The Membrane-Anchored Matrix Metalloproteinase (MMP) Regulator RECK in Combination with MMP-9 Serves as an Informative Prognostic Indicator for Colorectal Cancer

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ABSTRACT

Purpose: RECK, a membrane-anchored regulator of matrix metalloproteinases (MMPs), is widely expressed in healthy tissue, whereas it is expressed at lower levels in many tumor-derived cell lines. Studies in mice and cultured cells have shown that restoration of RECK expression inhibits tumor invasion, metastasis, and angiogenesis. However, the clinical relevance of these findings remains to be fully documented. Here we examined the expression of RECK and one of its targets, MMP-9, in colorectal cancer tissue.

Experimental Design: The RECK and MMP-9 expression levels in colorectal cancer samples from 53 patients were determined by immunohistochemical techniques. The expression level of each protein was scored, and the patients were divided into two groups based on these scores. In 33 cases, we performed gelatin zymography to estimate the degree of MMP-2 and MMP-9 activation. Microvessel density and vascular endothelial growth factor (VEGF) expression were also evaluated histologically.

Results: RECK protein was detected in 30 of 53 (56.6%) specimens. Importantly, patients with tumors expressing relatively high levels of RECK (high-RECK group) had a significantly lower risk of recurrence than did patients with tumors expressing relatively low levels of RECK (low-RECK group; P = 0.011). Moreover, RECK-dominant (RECK score ≥ MMP-9 score) patients showed a significantly lower incidence of recurrence than did MMP-9-dominant patients (P = 0.0003). Multivariate analysis revealed that the RECK/MMP-9 balance was an independent prognostic factor (P = 0.0122). The expression of VEGF and microvessel density were inversely correlated with the level of RECK expression.

Conclusions: RECK/MMP-9-balance is an informative prognostic indicator for colorectal cancer. Our data also suggest that RECK suppresses tumor angiogenesis, probably by limiting the availability of VEGF in tumor tissues.

INTRODUCTION

The RECK (reversion-inducing cysteine-rich protein with Kazal motifs) gene was isolated through an expression cloning approach designed to isolate genes that induce morphological reversion when expressed in a γ-Ki-ras-transformed NIH3T3 cell line (1–3). The RECK gene encodes a membrane-anchored glycoprotein (molecular weight, 110,000) that is associated with the cell membrane through a COOH-terminal glycosylphosphatidylinositol modification and contains multiple epidermal growth factor-like repeats and serine protease inhibitor-like domains (4). RECK is widely expressed in normal tissue and nonneoplastic cell lines, whereas its expression is strongly suppressed in several tumor-derived cell lines and in fibroblasts transformed with various oncogenes (4, 5). Restoration of RECK expression in malignant cells reduces their pro-matrix metalloproteinase (MMP)-9 secretion and suppresses their invasive, metastatic, and angiogenic activities (6).

Colorectal cancer is one of several neoplastic diseases that can be treated effectively with surgery; the 5-year survival rate after curative resection in combination with lymphadenectomy reaches 70–80%. In the remaining cases, the primary cause of recurrence and mortality (up to 75%) is blood-borne metastasis to the liver (7–9). It is therefore important to develop new strategies for colorectal cancer treatment that target metastasis.

In this study, we analyzed the expression levels of RECK and MMP-9 in colorectal cancer tissue to assess their value as prognostic indicators. We also analyzed vascular endothelial growth factor (VEGF) expression and microvessel density (MVD) because RECK and MMP-9 have been implicated in tumor angiogenesis (6, 10). Our results indicate that the level of RECK expression in colorectal tumors is inversely correlated with certain metastasis-related clinicopathological features as well as angiogenesis-related parameters and that the balance between the expression levels of RECK and MMP-9 has a strong impact on prognosis.
MATERIALS AND METHODS

Dot Blot Hybridization. A matched tumor/normal expression array (Clontech Laboratories, Inc.) was hybridized with human RECK cDNA (4) or a control ubiquitin probe following the manufacturer’s protocol. Autoradiographic images were digitized with a scanner, and the hybridization signal intensity was quantified using National Institutes of Health Image software. Loading variation among samples was normalized using ubiquitin as an internal control.

Patients and Tumor Samples. Tumor specimens were obtained from 53 patients who underwent surgery for treatment of colorectal cancer between 1996 and 1997 at Department of Surgery, Nara Medical University. All patients underwent complete resection with lymphadenectomy, which was the standard surgical procedure for colorectal cancer patients at this facility. The study included 38 men and 15 women, and patient age ranged from 24 to 80 years (mean age, 63 years). None of the patients received radiation therapy before surgery. The location of the primary lesion and the corresponding number of patients were as follows: rectum, 18 patients; sigmoid colon, 14 patients; descending colon, 1 patient; transverse colon, 3 patients; ascending colon, 11 patients; and cecum, 6 patients. In 10 patients who had liver metastasis at diagnosis, simultaneous partial resection of metastatic liver lesions was also attempted. After surgery, patients were followed-up with serum carcinoembryonic antigen monitoring, abdominal ultrasonographic examination every 3 months, and chest radiography every 6 months. When recurrence was suspected, a more detailed examination using abdominal computed tomography was performed. Recurrence-free survival was defined as the period between the date of the initial operation and the date when tumor recurrence was detected. The follow-up period ranged from 5.7 to 64.2 months (median follow-up, 44.3 months).

Tumor specimens were collected after obtaining informed consent from the patients in accordance with our institutional guidelines as well as the Declaration of Helsinki. A portion of the specimens was fixed in 10% formaldehyde neutral buffered solution, embedded in paraffin, and used for immunohistochemistry. Another portion was frozen immediately, stored at −80°C, and used for gelatin zymography.

Immunohistochemistry. For immunohistochemical analysis, 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded blocks and placed on silan-coated slides. After deparaffinization, sections were incubated in 3% H2O2 for 20 min to inactivate endogenous peroxidase. Deparaffinized and rehydrated specimens were heated in 10 mM citrate buffer (pH 6.0) for 10 min in an autoclave at 105°C. After being cooled to room temperature for 30 min, specimens were incubated with normal horse serum for 20 min at room temperature followed by incubation with monoclonal antibodies against RECK (5B11D12; 1:40; a gift from Amgen, Thousand Oaks, CA), VEGF (c-1; 1:150; Santa Cruz Biotechnology, Inc.), or MMP-9 (56-2A4; 1:100; Fuji Chemical Industries) for 1 h at 4°C (RECK and VEGF) or 1 h at room temperature (MMP-9). To exclude the possibility of background staining by the secondary antibodies, adjacent sections from the same tumors were similarly treated with nonspecific mouse IgG (DAKO). The sections were washed three times for 5 min in PBS and incubated for 1 h with biotinylated antimouse IgG secondary antibodies. After rinsing, immune complexes were visualized by the standard avidin-biotin-peroxidase complex (ABC) method using the ABC kit (VECTASTAIN; Vector Industries, Inc.) and the chromogen 3,3’-diaminobenzidine. Nuclei were counterstained with hematoxylin. The specificity of anti-RECK antibody has been confirmed by three experiments: (a) immunoblot assay with cells transfected with RECK cDNA (4); (b) immunohistochemical comparison between tissues from the wild-type and RECK-deficient mice; and (c) immunohistochemical comparison between tumors formed in nude mice after injection of HT1080 cells (with minimal expression of RECK) and cells overexpressing RECK (6). The specificity of the anti-MMP-9 antibody and its applicability for immunostaining have also been established by immunoblot assay and immunohistochemical staining of the rheumatoid synovium (11, 12). In examining the slides with heterogeneous staining, we first find the most strongly stained area at low magnification (×40), and at least 1000 tumor cells in that area were evaluated at high-power magnification (×200). To facilitate objective evaluation, we determined both the proportion of stained cells (PS) and the intensity of staining (IS). The PS among cancer cells was graded into four levels as follows: undetectable, PS grade 0; detectable but <33%, PS grade 1; 34–66%, PS grade 2; and >67%, PS grade 3. IS was also graded into four levels, as follows: undetectable, IS grade 0; weak, IS grade 1; moderate, IS grade 2; and strong, IS grade 3. The final score for each tumor was obtained by multiplying the IS and PS grades. The scoring was made independently by two investigators who were not aware of the identity of each sample or the scores assigned by the other investigator, and the data from the two investigators were averaged. In >94% of all cases, the scores assigned by the two investigators were identical. Final scores of ≥6 were defined as high, and those of <6 were defined as low.

Determination of Microvessel Density. Blood vessels were visualized by immunohistochemical staining with an anti-CD34 monoclonal antibody (QBend; 1:50; DAKO) following essentially the same protocol as described above, except that the final incubation was for 1 h at room temperature. MVD was determined by counting the number of vessels in one ×400 field from each of three selected areas in a tumor that showed the highest staining density as assessed by an initial scan at lower magnification (×40; Ref. 13). MVD analysis of each sample was performed independently by two investigators, neither of whom had any knowledge regarding the clinical outcomes and clinicopathological features of the disease.

Zymography. Gelatin zymography was performed as described by Haerron et al. (14). Briefly, frozen colon tissue was homogenized, and the tissue extracts (200 μg protein/lane) were subjected to electrophoresis at room temperature through a 10% polyacrylamide gel copolymerized with 0.5 mg/ml gelatin. The gel was washed twice with 50 mM Tris-HCl buffer (pH 7.6) containing 2.5% Triton X-100 and 0.02% NaN3 and incubated overnight at 37°C in the same buffer supplemented with 10 mM CaCl2 and 1 mM ZnCl2. The gel was then fixed and stained in 50% methanol containing 10% acetic acid and 0.1% Coomassie Blue R-250 and subsequently de-stained in 30% methanol containing 5% acetic acid. Proteins with gelatinolytic activity appeared as clear bands against a blue background. The relative
RECK considered significant when evaluating the difference between survival rates. Differences were expressed as the ratio of intensity (RECK signal/ubiquitin signal) was plotted (the horizontal line indicates the mean). Note that the data sets for nontumor tissues (mean = 0.48) are significantly higher (paired t test) than those for tumor tissues (mean = 0.074), indicating frequent down-regulation of RECK mRNA in colorectal cancer. The samples indicated by arrows gave lower hybridization signals with the ubiquitin probe in tumors and were therefore considered noninformative and excluded from the statistical analysis.

Statistical Analysis. Clinicopathological characteristics were compared with the expression levels of RECK and MMP-9 using the χ² test. MVDs among colorectal cancer tissues were compared by the Wilcoxon-Mann-Whitney U test. Univariate and multivariate survival analyses were performed using Cox’s proportional hazard model. The Kaplan-Meier method was used to draw survival curves, and the log-rank test was performed to evaluate the difference between survival rates. Differences were considered significant when P was < 0.05.

RESULTS

RECK mRNA Expression in Colorectal Cancer Tissue. RECK mRNA levels were compared between several colorectal cancer tissues and the surrounding nontumor tissues by dot blot hybridization (Fig. 1). In eight of nine informative cases of colon cancer (89%) and in five of five cases of rectal cancer (100%), RECK was expressed at lower levels in tumors than in the surrounding nontumor tissues, suggesting that RECK expression is down-regulated during colorectal carcinogenesis. RECK expression levels varied widely between tumor specimens. To determine whether this variation corresponds to disease outcome and/or RECK protein localization, we performed an immunohistochemical examination of the specimens for which clinicopathological information was available.

RECK and MMP-9 Protein Expression in Colorectal Cancer Tissues. Immunohistochemical analysis revealed a wide variation in RECK and MMP-9 protein expression levels among colorectal cancer specimens. To facilitate statistical analysis, we scored the levels of RECK and MMP-9 expression taking two variables into account: (a) IS and (b) PS. The IS was graded into four levels (grades 0–3), and representative examples are shown in Fig. 2. The PS was also graded into four levels (see “Materials and Methods” for details). These two scores were multiplied to obtain a final score for each tumor, and the final scores by two investigators (who examined slides independently, without knowledge of the scores assigned by the other investigator or the identity of the slides) were averaged. Scores of ≥6 were defined as high, and scores of <6 were defined as low.

RECK was detected in the cytoplasm and plasma membrane of normal colorectal mucosa cells and at lower levels in some tumor cells, which is consistent with the data on the levels of RECK mRNA described above. In contrast, MMP-9 was abundantly expressed only in the cytoplasm of some tumor cells. Thirty of 53 specimens (56.6%) were classified as expressing relatively high levels of RECK (high-RECK group), and 29 of 53 specimens (54.7%) were classified as expressing relatively high levels of MMP-9 (high-MMP-9 group).

Correlation between the Levels of RECK and MMP-9 Expression and Clinicopathological Factors. The level of RECK expression in colorectal cancer specimens was correlated with several clinicopathological factors (Table 1). First, there were no poorly differentiated cases in the high-RECK group (n = 30), whereas four poorly differentiated cases were found in the tumors expressing relatively low levels of RECK (low-RECK group; n = 23; P = 0.018). Second, lymph node metastasis occurred more frequently in the low-RECK group (61%) than in the high-RECK group (33%; P = 0.046). Third, the level of RECK expression was inversely correlated with Dukes’ classification (P = 0.018) and venous infiltration (P = 0.046). In contrast, the level of MMP-9 expression was not correlated with any of these clinicopathological factors.

As shown in Fig. 3, patients expressing relatively high levels of MMP-9 had a higher incidence of recurrence than patients expressing relatively low levels of MMP-9, although the statistical significance was on borderline (P = 0.053). In contrast, high-RECK patients had a significantly lower risk of recurrence than low-RECK patients (P = 0.011).

Because RECK is a negative regulator of MMP-9, we speculated that tumors expressing high levels of RECK and low levels of MMP-9 might exhibit the lowest risk of recurrence. To test this hypothesis, we divided the samples into two groups based on their RECK and MMP-9 scores: (a) a group in which the RECK score was greater than or equal to the MMP-9 score (RECK-dominant group); and (b) a group in which the RECK score was less than the MMP-9 score (MMP-9-dominant group). The RECK-dominant group (n = 36) showed a significantly lower incidence of recurrence than did the MMP-9-dominant group (n = 17; P = 0.0003; Fig. 3C). A multivariate analysis indicated that this combined criterion (i.e., RECK/MMP-9 balance) is an independent factor (P = 0.0122) that is comparable.
Fig. 2  Immunohistochemical staining of colorectal cancer specimens with antibodies against RECK and MMP-9. Examples of tumors expressing different levels of RECK (A–D) or MMP-9 (E–H) are shown. IS scores were as follows (see “Materials and Methods” for grading methods): 3 (A and E), 2 (B and F), 1 (C and G), and 0 (D and H). Immune complexes were visualized with ABC reagents (brown), and the sections were counterstained with hematoxylin (blue). Scale bars, 100 μm.
with age, lymph node metastasis, venous infiltration, and Dukes' classification (Table 2).

**Detection of Latent and Activated MMP Molecular Species in Colorectal Cancer Tissue.** The relative amounts of latent and activated MMP-9 and MMP-2 molecular species were assessed by gelatin zymography with extracts of tumors and normal colon mucosa from 32 patients whose frozen tissues (in good condition) were available (four typical cases are shown in Fig. 4). Gelatinolytic bands of around 92,000 (pro-MMP-2), and 84,000 (active MMP-9), M 72,000 (active MMP-9), M 66,000 (active MMP-2) with various intensities were detected. Among the colon carcinoma samples, active MMP-9 (M 84,000) was detected in all cases, whereas its latent form was detected in 20 colon carcinoma samples. Both of these MMP-2 bands were undetectable in all normal mucosa samples. No correlation was found between the level of RECK expression and the level of VEGF expression (Fig. 5E; $P = 0.0037$).

Second, we investigated the relationship between MVD and RECK expression. The MVD in tumors of the high-RECK group (mean ± SD, 160 ± 21) was significantly lower than that in tumors of the low-RECK group (265 ± 28; $P = 0.006$), indicating a significant inverse correlation between the level of RECK expression and MVD (Fig. 5F).

**DISCUSSION**

In this study, we found that *RECK* mRNA is expressed at lower levels in the majority of the colorectal cancer tissues examined as compared with the surrounding nontumor tissues. *RECK* transcription is negatively regulated by oncogenic signaling (5), and an activating mutation of the K-RAS oncogene is frequently found in colorectal tumors (15–17). Thus, the most simple interpretation of our finding is that in these tumors, RECK is down-regulated as a result of mutations in oncogenes.

Our immunohistochemical data indicate that the level of RECK expression in colorectal tumors may be useful for predicting disease outcome, i.e., the risk of recurrence. RECK was initially isolated as a transformation suppressor gene against activated RAS, and nude mice inoculated with HT1080 fibrosarcoma cells that had been transfected with a RECK expression vector showed lowered lung and lymph node metastasis (4), greatly reduced tumor angiogenesis, and a prolonged life span (6) compared with animals inoculated with control HT1080 cells. Our present data strongly support the idea that the level of (residual) RECK expression has a strong impact on the invasive and angiogenic potentials of tumor cells in human tumors. Recent reports indicate a positive correlation between RECK expression in tumors and survival of patients with other types of neoplasms.

### Table 1 Relationship between RECK or MMP-9 expression and clinicopathological features

<table>
<thead>
<tr>
<th>Variables</th>
<th>RECK expression</th>
<th>MMP-9 expression</th>
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<td>Histological grade</td>
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**NOTE.** Statistically significant $P$ values ($P < 0.05$) are shown in bold.

Abbreviations: Well/mod, well or moderately differentiated; Poor, poorly differentiated.
tumors, such as pancreatic cancer (18) and breast cancer (19). The correlation may therefore be a common feature among many tumors, and this seems consistent with the previous findings that the \textit{RECK} gene expression is down-regulated by a variety of oncogenes (4, 5).

\textit{RECK} is believed to exert its biological effects by regulating multiple MMP family members such as MMP-2, MMP-9, and membrane type 1-MMP (4, 6). This idea is further supported by our finding that the balance between \textit{RECK} and MMP-9 strongly correlates with prognosis. Our zymographic data indicate that active MMP-9 is the most abundant gelatinase species in colorectal cancer homogenates (Fig. 4), which helps to explain why MMP-9 expression in conjunction with \textit{RECK} expression has such a prominent impact on prognosis.

Furumoto \textit{et al.} (20) reported a similar positive correlation between \textit{RECK} expression level and prognosis in hepatocellular carcinoma (HCC). The profile of \textit{RECK} expression in HCC is somewhat different from that in colorectal cancer. For example, \textit{RECK} down-regulation is infrequent among HCC samples, and \textit{RECK} expression level is often higher in tumors than in nontumor tissues. Moreover, a positive correlation between the levels of \textit{RECK} and MMP-9 mRNA was found in HCC, whereas in colorectal tumors, \textit{RECK} and MMP-9 appear to be independently regulated and disease outcome appears to depend on the balance between them. These differences probably reflect the existence of tissue-specific regulatory circuits for \textit{RECK} and MMP-9.

The simplest model for the relationship between \textit{RECK} and MMP-9 in the determination of malignancy in colorectal tumors is that \textit{RECK} regulates MMP-9 activity. Takahashi \textit{et al.} (4) showed that when expressed in HT1080 cells, \textit{RECK} inhibits

\begin{table}
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\begin{tabular}{|l|l|l|l|}
\hline
Variable          & 95\% CI  & Hazard ratio & \( P \)  \\
\hline
Age               & 0.399-3.796  & 1.231  & NS  \\
Tumor invasion    & 0.166-1.836  & 1.815  & NS  \\
Lymph node metastasis & 0.045-1.741  & 3.559  & NS  \\
Venous infiltration & 0.193-2.859  & 1.346  & NS  \\
Dukes' stage      & 0.003-0.495  & 26.316 & 0.0125  \\
\textit{RECK} vs MMP-9 & 0.051-0.695  & 5.319  & 0.0122  \\
\hline
\end{tabular}
\caption{Multivariate proportional hazard model analysis for disease-free survival.}
\end{table}

Abbreviations: CI, confidence interval; NS, nonsignificant (\( P > 0.05 \)).

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Disease-free survival of patients with different expression levels of MMP-9 and \textit{RECK}. \textit{A} and \textit{B}, postoperative survival of patients with tumors expressing high (dashed line) or low (solid line) levels of MMP-9 (\textit{A}) or \textit{RECK} (\textit{B}). \textit{C}, postoperative survival of patients with \textit{RECK}-dominant tumors (dashed line) or MMP-9-dominant tumors (solid line). See text for classification methods.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{fig4}
\caption{Gelatinolytic activities in tissue homogenates. Gelatin zymography was performed using pairs of tissue homogenates prepared from normal colon mucosa (N) and colon cancer tissues (T). Four typical cases are shown. Positions of the major gelatinolytic bands are indicated on the left (P). Histochemically determined \textit{RECK} scores are shown below the panels.}
\end{figure}
the secretion of pro-MMP-9 into the culture supernatant without affecting the level of MMP-9 mRNA. Processing of pro-MMP-9, however, is inefficient in HT1080 cells, so that the effects of RECK on this process have never been assessed. Our zymographic data indicate that there is no correlation between the level of RECK and the extent of pro-MMP-9 processing (Fig. 4), which suggests for the first time that, at least in colorectal tumor tissues, RECK does not affect this process. Our data, however, do not preclude the possibility that RECK suppresses secretion of MMP-9 because we used tissue homogenates, rather than extracellular fluids, for gelatin zymography, and therefore no information on subcellular localization of these MMPs could be obtained.

Our present findings are also consistent with the model in which MMP-9 and RECK act in opposite ways on a common target molecule(s) or system(s), so that a decrease in one molecule and an increase in the other have synergistic effects. Obvious candidates for such targets are extracellular matrix (ECM) components and cytokines. In fact, MMP-9 has been proposed to play a role in “angiogenic switch;” it frees ECM-bound VEGF and helps tumor cells to recruit microvessels (10). Because RECK is known to inhibit several MMPs and help maintain ECM integrity, it is reasonable to speculate that high RECK expression would pose negative effects on microvessel recruitment. It is unclear, however, why RECK expression and the level of VEGF immunoreactivity are inversely correlated among the tumor samples. One possibility is that high RECK expression may result in a more rigid ECM, thereby limiting the accessibility of antibodies to the tissue-associated VEGF. Alternatively, RECK may somehow suppress the production of

Fig. 5 A–D, immunohistochemical staining of colorectal cancer specimens with antibodies against VEGF. Examples of tumors that express different levels of VEGF. IS scores were as follows: 3 (A); 2 (B); 1 (C); and 0 (D). E and F, relationship between RECK expression and angiogenesis. E, summary of VEGF staining. The number of cases with indicated status of RECK and VEGF expression is presented. A significant inverse correlation was detected between the level of RECK expression and the level of VEGF expression. The P values were derived using Fisher’s exact probability test. F, relationship between the level of RECK expression and MVD. A significant inverse correlation was also detected between the level of RECK expression and MVD.
VEGF. In any event, our findings implicate decreased availability of VEGF in RECK-mediated suppression of angiogenesis. In conclusion, the balance between the levels of RECK and MMP-9 expression may serve as an informative prognostic indicator in colorectal cancer. Our data, together with previous experimental findings in vitro and in mice, strongly support the hypothesis that RECK is a suppressor of malignancy in human cancer.

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