 67 years (range, 46–81 years). We chose only those patients who had survived ≥60 days after surgery to exclude perioperative mortality-related bias. Follow-up data were updated on December 31, 2000 [median follow-up was 11.7 months (range, 2.2–54.7 months)]. At that time, 33 patients had died of pancreatic cancer, 6 had died because of other factors, and 11 were alive. Histopathologic diagnosis was confirmed by the Department of Pathology, Kyoto University Hospital. Pancreatic cancer was staged according to the Tumor-Node-Metastasis (Unio Internationale Contra Cancrum) system (9), and the following clinicopathologic features were recorded: (a) tumor size; (b) histological grade of malignancy; (c) serosal invasion; (d) retroperitoneal invasion; (e) lymphatic infiltration; (f) venous infiltration; and (g) clinical tumor stage in accordance with the classification of pancreatic cancer as defined by the Japan Pancreas Society (10). We defined “moderate” status as the score 0 (none) or the score 1 (suspect) and “severe” status as the score 2 (clearly observed) of the Japan Pancreas Society classification. In gelatin zymography, 33 samples of invasive ductal carcinoma of the pancreas were examined.

Tumor specimens were collected after obtaining the patients’ informed consent in accordance with the institutional guidelines. Samples for zymography were immediately frozen in liquid nitrogen at the time of surgery and stored at −80°C. Tissue samples for immunohistochemistry were fixed in 4% paraformaldehyde or 10% formaldehyde in PBS, embedded in paraffin, and cut into consecutive, 4-μm-thick sections.

Immunohistochemistry. The serial sections were deparaffinized in three changes of xylene, rehydrated in descending concentrations of ethanol, and then washed three times for 5 min each with double-distilled water. After rehydration, the sections were placed in 0.01 M sodium citrate buffer (pH 6.0) for 20 min at 95°C and then incubated for 30 min at room temperature in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity. The sections were then incubated for 30 min at room temperature with PBS (pH 7.4) containing 5% NGS and 1% BSA (fraction V; Sigma, St. Louis, MO), followed by overnight incubation at 4°C with anti-RECK monoclonal antibody (5B11D12; Amgen, Thousand Oaks, CA; Refs. 4 and 6) diluted 1:50 in PBS containing 2% NGS and 0.1% BSA. To exclude the possibility of background staining by the secondary antibodies, adjacent sections from the same tumors were similarly treated with nonspecific mouse IgG. The sections were washed three times for 5 min in PBS with O.I.V. Tween 20 (Wako Pure Chemical Industries, Osaka, Japan) and incubated for 60 min with the biotinylated goat antimouse IgG secondary antibodies (Dakopatts, Stockholm, Sweden) diluted 1:500 in PBS containing 2% NGS and 0.1% BSA. The slides were again washed three times in PBS with O.I.V. Tween 20 for 5 min each and incubated for 30 min in streptavidin-peroxidase complex. After washing, immune complexes were visualized using dianaminobenzidine-tetrahydrochloride (0.03%) as a chromogen, and the sections were counterstained with Mayers hematoxylin and mounted.

The intensity of anti-RECK staining was roughly graded into three classes: (a) 0 = undetectable; (b) 1+ = weak staining; and (c) 2+ = strong staining. The proportion of positively stained cells among cancer cells was also graded into three classes; tissue sections with <5% reactive tumor cells were considered as negative, and those with 5–50% and >50% reactive cells were defined as 1+ and 2+, respectively. The staining score was obtained by multiplying the staining intensity score by the score of the proportion of the positively stained cells. Because this staining considered scores 2 and 4 to be the most significant prognostic effects in overall survival, the cutoff point of the low and high expression was defined between 1 and 2. Therefore, the specimens with a score of no <2 were regarded as positive, and those with a score of no >1 were regarded as negative. The expression of RECK was independently evaluated by two investigators (T. M. and M. K.) without knowledge of the patients’ clinicopathologic features.

Gelatin Zymography. Portions (10–30 mg) of each sample were homogenized in 300 μl/mg sample buffer [63 mM Tris-HCl (pH 6.8), containing 5% glycerol, 1% SDS, 1% g-bexate mesilate-synthesized protease inhibitor (FOY; Ono Pharmaceutical, Osaka, Japan), and 0.02% bromphenol blue] with a microtubhe homogenizer (PELLLET PESTLE; Kontes, Vineland, NJ). A constant value of sample buffer-to-weight of tissue ratio (volume/weight) was used to allow equal sample loading. The samples were microcentrifuged at 10,000 × g for 5 min. Proteins in each sample (10 μl) were separated by electrophoresis through polyacrylamide (10%) gel containing 10% SDS and 1 mg/ml gelatin. The gels were incubated with 1% Triton X-100 with gentle agitation for 30 min at room temperature and washed with developing buffer [10 mM Tris base, 40 mM Tris HCl, 200 mM NaCl, 5 mM CaCl2, and 0.02% Brij 35] for 30 min. The gels were then incubated with fresh developing buffer overnight at room temperature. The gels were stained with Coomassie solution (0.5% Coomassie blue R250, in 30% methanol, and 10% acetic acid) for 15 min and destained with the same solution without Coomassie blue. The gels were dried with
a gel dryer (multigel dryer; Daiichi Pure Chemicals Co. LTD., Tokyo, Japan) for 2 days at room temperature.

**Densitometrical Analysis of MMP-2 Activation.** Zymographic images on transilluminated gels were scanned using a densitometer (Argus 50; Hamamatsu Photonics Co., Hamamatsu, Japan). The extent of MMP-2 activation was calculated as the intensity ratio of the Mr 66,000 band per the total intensity of the Mr 72,000 and 66,000 bands. A preliminary study was performed to evaluate the accuracy of the zymographic procedures and validate the reproducibility of the activation ratio calculation. When varying amounts of an identical sample were loaded on the gel, zymographic band intensities of the MMP-2 latent forms and activated form each correlated well with the amount of protein loaded, and the MMP-2 activation ratio was almost constant. When an intra-assay variance test was performed in two samples with high and low activation ratio, intra-assay variance in eight repetitions was 6.5 and 9.5%, respectively.

**Statistical Analysis.** Clinicopathological characteristics were compared with RECK expression (high and low) using the \( \chi^2 \) or Fisher’s exact probability test. The Kaplan-Meier method was used to calculate the survival curves, and the Log-rank test was performed to compare differences in the survival rates of patients who were subjected to curative surgery. Seven patients subjected to noncurative surgery were excluded. The comparative evaluations between RECK expression and MMP-2 activation were performed by the Kruskal-Wallis test. All analyses were done using JMP software (version 3.02) for Macintosh. A \( P < 0.05 \) was considered significant.

**RESULTS**

**RECK Expression in Pancreatic Cancer and Normal Pancreas.** Representative results of the immunochemical staining for RECK in pancreatic cancer and normal pancreas are shown in Fig. 1. The RECK staining was mainly found in the cytoplasm of cancer cells, often in a granular pattern. Of the 50 invasive ductal adenocarcinomas examined, 26 (52%) were stained positive for RECK, and 24 (48%) were negative. Some sections showed labeling of the majority of cancer cells, whereas in others, only small areas of the tumor were found to be positive for RECK. The pattern of the RECK staining cancer cells seemed to have no correlation with the morphological status of the pancreatic cancer cells. In the 26 cases defined as positive, 3 cases showed the RECK expression of 5–50% of the...
cancer cells, and 23 cases showed 50–100% of the cancer cells, whereas the intensity score was weak staining in 4 cases and strong in 22 cases. RECK expression was detected both in the cytoplasm and on the plasma membranes of the cancer cells. The positive staining was also observed in noncancerous pancreatic tissues in acinar cells and faintly in islet cells but not in ductal cells (Fig. 1d). No staining was observed when nonspecific mouse IgG was used as a primary antibody (Fig. 1b).

**Correlation of RECK Protein Expression with Survival and Clinicopathological Features of Patients.** In a comparison between the patients with RECK-positive tumors and patients with RECK-negative tumors, the 2-year survival rate of the RECK-positive group was found to be 42%, whereas that of the RECK-negative group was 0%, indicating significantly better survival of the RECK-positive group \( (P = 0.0463 \text{ by Log-rank test; Fig. 2}) \). The Fisher’s exact test indicated a significant inverse correlation between RECK expression and serosa invasion \( (P = 0.0387) \). The “t factor,” an indicator of total invasiveness of pancreatic cancer determined based on the status of serosal, retroperitoneal, portal, arterial, duodenal, and choledochal invasion, was also inversely correlated with RECK expression \( (P = 0.0438) \). No other factors, such as age, sex, tumor size, histological grade, lymph node metastasis, distant metastasis, and tumor stage, showed significant difference between these two groups (Table 1). These results imply invasion-inhibitory effects of RECK in pancreatic cancer.

Evaluation of the data by the Cox’s proportional hazard model indicated that RECK expression was not an independent variable affecting overall survival, which is consistent with our hypothesis that RECK affects the progression of the disease by affecting another variable, i.e., invasiveness.

**Correlation of the RECK Protein Expression and MMP Activation in Pancreatic Cancer.** As RECK expression was found to be inversely correlated with tumor invasiveness of pancreatic cancer, we further analyzed its involvement in MMP activation.

![Gelatin zymography](image-url)
activation. Fig. 3 shows typical zymographic patterns obtained, in this case, using the normal pancreas and four pancreatic carcinoma tissue specimens. With normal pancreatic tissues, gelatinolytic bands were usually detected at $M_r$ 130,000; 92,000 (pro-MMP-9); and 72,000 (pro-MMP-2). Pro-MMP-9 ($M_r$ 92,000) was detected in all 33 samples of pancreatic carcinoma, and its activated form ($M_r$ 82,000) was observed in 9 samples (27%). Pro-MMP-2 ($M_r$ 72,000) was also detected in all 33 pancreatic cancers, and the level of its activated form ($M_r$ 66,000) varied widely; the MMP-2 activation ratio [$M_r$ 66,000/($M_r$ 72,000 + 66,000)] ranged from 0.12 to 0.78. The Kruskal-Wallis test detected a significant inverse correlation between RECK expression and the MMP-2 activation ratio in pancreatic cancer ($P = 0.0374$; Fig. 4). In contrast, no correlation was detected between RECK expression and MMP-9 activation (Table 2).

**DISCUSSION**

In recent years, there has been an increase in the survival rates of pancreatic cancer patients. Yet, their prognosis is very poor, even after curative resection (11, 12). One of the reasons for poor prognosis is high frequency of invasion and metastasis (13). A number of reports has demonstrated that degradation of the extracellular matrix, particularly basement membranes, is mandatory for the tumor invasion and metastasis (14). The extracellular matrix, which consists of various types of collagen, laminin, fibronectin, elastin, and proteoglycans, is degraded by proteases, including MMPs (15, 16). In pancreatic cancer, it is recognized that MMPs play an important role for tumor invasion and metastasis (17). MMPs are secreted from cancer cells and/or adjacent stromal cells as latent forms and converted into active forms (18). The activities of MMPs in cancer and inflammatory tissues are thought to be regulated by TIMP-1 to TIMP-4. The unbalance among MMPs and TIMPs may confer invasive potential to cancer cells (18).

In the present study, we found that pancreatic tumors positive for RECK staining tended to be less invasive than RECK-negative tumors ($P = 0.0468$) and that the patients with RECK-positive pancreatic tumors tend to survive longer than the patient with RECK-negative tumors ($P = 0.0438$). These results are consistent with the previous observation by Takahashi et al. (4) that RECK-transfected melanoma and fibrosarcoma cells showed reduced invasiveness. We also found that RECK expression showed significant inverse correlation with MMP-2 activation but not with MMP-9 activation in pancreatic cancer tissues. Because activation of MMPs has been implicated in tumor invasion and metastasis (19, 20), the lower MMP-2 activation may account for the lower invasiveness of RECK-positive tumors. Our findings in pancreatic tumors are consistent with the recent observation by Oh et al. (6) that RECK regulates MMP-2 activation by inhibiting MT1-MMP and active MMP-2 in vitro. Oh et al. have also shown that artificial expression of RECK in fibrosarcoma cells results in the inhibition of blood vessel branching in tumor xenografts and longer survival of the host animals (6). Hence, suppression of tumor angiogenesis by RECK may also account for our finding that patients with RECK-positive tumors tend to show better prognosis.

We found that RECK protein was expressed not only in nontumorous tissues of the pancreas but also in tumors tissues, and 52% of them showed strong expression of RECK protein. The expression of the RECK protein in cancer tissues is inconsistent with the fact that the expression of the RECK gene is suppressed in several tumor-derived cell lines and ras-transformed fibroblasts (5) and our Western blot analysis showing no RECK expression in six pancreatic cancer cell lines (data not shown). Although the exact reason for this discrepancy remains to be clarified, possible explanations would include: (a) selection of RECK-negative populations during propagation in vitro; and (b) environmental factors present only in vivo (or in vitro), which have positive or negative effects on RECK expression. In this respect, it is of particular interest to examine whether RECK plays any roles in the formation of abundant stromal desmoplasia often found around pancreatic cancer tissues (21).

A critical difference between RECK and TIMPs, another group of endogenous MMP inhibitors, lies in their localization; RECK is membrane anchored (4), whereas TIMPs are secreted. Membrane anchoring allows RECK to be concentrated on the plasma membrane and effectively regulate local proteolytic events at the cell surface, where proteolytic processing of MMPs occurs (22). The lack of RECK is lethal in mouse embryos (6), whereas the lack of TIMP-1 or -2 has little effect on development (23, 24), suggesting a larger impact of RECK than each TIMP on embryonic development. Functional relationships be-

**Table 2** Comparison of MMP-9 activation and RECK protein in pancreatic cancer tissues

<table>
<thead>
<tr>
<th>Category</th>
<th>RECK stain</th>
<th>Negative $n = 14$</th>
<th>Positive $n = 19$</th>
<th>$P^{*}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>Not activated</td>
<td>10</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Activated</td>
<td>4</td>
<td>5</td>
<td>1.000</td>
</tr>
</tbody>
</table>

$^{*} P$ was obtained by Fisher’s exact probability test.

**Fig. 4** RECK expression and MMP-2 activation in pancreatic cancer tissues. The tumors with RECK expression ($n = 14$) show a significantly lower MMP-2 activation ratio than the tumors without RECK expression ($n = 19$). $P (0.0374)$ was derived using the Kruskal-Wallis test. Error bar represents SD.
between these different groups of MMP regulators during carcinogenesis will be an interesting subject for future studies.

In conclusion, we found a significant correlation between RECK expression in pancreatic cancer tissues and survival of the patients. An inverse correlation between RECK expression and MMP-2 activation in the tumor tissues, as well as their invasive potentials, was also found. These data are consistent with the model that RECK plays an active role in suppressing malignant phenotypes of pancreatic cancer cells. Practically, RECK expression can be a good prognostic indicator in pancreatic cancer patients, and therapeutic strategies based on RECK or its mechanism of action may be of value in the treatment of this disease.

REFERENCES


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