Receptor for Hyaluronan-mediated Motility and CD44 Expressions in Colon Cancer Assessed by Quantitative Analysis Using Real-time Reverse Transcriptase-Polymerase Chain Reaction

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Receptor for hyaluronan (HA)-mediated motility (RHAMM) is a receptor for HA-mediated motility and its expression is correlated with malignancy of ras-transformed cells in that binding of HA to this receptor activates their migratory ability. CD44, a cell surface receptor for HA is also implicated in metastatic behavior of some cancer cells. In this study we examined the relationships of cancer progression with mRNA levels of RHAMM, CD44 (all forms), and exon 6 of CD44 using the real-time reverse transcriptase-polymerase chain reaction method in specimens of colon cancers at different diagnostic stages from 30 patients. Increased mRNA levels of RHAMM were observed in 29 specimens (97%), CD44s (all forms) in 21 specimens (70%), and its exon 6 in 19 specimens (63%) in comparison with those in the corresponding noncancerous tissue specimens. A statistically significant correlation between RHAMM expression and cancerous specimens at any of Dukes’ stages A, B, and C was found, and the overexpression of CD44 mRNAs was confirmed in specimens at Dukes’ stage C. Thus, our present study for the first time suggests that RHAMM expression may be a clinically useful indicator of colon cancer.

Key words: RHAMM — CD44 — Colon cancer — Real-time RT-PCR — Hyaluronan

HA is a high-molecular-weight linear molecule composed of repeating disaccharide units of glucuronosyl-N-acetylglucosamine, and is a major component of the extracellular and pericellular matrices. Although this macromolecule is a passive structural component in some connective tissues, such as cartilage to support tissue architectures and in the capsule of certain strains of bacteria, it also plays regulatory roles in basic cellular activities such as cell adhesion, cell migration, cell-cell recognition, and cell differentiation. Thus, it participates in the biological processes of embryogenesis, tumor invasion and metastasis, wound healing, and inflammation.1,4 It has been proposed that HA acts on cells by binding to a variety of HA-binding proteins to form extracellular environments rich in HA and by signaling through interaction with HA receptors on cell surfaces. Two different types of HA receptors on cell surfaces, CD44 and RHAMM, have so far been characterized and cloned.5 CD44 is an almost ubiquitously expressed transmembrane glycoprotein and has been shown to mediate interaction of cells with HA in lymph node homing, lymphocyte activation, cell migration, inflammation, and tumor metastasis.4 Interestingly, although it is still uncertain how HA-binding is involved in the process, expression of CD44 proteins has been shown to be linked to the metastatic abilities of tumor cells.5,7 Numerous studies by RT-PCR and/or immuno-histochemical analysis have revealed aberrant expression of CD44 variant forms which additionally contain some of exons 6 to 15 (V1 to V10), especially exon 11 (V6), in various cancer tumors such as colorectal carcinoma.8,11 These splicing variants of CD44 may play a crucial role in the progression of several tumors, particularly in the process of metastasis. Matsuura et al.10 also reported the expression of splicing variants containing exon 6 (V1) in cancer tissues.10 However, their relationship to tumor progression has remained uncertain. Another HA receptor, RHAMM was originally identified as part of a multimeric complex (HARC) that mediates HA-induced motility of H-ras-transformed fibro-
blasts. It has subsequently been demonstrated that overexpression of the RHAMM gene by transfection into fibroblasts is transforming and causes spontaneous metastasis. Significant overexpression of RHAMM was found in primary sites and lymph node metastases of human breast cancer patients. Elevated levels of tumor-associated HA have been shown to be correlated with invasiveness and metastatic behavior. We previously showed that introduction and expression of the HA synthase gene into mutant mammary carcinoma cells defective in HA synthesis restored their metastatic ability. Therefore, it seems important to investigate if there are relationships among the expressions of these molecules, HA, CD44 and RHAMM, in connection with tumor invasion and metastasis. We have recently observed a statistically significant relationship between the upregulated expression of HAS1, one of three HAS and Dukes’ high grade in human colon cancers (unpublished observation). In this study, we have investigated the expressions of RHAMM, CD44 (all forms), and their exon 6 and their relationships among various colon cancer specimens at different Dukes’ stages and with different clinical backgrounds. For this purpose, we took advantage of “real-time RT-PCR,” a newly developed method for the quantitation of mRNAs in small biopsy specimens. The results suggest that overexpression of RHAMM detected by this method may be clinically useful as an indicator of colon cancer progression and support a significant role of the expression of CD44s in colon cancer progression. No significant relationship of exon 6 of CD44 was found.

MATERIALS AND METHODS

Tumor samples Cancerous and noncancerous tissues of the human colon were obtained from 30 colon cancer patients with informed consent as surgically resected specimens at Fussa Hospital (Tokyo). Noncancerous tissues comprising histologically the tunica mucosa and muscularis mucosae, being devoid of the muscularis propria, were obtained from areas adjacent to the cancerous regions. The cancerous and noncancerous tissues were frozen in liquid nitrogen immediately after surgical resection, and stored in liquid nitrogen until RNA extraction and preparation of tissue lysates for real-time RT-PCR.

Clinical information after clinical and pathological diagnosis of the 30 patients, for example, sex, age, location of primary tumor, pathological differentiation, lymph node metastasis, distant metastasis and Dukes’ classification of the cancer [Dukes’ A (n=9), disease limited to the bowel; Dukes’ B (n=10), extension through the deep muscle without metastasis; Dukes’ C (n=10), tumors with regional and distant metastasis], is summarized in Table I. Real-time RT-PCR analysis Real-time RT-PCR analysis was performed according to the reported method.

Briefly, within a gene-specific PCR oligonucleotide primer pair, an oligonucleotide probe labeled with a reporter fluorescent dye (FAM) at the 5’-end and a quencher fluorescent dye (TAMURA) at the 3’-end were designed. Fluorescence intensity produced during PCR amplifications was monitored by the sequence detector directly in the reaction tube (“real time”). A computer algorithm compares the amount of reporter dye emission with the quenching dye emission and calculates the threshold cycle number (Ct), when signals reach 10 times the standard deviation of the baseline, from which the amounts of various mRNAs levels of various genes tested were obtained.

Total RNA samples (200 ng for each) were added to a 50 µl RT-PCR reaction (PCR-Access, Promega, Madison, WI). The “reaction master mixture” was prepared according to the manufacturer’s protocol to give final concentrations of 1x avian myeloblastosis virus Tfl reaction buffer, 0.2 mM dNTP, 1.5 mM MgSO4, 0.1 unit/ml avian myeloblastosis virus reverse transcriptase, 0.1 unit/ml Tfl DNA polymerase, 200 nM concentration of the primers, and 100 nM concentration of the corresponding probe, as described by Gibson et al. Primers and probes for real time PCR analysis of RHAMM, CD44s, and exon 6 mRNAs were designed by the Oligo version 4.0 program (National Bioscience, Plymouth, MN), according to Heid et al. Sequences of all oligonucleotides used are shown in Table II. For the mRNA analyses of RHAMM, CD44s (all forms), and the exon 6, we used RHAMM 316F and 619R, CD44s 292F and 579R, and exon 6 23F and 129R as primer sets, and RHAMM TaqMan FP, CD44s TaqMan FP, and its exon 6 TaqMan FP were used as the probes, respectively. The GAPDH primer and probe (TaqMan GAPDH detection reagents) were purchased from Perkin-Elmer and Applied Biosystems (Perkin-Elmer Corp., Norwalk, CT). RT-PCR reactions and the resulting relative increases in reporter fluorescent dye emission were monitored in real time by the 7700 sequence detector (Perkin-Elmer). Signals were analyzed by the sequence detector 1.0 program (Perkin-Elmer). The conditions for PCR were as follows; 1 cycle at 50°C for 2 min, to allow the uracil N-glycosylase to act, 1 cycle at 60°C for 30 min, 1 cycle at 95°C for 5 min, 50 cycle at 95°C for 20 s, and 60°C for 1 min. The relative amounts of RHAMM, CD44s, and the exon 6 mRNA in one sample was obtained from standard curves. Standard curves were obtained with the following amounts of human heart total RNA; 4000, 2000, 1000, 500, 250, 125, and 62.5 ng (Clontech, Palo Alto, CA). Comparison of the amount of each mRNA among different specimens and between those mRNA amounts in one specimen was done by using the values obtained by normalizing the amount of each mRNA divided by that of the GAPDH mRNA in each specimen (designated as “relative expression coefficient” in this study).
Expression of RHAMM in Colon Cancer

Statistical analyses were done by using Student’s t test. The criterion of significance was $P<0.05$.

RESULTS AND DISCUSSION

Colon cancers are thought to be favorable systems to investigate expression and regulation of molecules involved in the processes of tumor progression, because it is generally easy to obtain cancerous and associated non-cancerous parts with little mutual contamination. We thus prepared total RNAs from both parts from 30 colon cancer patients and quantitatively analyzed the relative amounts of mRNAs for RHAMM, CD44s (including all forms), and exon 6 of CD44 by "real-time RT-PCR." This new method has been developed to perform accurate real-time quantitative PCR reaction for determining relative amounts of mRNAs. To determine the dynamic range of real-time quantitative PCR, we made serial dilutions of human heart t-RNA as described in "Materials and Methods." All standard curves were linear over 4 orders of magnitude, with $R^2$ values greater than 0.99.

The relative expression coefficients obtained in this study (see "Materials and Methods") have enabled us to compare the relative amounts of mRNAs of RHAMM, CD44 (all forms), and exon 6 of CD44 between the can-

Table I. Characteristic of Patients

| Case No. | Age | Sex | Location | n | M | Histological features | Dukes' stage | RHAMM | CD44s | CD44 exon 6 |
|----------|-----|-----|----------|---|---|----------------------|-------------|-------|-------|------------|
| 1        | 49  | M   | R        | 0 | 0 | well                 | A           | 5.49  | 10.2  | 1.86      |
| 2        | 73  | M   | D        | 0 | 0 | well                 | A           | 8.87  | 16.7  | 1.88      |
| 3        | 50  | F   | R        | 0 | 0 | mod                  | A           | 6.47  | 13.6  | 2.10      |
| 4        | 77  | F   | S        | 0 | 0 | mod                  | A           | 14.4  | 20.3  | 1.41      |
| 5        | 67  | F   | R        | 0 | 0 | mod                  | A           | 15.6  | 19.8  | 1.27      |
| 6        | 82  | F   | Ce       | 0 | 0 | well                 | A           | 1.13  | 8.98  | 7.94      |
| 7        | 79  | F   | Ce       | 0 | 0 | well                 | A           | 4.87  | 16.7  | 3.43      |
| 8        | 55  | M   | S        | 0 | 0 | mod                  | A           | 1.65  | 11.7  | 7.09      |
| 9        | 75  | M   | R        | 0 | 0 | mod                  | A           | 1.60  | 2.69  | 1.68      |
| 10       | 75  | F   | A        | 0 | 0 | mod                  | B           | 0.52  | 4.28  | 8.23      |
| 11       | 62  | M   | Ce       | 0 | 0 | mod                  | B           | 1.74  | 8.63  | 4.96      |
| 12       | 54  | F   | R        | 0 | 0 | mod                  | B           | 2.40  | 5.43  | 2.26      |
| 13       | 78  | F   | A,T      | 0 | 0 | mod                  | B           | 13.8  | 24.5  | 1.78      |
| 14       | 75  | F   | A        | 0 | 0 | mod                  | B           | 1.58  | 6.43  | 4.07      |
| 15       | 64  | M   | R        | 0 | 0 | mod                  | B           | 2.46  | 12.1  | 4.92      |
| 16       | 78  | M   | R        | 0 | 0 | well                 | B           | 6.42  | 1.50  | 0.23      |
| 17       | 54  | M   | A        | 0 | 0 | mod                  | B           | 1.25  | 6.15  | 4.92      |
| 18       | 67  | M   | R        | 0 | 0 | mod                  | B           | 1.40  | 16.4  | 11.7     |
| 19       | 54  | M   | S        | 0 | 0 | mod                  | B           | 1.25  | 13.4  | 10.7     |
| 20       | 38  | M   | D        | 3 | 0 | poor                 | C           | 1.15  | 4.11  | 3.57      |
| 21       | 66  | M   | S        | 2 | 0 | poor                 | C           | 0.98  | 5.40  | 5.51      |
| 22       | 67  | M   | S        | 1 | 0 | mod                  | C           | 4.89  | 12.1  | 2.47      |
| 23       | 70  | M   | R        | 1 | 0 | poor                 | C           | 12.3  | 17.0  | 1.38      |
| 24       | 61  | F   | T        | 2 | 0 | mod                  | C           | 8.79  | 9.40  | 1.07      |
| 25       | 73  | M   | Ce       | 1 | 0 | well                 | C           | 7.30  | 9.60  | 1.32      |
| 26       | 65  | M   | S        | 1 | 0 | mod                  | C           | 2.35  | 10.6  | 4.51      |
| 27       | 49  | F   | S        | 2 | 0 | mod                  | C           | 0.85  | 11.6  | 13.6     |
| 28       | 68  | F   | S        | 1 | 0 | mod                  | C           | 2.75  | 17.8  | 6.47      |
| 29       | 73  | M   | R        | 1 | 0 | mod                  | C           | 0.42  | 5.50  | 13.1     |
| 30       | 63  | M   | R        | 2 | 0 | lung                 | C           | 0.75  | 0.79  | 1.05      |

*Location, location of primary tumor; A, ascending colon; Ce, cecum; D, descending colon; R, rectum; S, sigmoid colon; T, transverse colon.
*b) n, lymph node metastasis.
*c) M, distant metastasis.
*d) well, well differentiated; mod, moderately differentiated; poor, poorly differentiated.
*e) N, normal colon tissues; T, colon cancer tissues.
cerous and noncancerous parts of various specimens at different Dukes’ stages (A, B, and C). Total RNAs were extracted from noncancerous (○) and cancerous (●) parts. Equal amounts of total RNA (200 ng) were subjected to real-time RT-PCR analysis and the relative amounts of mRNAs of RHAMM and GAPDH in each sample were calculated using standard curves as described in “Materials and Methods.” Relative expression coefficient for each mRNA (the ordinate) was obtained by dividing the relative amount of RHAMM mRNA by that of GAPDH mRNA in each sample so that quantitative comparisons of RHAMM expression could be made among different samples. Each point is the mean value obtained from two independent experiments in which the difference was less than 10%. Statistically significant differences between the cancerous and noncancerous parts of the specimens at different Dukes’ stages (A, B, and C) were observed in the relative expression coefficient of RHAMM. Bars, mean. * P<0.05.

Fig. 1. Expression of RHAMM in colon cancers at Dukes’ stages A, B, and C. Total RNAs were extracted from noncancerous (○) and cancerous (●) parts. Equal amounts of total RNA (200 ng) were subjected to real-time RT-PCR analysis and the relative amounts of mRNAs of RHAMM and GAPDH in each sample were calculated using standard curves as described in “Materials and Methods.” Relative expression coefficient for each mRNA (the ordinate) was obtained by dividing the relative amount of RHAMM mRNA by that of GAPDH mRNA in each sample so that quantitative comparisons of RHAMM expression could be made among different samples. Each point is the mean value obtained from two independent experiments in which the difference was less than 10%. Statistically significant differences between the cancerous and noncancerous parts of the specimens at different Dukes’ stages (A, B, and C) were observed in the relative expression coefficient of RHAMM. Bars, mean. * P<0.05.
tered medicines. However, it remains to be examined because normal specimens are not often available. RHAMM was previously found to contribute to cell motility, invasive behavior, and cell proliferation in fibroblasts in vitro.12) RHAMM overexpression has been found in a few cultured tumor cell lines such as human malignant pancreatic cells,19) human lung small cell carcinomas,20) and human breast carcinomas.13) Our present study has for the first time shown higher expression levels of the RHAMM mRNA in various colon cancers obtained from patients at any Dukes’ stage. It has been demonstrated that RHAMM is crucial to control ras signaling.12) In addition, a recent study on breast carcinoma13) suggested that RHAMM is significantly associated with ERK expression and may function collaboratively with the MAP kinase cascade. Thus, its overexpression may be linked to overexpression of both ras and ERK. Consistent with those reports, our present results suggest that RHAMM overexpression is closely associated with human colon cancer. Thus, it may be of use as diagnostic indicator.

The CD44 (all forms) expression in cancerous parts increased on average 1.30 times at Dukes’ stage A, 0.99 times at Dukes’ stage B, and 1.89 times at Dukes’ stage C, compared with those in noncancerous parts at the corresponding Dukes’ stages (Fig. 2). On the other hand, the exon 6 of CD44 expression in cancerous parts only increased 1.14 times at Dukes’ stage A, 0.86 times at Dukes’ stage B, and 1.52 times at Dukes’ stage C, compared with those in noncancerous parts at the corresponding Dukes’ stages (Table I). It is of note that high expression of CD44s but not that of its exon 6 is statistically significant at Dukes’ stage C and there was also a significant difference between the specimens at Dukes’ stage B and those at stage C (Fig. 2). The ratio of CD44s expression in cancerous parts to that in noncancerous parts appears to be parallel to the ratio of the exon 6 of CD44 expression in cancerous parts to that in noncancerous parts at all stages (on average 1.17 at Dukes’ stage A, 1.18 at Dukes’ stage B, and 1.34 at Dukes’ stage C) and shows no statistically significant variation with Dukes’ stage. The involvement of expression of exon 6 (V1) in tumor cell malignancy has been suggested by Matsumura et al.10) although there are many reports on aberrant expressions of other variant forms in various carcinoma cell lines21) and several human cancer tissues including human colorectal carcinoma.8) However, this study using the real-time RT-PCR does not support Matsumura’s proposal, at least for human colorectal cancers. The overexpression of CD44 (all forms) is significantly related to the progression of this cancer. The difference between our results and the others might be due to our use of the real-time RT-PCR method which we believe to be more reliable, but simultaneous analyses on the same specimens using various methods may be needed to reach a firm conclusion.

Our findings suggest that there are changes of expressions of two different HA-receptors, RHAMM and CD44s, as well as those of three different HASs, HAS1, HAS2 and HAS3, in the processes of tumor progression. It appears that RHAMM is upregulated, CD44 and its variant forms are expressed and upregulated, and HA synthesis is upregulated by overexpression of HASs. It has been shown that expression of antisense CD44 variant 6 inhibits colorectal tumor metastasis.9) We have recently found that HA synthesis could be modified or inhibited by the manipulation of HAS genes (unpublished observations). Further studies should provide new methods to inhibit and/or suppress tumor progression and metastasis.

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