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The Ratio of Splicing Variants of MGC-24/CD164, a Sialomucin, Correlates with the Metastatic Potential of Colorectal Carcinomas[1]

Takanori Matsui,*,† Nobuyuki Kurosawa,* Kenji Hibi,† Seiji Akiyama,† Yasushi Kasai,† Junichi Sakamoto,‡ Katsuki Ito,† Akimasa Nakao,† and Takashi Muramatsu*,[2]

*Departments of *Biochemistry and †Surgery II, Nagoya University School of Medicine, 65-Tsurumai-cho, Showa-ku, Nagoya 466-8550; and ‡Department of Surgery, Aichi Prefectural Hospital, 18 Kuriyado, Kakemachi, Okazaki 444*

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MGC-24/CD164 is a sialomucin expressed in many normal and cancerous tissues. In humans, soluble and transmembrane forms of MGC-24 are produced by alternative splicing. The total MGC-24 RNA level was found to be lower in human colorectal carcinomas as compared with the adjacent normal mucosal tissues. Lower MGC-24 mRNA levels in colon carcinomas and in the adjacent normal mucosa epithelium correlate with lymphatic vessel invasion by the carcinoma. The ratio of the soluble form to the transmembrane form of the mRNA in colorectal carcinomas was determined by ribonuclease protection assay. Higher ratios were correlated with less venous invasion and less remote metastasis, which became evident during postoperative observation.

Key words: CD164, colorectal carcinomas, metastasis, sialomucin, vascular invasion.

Mucins and the carbohydrate epitopes they carry play important roles in intercellular communication. For example, selectins, a family of carbohydrate-recognizing proteins that regulate leukocyte trafficking, bind to carbohydrate epitopes on mucin-like glycoproteins such as GlyCAM-1 and MAdCAM-1 (*1–3*). Mucin expression also influences tumor progression and metastasis. The mature form of MUC-1, which is a mucin molecule with a transmembrane domain, is expressed more abundantly in colon cancer cells than in normal mucosal cells, and more in metastatic lesions than in primary tumors (*4*). In gastric carcinomas, patients with MUC-1 positive tumors show a worse prognosis than those with MUC-1 negative tumors (*5*). MUC-1 exerts its effect by suppressing the interaction between immune cells and target cancer cells, and also by interfering with the functions of cadherins and integrins leading to increased mobility of cancer cells (*6*, *7*). On the other hand, the expression of a soluble mucin, MUC2, is suppressed in the liver and lymph node metastasis of colorectal carcinomas (*8*). MUC2 antigen expression in gastric carcinomas is a prognostic factor associated with a favorable outcome in patients. Furthermore, various carbohydrate epitopes on mucins influence the metastatic potential of human carcinomas (*9*).

MGC-24 is a sialomucin found as a carrier of peanut agglutinin–binding sites (T antigen) in human gastric carcinoma cells (*10*). Although MGC-24 was originally described in the soluble form, a transmembrane form has recently been found as a marker of hematopoietic progenitors and bone marrow cells, and has been given the CD number CD164 (*11*, *12*). This transmembrane molecule is thought to regulate hematopoiesis negatively. This study was performed to clarify whether the ratio of the soluble form of MGC-24 to the transmembrane form influences the behavior of colon cancer cells.

MATERIALS AND METHODS

Tumor Specimens—Tumor specimens were obtained from 92 patients with advanced colorectal carcinoma that had at least invaded the muscle layer, who underwent surgery at the University Hospital, Nagoya University School of Medicine. Cases of Recurrence involving tumors too small to obtain specimens in sufficient amounts, or of non-epithelial malignancies (*e.g.* lymphoma and leiomyosarcoma) were excluded from this study. Informed consent was obtained from each patient. The average age of the patients was 61.6 ± 10.7. Fifty-one patients were male, and 41 were female. Table I shows clinicopathological findings of the tumors used for Northern blotting analysis and ribonuclease protection assay. Normal mucosal tissues, which were separated by at least 10 cm from the carcinoma, were also collected. To obtain pure mucosal tissues, blunt dissection at the submucosal layer was performed and the musculoserosal layer was removed. These procedures were performed immediately after surgical resection. All specimens were frozen in liquid nitrogen as rapidly as possible and stored at −80°C until RNA preparation.

Clinicopathological Analysis—Liver metastasis and peritoneal dissemination were examined during surgery. Resected tumor and lymph nodes were examined microscopically for pathological diagnosis including histological type, depth of main tumor invasion, lymphatic and blood vessel invasion, and lymph node metastases.

RNA Preparation—Total RNA was isolated from frozen surgical specimens according to the acid-guanidinium isothiocyanate extraction method of Chomczynski and Sacchi (*13*).

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[2] To whom correspondence should be addressed. Tel: +81-52-744-2059, Fax: +81-52-744-2065, E-mail: tmurama@tsuru.med.nagoya-u.ac.jp

Northern Blotting Analysis—For Northern blotting, aliquots containing about 10 μg of total RNA were electrophoresed in 1% agarose/formaldehyde gels and transferred onto nylon filters. The blots were hybridized with α-^{32}P–labeled DNA probe for MGC-24 or glyceraldehyde 3-phosphate dehydrogenase (G3P). The membranes were prehybridized in 50% formamide, 4× SSC, 1× Denhardt's solution, 1% SDS, and 100 μg/ml salmon sperm DNA at 42°C for 1 h, and then hybridized with α-^{32}P–labeled DNA probes for 12 h under the same conditions used for prehybridization. The MGC-24 probe was a 2.4 kb *Bam*HI-digested cDNA fragment of MGC-24(10) in the SK(−) vector [pMGC-24SK(−)] labeled with a Multiprime labeling kit (Amersham). The G3P DNA probe was a 435 bp fragment amplified by PCR with primers 5′-GACCACAGTCCATGCATCAC-3′ and 5′-GTAGCCAAATTCGTTGTCATACC-3′. After hybridization, the blots were washed three times with 2× SSC and 1% SDS at 65°C for 30 min.

Ribonuclease Protection Assay—To analyze alternative splicing of MGC-24, 43 of the 92 samples (Table I) were examined by ribonuclease protection assay. A 215-bp cDNA fragment corresponding to the COOH-terminal region of MGC-24S (nucleotides 452–666) (*10*) was amplified by PCR with the primers 5′-AGAGGAATTCAAACCCACAGTTCAG-3′ and 5′-AGAGCTCGAGACACTAATGGTATCC-3′ (nucleotide sequences added to form restriction enzyme sites are underlined). The amplified DNAs were digested with *Eco*RI and *Xho*I and then ligated into the corresponding sites of pBluescript SK(+). After linearization of the plasmids with *Not*I, antisense RNA probes were produced using T7 RNA polymerase and α-^{32}P–UTP. The RNA probe of 283 nucleotides including 68 nucleotides derived from multi-cloning sites was hybridized with 10 μg of total RNA in 30 μl of hybridization buffer (80% formamide, 40 mM of PIPES, pH 6.4, 400 mM of NaCl, and 1 mM EDTA) at 45°C for 12 h. Free probes were then digested with ribonuclease in digestion buffer (310 mM Tris HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 40 μg/ml of ribonuclease A, and 2 μg/ml of ribonuclease T1) for 30 min at 30°C. Protected probes were separated in denaturing polyacrylamide gels (*14*).

Radioactivity Quantification—Radioactivity was visualized and quantified using a BAS 2000 Radio Image Analyzer (Fuji Film). Probe activities and the incubation periods for each step (prehybridization and hybridization, membrane wash, and exposure to imaging plates) were equal in all membranes to allow quantitative comparisons without membrane bias.

Statistical Analysis—The hybridization signals, quantified as described, were normalized to the signal of G3P. As these represent "ratio" values, we used the logarithms of these values for statistical analysis. The ratio of the soluble to transmembrane forms was treated in the same way. Paired t and *Z*-tests were used to compare carcinoma and normal tissues. To examine the relationship with the clinicopathological findings, unpaired *t*-tests were also performed. Calculations were performed using StatView J-4.5 (Abacus Concepts).

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TABLE I. **Profiles of 92 patients analyzed for the expression of MGC-24 mRNA by Northern blotting.**

Pathological diagnosis					No. of cases
Well differentiated adenocarcinoma					7 (3)
Moderately differentiated adenocarcinoma					80 (36)
Poorly differentiated adenocarcinoma					2 (1)
Mucinous cell carcinoma					3 (3)
	Clinicopathological profiles				
	0	1	2	3	4
H	83 (38)	4 (1)	1 (0)	4 (4)	
P	90 (41)	0 (0)	1 (1)	1 (1)	
M	90 (41)	2 (2)			
N	52 (23)	24 (10)	7 (5)	5 (2)	4 (3)
ly	12 (7)	35 (18)	34 (13)	11 (5)	
v	41 (19)	47 (23)	3 (2)	1 (1)	
Region in the colon and rectum					
Right side					
Cecum			8		(4)
Ascending colon			14		(7)
Transverse colon			4		(2)
Total			26		(13)
Left side					
Descending colon			5		(3)
Sigmoid colon			12		(6)
Rectum			48		(21)
Proctos			1		(0)
Total			66		(30)

Numbers of cases are shown in the table. Numbers in parentheses indicate cases analyzed for the ratio of soluble to membrane-bound form of MGC-24 mRNA. Clinicopathological profiles were assessed according to the rules established by the Japanese Society for Cancer of the Colon and Rectum (*16*) (H, liver metastasis; P, peritoneal dissemination; M, other metastasis; n, lymph node metastasis; ly, lymphatic vessel invasion; v, venous invasion). 0 is negative and 1–4 are positive; larger numbers show advanced metastasis or invasion.

RESULTS

Total mRNA Level of MGC-24 in Colorectal Carcinomas—We performed Northern blotting analysis of MGC-24 mRNA using the soluble form as a probe (Fig. 1). Since the size of the transmembrane form of the mRNA is indistinguishable from that of the soluble form (*11*), this method should have detected the total level of MGC-24 mRNA. Total MGC-24 mRNA expression was generally lower in colorectal carcinomas relative to the corresponding normal mucosa (Fig. 2). Log (intensity of hybridization to MGC-24 probe)/(intensity of hybridization to G3P probe) was −0.961 ± 0.049 in carcinoma specimens, while the value was −0.650 ± 0.049 in the adjacent normal mucosa. This difference was statistically significant ($p < 0.0001$). Furthermore, the level of MGC-24 mRNA in the cancerous tissue correlates well with that in the adjacent normal tissue (Fig. 2B).

We found no significant differences in total MGC-24 mRNA expression in carcinoma specimens with respect to stage, the presence or absence of hepatic metastases, lymph node metastases, or peritoneal dissemination. The only

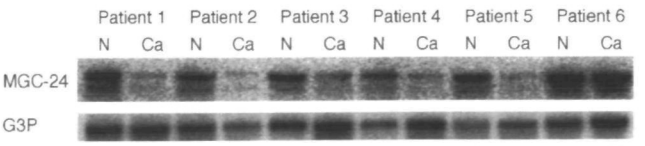

Fig. 1. **Northern blot analysis to detect MGC-24 mRNA.** The two bands were each inferred to have arisen due to differences in poly A addition sites (1,610 and 2,420). N, normal adjacent mucosa; Ca, cancerous tissue. G3P, glyceraldehyde 3-phosphate dehydrogenase.

meaningful correlation observed was that carcinomas with lymphatic vessel invasion had significantly lower MGC-24 levels than those without lymphatic vessel invasion ($p = 0.0159$) (Table II). Normal tissue from patients with carcinomas invading lymphatic vessels also showed lower MGC-24 values than similar tissues from patients with non-invading carcinomas ($p = 0.0145$) (Table II).

Ratio of the Soluble to the Transmembrane Form of MGC-24 in Colorectal Carcinomas—Since the total MGC-24 mRNA level yielded only limited information, we next performed ribonuclease protection assays to determine the ratio of the mRNA encoding the soluble form to that encoding the transmembrane form. Since the ribonuclease protection assay requires relatively large amounts of RNA, only about half of the RNA specimens could be analyzed by this method (Table I). An example of the ribonuclease protection assay and its scheme are shown in Fig. 3. The ratio of the soluble to the transmembrane form in cancer specimens also correlates with the value in the adjacent normal tissue (Fig. 4).

We found that the presence of venous invasion was correlated with a lower soluble/transmembrane ratio (MGC-24S/MGC-24M) in carcinoma ($p < 0.05$) (Fig. 5A). The value in the adjacent normal mucosal tissue showed no such correlation (Fig. 5A). Lymphatic vessel invasion showed a similar correlation, but it was not statistically significant, probably because the number of specimens was insufficient (Fig. 5B). The absolute amounts of MGC-24S and MGC-

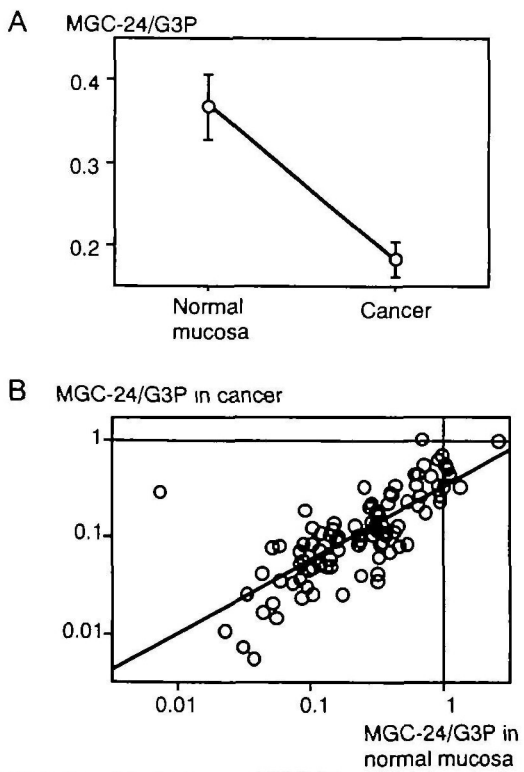

Fig. 2. **Relationship between MGC-24 mRNA levels in colon carcinoma and those in adjacent normal colonic mucosa.** A: The intensity of the MGC-24 signal/the intensity of the G3P signal was lower in carcerous tissue as compared to the normal adjacent mucosa (*t*-test; $p < 0.0001$). B: The intensity of the MGC-24 signal/the intensity of the G3P signal in cancer specimens was correlated with the intensity in normal adjacent tissues. (*Z*-test: $r = 0.757$, $p < 0.0001$).

TABLE II. **Lower MGC-24 mRNA levels in colon carcinomas and the adjacent normal mucosal epithelium as correlated with lymphatic vessel invasion by the carcinomas.**

Sources of RNA	Log (MGC-24 mRNA)/(G3P mRNA) in patients with colon carcinomas that	
	Invaded lymphatic vessels	Did not invade lymphatic vessels
Colon carcinoma	−1.007±0.052	−0.661±0.113
Normal colonic mucosa	−0.695±0.053	−0.346±0.087

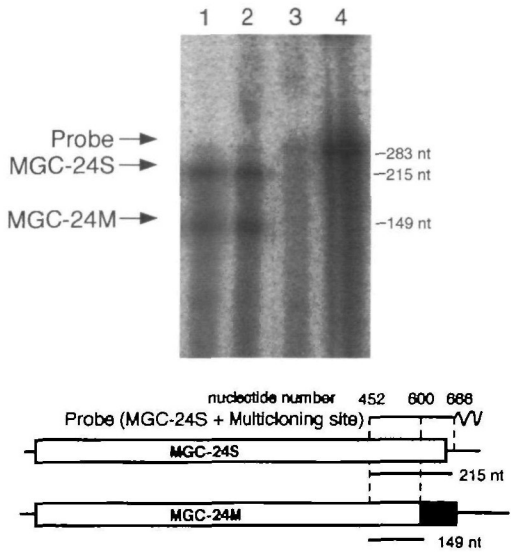

Fig. 3. **Ribonuclease protection assay to determine the ratio of soluble MGC-24 (MGC-24S) mRNA to transmembrane-type MGC-24 (MGC-24M) mRNA.** Protected fragments of the probe were separated by polyacrylamide gel electrophroresis. Positions of the probe (283 nt), the protected band of MGC-24S (215 nt), and MGC-24M (150 nt) are shown by arrows. The shaded area in MGC-24M shows the translated region with a sequence different from that of MGC-24S. Lane 1, RNA from normal adjacent mucosa; lane 2, RNA from cancer, the samples are paired. lane 3, tRNA, lane 4, probe. Shown below are schemes of the probe, MGC-24S, MGC-24M, and probes protected from them.

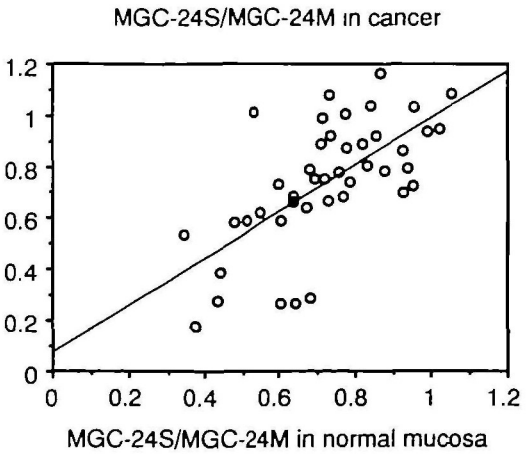

Fig. 4. **Ratio of MGC-24S/MGC-24M in cancer correlated with the value in adjacent normal mucosa (*Z*-test: $r = 0.654$, $p < 0.0001$).** S, soluble; m, membrane-bound.

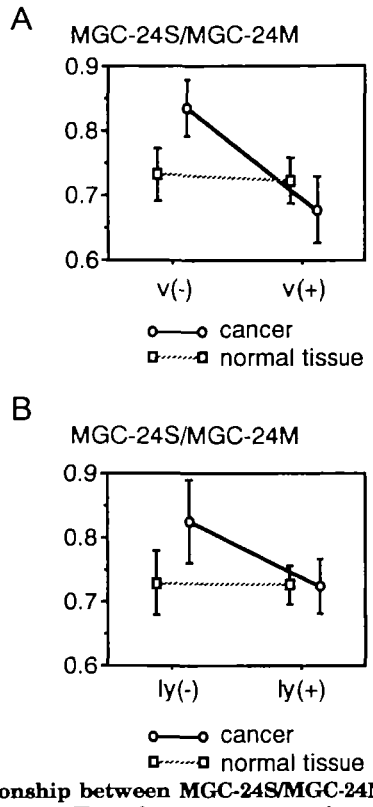

Fig. 5. **Relationship between MGC-24S/MGC-24M values and tumor invasion.** A: The values in cancer are lower in cases with venous invasion [v(+)] as compared to cases without tumor invasion [v(−)] (unpaired *t*-test: $p = 0.033$). B: The value in cancer is also decreased in cases with lymphatic vessel invasion [ly(+)] as compared to cases without lymphatic vessel invasion [ly(−)], but the difference was not significant (unpaired *t*-test; $p = 0.275$). S, soluble; M, transmembrane.

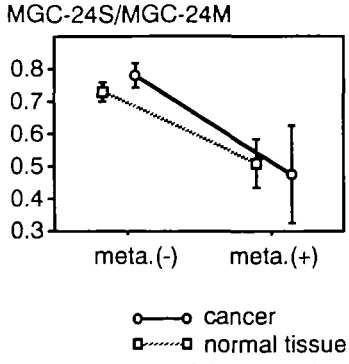

Fig. 6. **Relationship between MGC-24S/MGC-24M values and remote metastasis.** Among patients who underwent curative surgery, patients with remote metastasis during observation showed significantly lower MGC-24S/MGC-24M ratios as compared with patients without remote metastasis during the follow up period. The difference was seen not only in the cancerous tissue ($p = 0.02$) but also in normal mucosa ($p = 0.003$). S: soluble, M: transmembrane. meta.(+), metastasis occurred; meta.(−), metastasis did not occur.

TABLE III. **Possible difference in the MGC-24/G3P value and MGC-24S/MGC-24M value between the right side and the left side of the colon and rectum.**

MGC-24/G3P	Right side	Left side	
Normal	0.297±0.060	0.394±0.051	$p>0.05$
Cancer	0.143±0.033	0.198±0.026	$p>0.05$
MGC-24S/M	Right side	Left side	
Normal	0.627±0.052	0.761±0.029	$p>0.05$
Cancer	0.722±0.086	0.747±0.041	$p>0.05$

The right side and left side are defined in Table I.

24M were not correlated with vascular invasion (data not shown).

Among 36 cases in which no evidence of remote metastasis was detected until surgery, two liver metastases and one lung metastasis became evident during subsequent observation. The occurrence of this remote metastasis also correlated with a lower soluble to transmembrane form ratio in carcinomas (Fig. 6). A similar tendency was also found for the ratio in normal tissues (Fig. 6).

Finally, we examined whether the ratio of MGC-24S/MGC-24M or the level of total MGC-24 correlates with the site of the carcinoma in the colon and rectum. However, no significant difference between the values in the distal and proximal portions, either in the normal region or the cancerous region (Table III).

DISCUSSION

The total MGC-24 mRNA level was lower in colon carcinomas as compared to normal colonic mucosa. Furthermore, the lower MGC-24 mRNA levels in colon carcinomas were correlated with a tendency for invasion into lymphatic vessels. The same tendency was also observed in the corresponding normal mucosa. Thus, lymphatic vessel invasion appears to be influenced by the degree of MGC-24 expression in normal mucosa. If the level of MGC-24 expression is genetically determined, this may be a factor affecting patient prognosis.

On the other hand, the total MGC-24 mRNA level is not correlated with venous invasion or the occurrence of remote metastasis. Thus, we performed ribonuclease protection assays to determine the ratio of the soluble to transmembrane forms of the mRNA. The occurrence of venous invasion was found to be correlated with a lower soluble to transmembrane ratio. Since venous invasion precedes remote metastasis, the correlation of both phenomenon is considered reasonable.

One remaining question is in which normal cells is MGC-24 strongly expressed. This point is important when considering the physiological function of MGC-24 as a whole, MGC-24M and MGC-24S. Further work along this line is required.

The transmembrane form of MGC-24 has been suggested to act as a cell adhesion molecule (*11*). It is possible that the transmembrane form also participates in the adhesion of tumor cells to host tissues, such as blood vessels, and is involved in tumor metastasis. The soluble form might suppress tumor metastasis by counteracting the transmembrane form. Furthermore, carbohydrate epitopes in the soluble form can be speculated to inhibit cell adhesion processes based on carbohydrate protein interactions. The development of immunoassay systems that can discriminate between the soluble and transmembrane forms of MGC-24 may provide a way to predict the prognosis of colorectal carcinomas. Furthermore, the introduction of soluble MGC-24 either as a gene or as a protein may be useful as a

method for suppressing tumor invasion.

Recently, we have found that mice produce only the transmembrane form of MGC-24 (*15*). It is possible that the soluble form of MGC-24 evolved as a counteractive defense against tumor invasion.

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