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Increased Midkine Gene Expression in Human Gastrointestinal Cancers

Kuniaki Aridome,[1,2] Jun-ichiro Tsutsui,[2] Sonshin Takao,[1] Kenji Kadomatsu,[2,3] Masayuki Ozawa,[2] Takashi Aikou[1] and Takashi Muramatsu[2,3]

[1]*First Department of Surgery and* [2]*Department of Biochemistry, Kagoshima University, Faculty of Medicine, 8-35-1 Sakuragaoka, Kagoshima 890 and* [3]*Department of Biochemistry, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466*

Midkine (MK) is a product of a retinoic acid-responsive gene, and is a novel growth differentiation factor. We examined the expression of the MK gene in specimens of 47 surgically removed human carcinomas of the gastrointestinal organs, namely, gastric, colorectal, hepatocellular, pancreatic, esophageal, ampullary duodenal and bile duct carcinomas. In most cases, the MK mRNA level was higher in cancer specimens than in the corresponding non-cancerous tissues. Furthermore, MK mRNA was more highly expressed in the colon adenocarcinoma lesion than in the adenoma lesions, in the two familial polyposis cases. While MK mRNA was not detected in the normal liver, it became detectable in cirrhotic tissues in 2 of 4 cases, and its expression was increased in the cancerous tissues. Thus, the increase of MK mRNA level is a phenomenon seen in many human gastrointestinal carcinomas. The increased expression of the MK gene in gastric carcinoma was significantly more prominent in well and moderately differentiated adenocarcinomas than in poorly differentiated adenocarcinomas and signet ring cell carcinomas.

Key words: Gastric carcinoma — Gastrointestinal cancer — Growth factor — Midkine

Midkine (MK) is a growth factor found as a product of a retinoic acid-responsive gene.[1] MK is a basic, cysteine-rich polypeptide[1,2] and is a member of a novel protein family[3] together with heparin-binding growth-associated molecule[4]/pleiotrophin.[5] MK promotes survival and neurite outgrowth of embryonic neuronal cells, and is mitogenic to certain fibroblastic cell lines and neuroectoderm-derived cells.[6-10]

Aberrant expression of growth factors is often associated with tumorigenesis, and abnormalities in the expression of growth factors and their signal transduction systems are considered to contribute to tumorigenesis.[11-13] Therefore, we are interested in the expression of the MK gene in human carcinomas. We have found that MK gene expression is invariably increased in surgical specimens of Wilms' tumor as compared with corresponding non-tumorous kidney tissues.[14] The anti-MK antibody partially inhibits the growth of Wilms' tumor cells.[10] MK gene expression is also increased in the majority of hepatocellular carcinomas.[14] High levels of MK gene expression have been observed in various cell lines and nude-mice transplanted lines of human carcinomas.[14] We describe here a study of the MK mRNA expression in human gastrointestinal cancers, conducted to examine whether increased MK gene expression is a phenomenon observed in many human carcinomas.

MATERIALS AND METHODS

Surgically resected specimens Specimens were resected from 47 patients, who had surgery at the University Hospital, Kagoshima University from July 1992 to November 1993. They were immediately frozen and kept at -80°C. Non-cancerous tissues (mucosal tissues from gastric, colorectal and esophageal cancer patients) were resected from the same patient; the distance from the cancerous region was 5–10 cm for the stomach, 5–15 cm for the colon and rectum, 3–5 cm for the liver, about 2 cm for the pancreas, and about 5 cm for the esophagus. Table I shows the age and sex of the patients and pathological information concerning the carcinomas. The mean age of the patients was 61.5 years and the male/female ratio was 1.9.

RNA preparation and Northern blot analysis Total cellular RNA was prepared from about 0.5 g of frozen tissues or cells by acid guanidium thiocyanate phenol-chloroform extraction.[15] For Northern blot analysis, 20 μg of total cellular RNA was denatured with glyoxal, electrophoresed through 1% agarose gel, and transferred to a Hybond nylon membrane (Amersham). The membrane was prehybridized and hybridized with a ^{32}P-labeled probe. Northern hybridization was performed overnight at 42°C in 50 m*M* Tris-HCl buffer, pH 8.0, containing 1 *M* NaCl, 10 m*M* EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.2% polyvinylpyrolidine, 0.2% Ficoll, and 0.2% bovine serum albumin. The membrane was then washed twice with 2×SSC (1×SSC, 0.15 *M* NaCl containing 0.015 *M* sodium citrate) containing 0.1% SDS at 56°C for 10 min and twice with 0.2×SSC containing 0.1% SDS at 56°C for 30 min. It was exposed to X ray film at -80°C for two days.

Probes The MK probe was a 487-base-pair human cDNA fragment (nucleotide number 76–562),[16] and a

Table I. Clinical Features of 47 Human Cancers of the Digestive Tract

Case number	Sex	Age	TNM	Stage	Histology[a]	Other information
Gastric cancer						
1	M	72	pT2pN2pM0	IIIB	Well	
2	M	63	pT3pNp2M1	IV	Poor	Scirrhous type
3	F	66	pT3pN2pM0	IIIA	Sig	Scirrhous type
4	F	68	pT2pN2pM0	IIIA	Sig	
5	M	65	pT3pN2pM1	IV	Poor	Scirrhous type
6	F	81	pT2pN2pM0	IIIA	Moderate	
7	F	51	pT1pN2pM0	II	Well	
8	M	64	pT1pN0pM0	IA	Poor	
9	M	55	pT2pN2pM0	IIIA	Poor	
10	F	74	pT4pN2pM0	IIIB	Poor	
11	M	61	pT3pN2pM1	IV	Moderate	
12	F	43	pT1pN0pM0	IA	Poor	
13	F	72	pT2pN0pM0	IB	Well	
14	M	66	pT2pN0pM0	IB	Well	
15	M	58	pT3pN1pM1	IV	Sig	Scirrhous type
16	F	82	pT1pN0pM0	IA	Poor	
Colorectal cancer[b]						
17	F	74	pT4pN1pM0	III	Mucinous	Rectum
18	F	39	pT3pN1pM0	III	Well	Sigmoid, FP[d]
19	M	86	pT3pN2pM0	III	Well	Rectum
20	M	27	pT3pN1pM0	II	Well	Rectum
21	M	26	pT2pN0pM0	I	Well	Sigmoid, FP[d]
22	M	44	pT2pN0pM0	I	Well	Sigmoid
23	M	74	pT2pN0pM0	I	Well	Rectum
24	M	71	pT3pN1pM1	IV	Well	Sigmoid
25	M	59	pT2pN0pM0	I	Well	Rectum
26	F	65	pT3pN3pM0	III	Well	Cecum
27	M	66	pT3pN0pM0	II	Well	Sigmoid
28	M	67	pT3pN0pM0	II	Moderate	Rectum
29	F	72	pT2pN0pM0	I	Well	Anorectum
Hepatocellular carcinoma[c]						
30	M	55	pT4pN0pM0	IVA	Moderate	Liver cirrhosis
31	M	70	pTp2N0pM0	II	Moderate	Liver cirrhosis
32	M	64	pT3pN0pM0	II	Moderate	Chronic hepatitis
33	M	63	pT2pN0pM0	II	Moderate	Chronic hepatitis
34	M	65	pT3pN0pM0	III	Moderate	Liver cirrhosis
35	M	65	pT2pN0pM0	II	Well	Liver cirrhosis
36	F	67	pT4pN0pM0	IVA	Moderate	Normal liver
37	M	70	pT3pN0pM0	III	Moderate	Normal liver
38	M	65	pT4pN0pM0	IVA	Poor	Normal liver
Pancreatic cancer						
39	F	71	pT1bpN0pM0	I	Well	
40	M	58	pT1bpN0pM1	IVA	Poor	
41	M	67	pT3pN1pM0	III	Well	
42	M	45	pT1bpN1pM0	III	Well	
43	F	26	pT1bpN0pM0	I	SCT[e]	
Esophageal cancer						
44	M	46	pT4pN1pM0	III	Moderate	
45	M	66	pT2pN1pM0	IIB	Poor	
Ampullary duodenal cancer						
46	M	61	pT3pN1pM0	III	Well	
Liver metastasis from bile duct cancer						
47	F	55			Well	Normal liver

a) Well, moderate and poor stand for well, moderately and poorly differentiated adenocarcinoma (or hepatocellular carcinoma), respectively. Sig indicates signet ring cell carcinoma.
b) The site of the cancer is indicated in other information.
c) Status of the noncancerous region is shown in other information.
d) FP indicates familial polyposis as a complicating disease.
e) SCT indicates solid and cystic tumor.

2-kilobase-pair human β-actin cDNA (Clontech) was also used as a reference probe. They were ^{32}P-labeled with [α-^{32}P]dCTP (10 mCi/ml, DuPont/NEN Research Products) using a random oligonucleotide primer (Amersham).

Quantitative analysis of hybridization signals The MK hybridization signals were quantitated with an LKB UltroScan XL laser densitometer and normalized with respect to the signal of β-actin mRNA. In every Northern blot experiment, PA1 teratocarcinoma RNA was run as an internal control,[14] and the result was expressed in relation to the value for PA1 cells.

Heparin-Sepharose column chromatography Primary rectal cancer tissues (0.4 g) and corresponding normal tissues (0.4 g) obtained from a patient (case number 74) were homogenized and extracted with 4 ml of 0.01 *M* Tris-HCl buffer, pH 7.6 containing 1% Triton X-100, 1 *M* NaCl and 0.5 m*M* phenylmethylsulfonyl fluoride. After centrifugation at 10,000*g* for 30 min, the supernatant was diluted 5 times with 0.05 *M* Na phosphate buffer and applied to a column of heparin-Sepharose (0.5×0.5 cm). The column was washed with 20 ml of the buffer containing 0.5 *M* NaCl, and proteins were eluted with 5 ml of the buffer containing 1.5 *M* NaCl.

Western blotting Samples were subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli.[17] Proteins in the gels were transferred to nitrocellulose membranes according to the method of Towbin *et al.*[18] After incubation in 5% skim milk in phosphate-buffered saline (PBS) for 1 h at room temperature, the membranes were incubated with rabbit anti-MK-antibody (8 μg/ml) for 2 h at room temperature, washed with PBS containing 0.1% Tween 20, incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Jackson Immunoresearch Laboratories) and stained with 4-chloro-1-naphthol. A rabbit anti-MK antibody was prepared against the recombinant MK produced in *Escherichia coli*, and affinity-purified as described previously.[19]

Immunohistochemistry A specimen of rectal cancer tissue (case 23, Table I) was fixed for 1–2 days in 4% paraformaldehyde in 0.1 *M* Dulbecco's PBS (pH 7.2) [PBS(−)] at 4°C, embedded in paraffin and cut into 4 μm sections. Each paraffinized and rehydrated section was immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activities. Then it was treated with 5% 2-mercaptoethanol in 0.1% Tris-HCl buffer (pH 8.8) and incubated with 0.3% bovine serum albumin for 30 min to block the non-specific Fc-receptor in tissue. It was washed with 0.1 *M* PBS containing 0.3% Triton X-100 (PBS-T), then incubated with rabbit anti-MK antibodies (10 μg/ml) in PBS(−) containing 1% bovine serum albumin. The section was kept overnight at 4°C and then treated with biotinylated swine anti-rabbit immunoglobulins (DAKO) diluted 300 fold with PBS(−) for 1 h at room temperature. It was washed with PBS-T, peroxidase-conjugated streptavidin (DAKO) was added and color was developed with 3,3′-diaminobenzidine. For negative controls, the primary antibodies were omitted.

RESULTS

Comparison of MK RNA level between cancerous region and corresponding non-cancerous region MK gene expression was examined by Northern blot analysis using human MK cDNA as a probe, in 46 cases of surgically removed primary cancers of the gastrointestinal system, namely gastric, colorectal, hepatocellular, pancreatic, esophageal and ampullary duodenal carcinomas (Table I). MK mRNA was detectable in the primary tumor in 45 cases (Table II). The only negative case was a hepatocellular carcinoma (case 37). In 43 of the 45 positive cases, non-cancerous adjacent tissue was available. Thus, we quantitated the MK mRNA in both the cancerous and corresponding non-cancerous region for comparison. In these studies, the intensity of the signal was normalized to that of β-actin mRNA. Furthermore, for comparison of experiments performed at different periods, the value was expressed relative to that of PA-1 teratocarcinoma cells, which were run as an internal control in each experiment. We found that in 39 of the 43 cases, the primary tumors expressed more MK mRNA than the non-cancerous adjacent tissue (Table II). The exceptions were 3 cases of gastric carcinomas (case 12, equal expression; cases 15 and 16, less expression in the cancer) and one case of colorectal cancer (case 19).

Fig. 1A shows an example of Northern blot analysis. In a case of carcinoma of the rectum (case 23), MK mRNA was strongly expressed in the primary tumor, but not in the normal mucosa. The Western blotting experiment using heparin-binding proteins from these tissues verified that MK was actually expressed in the tumor tissue, but not in the normal mucosa (Fig. 1B). Of the two bands detected, the higher molecular weight band corresponded to MK. The lower molecular weight band is probably the degraded product, though we can not exclude the possibility that it is an immunologically cross-reactive protein.

To confirm the expression of MK protein in cancer cells, immunohistochemistry was performed on a well differentiated adenocarcinoma of the rectum (case 23, Table I). MK protein was strongly expressed in the cancerous region, predominantly in the cytoplasm of the cancer cells (Fig. 2A); when the first antibody was removed, scarcely any staining was seen (Fig. 2B).

Familial polyposis of the colon is known to develop to colon carcinoma with 100% probability, when left un-

Table II. MK Expression in Human Cancers and Corresponding Non-cancerous Regions

Case number	MK expression[a] in	
	Cancer	Non-cancerous region
Gastric cancer		
1	23.8	3.0
2	1.4	0.6
3	0.6	0.4
4	5.1	1.9
5	4.5	3.1
6	5.9	1.2
7	20.9	3.0
8	7.6	3.5
9	2.0	1.1
10	3.5	3.0
11	3.4	0.5
12	0.5	0.5
13	3.0	0.7
14	3.9	0.8
15	1.5	2.6
16	0.5	3.8
Colorectal cancer		
17	6.2	1.4
18	0.9	0
19	4.1	6.9
20	7.4	1.3
21	5.0	1.1
22	8.9	1.0
23	11.7	0
24	1.1	0.6
25	2.9	0.4
26	2.9	0.3
27	10.6	4.5
28	2.4	1.5
29	1.8	1.1
Hepatocellular carcinoma		
30	9.5	0
31	9.6	0
32	7.2	0.4
33	0.7	0
34	0.6	0.5
35	5.6	0.4
36	2.2	0
37	0	0
38	52.8	0
Pancreatic cancer		
39	8.7	3.8
40	5.4	0
41	1.9	0.5
42	3.4	0
43	6.5	0
Esophageal cancer		
44	11.9	0
45	5.8	0
Ampullary duodenal cancer		
46	6.9	—
Liver metastasis from bile duct cancer		
47	1.3	0

a) The hybridization signals were quantitated with a densitometer and normalized to the signal of β-actin mRNA. MK mRNA expression in cultured cells from the teratocarcinoma cell line (PA-1) was defined as 10, and each quantitated signal of MK gene was compared with this.

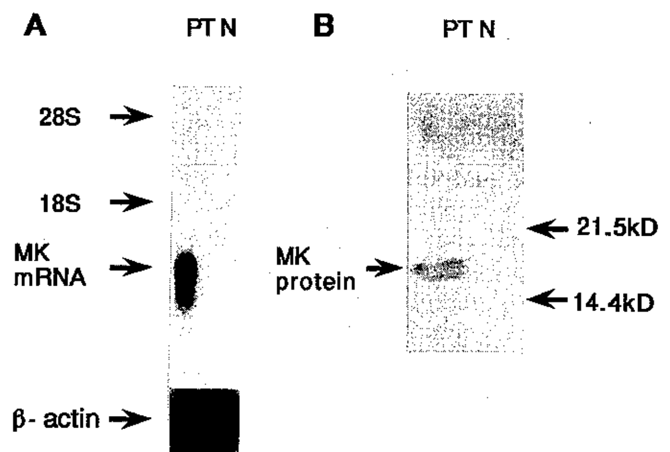

Fig. 1. Presence of MK mRNA and MK protein in primary tumor (PT) of rectal carcinoma (case 23, Table I), but not in the corresponding normal tissue (N). A, Northern blotting. Arrows show the positions of 28S and 18S ribosomal RNAs, MK mRNA and β-actin mRNA. B, Western blotting. Arrows show the positions of MK and protein standards, soybean trypsin inhibitor (21.5 kDa) and hen egg white lysozyme (14.4 kDa).

treated. In cases 18 and 21, the patients with familial polyposis had already developed colon carcinoma. The non-cancerous mucosa, which has the status of adenoma, expressed MK in case 21, but not in case 18. In case 21, the MK RNA level in the carcinoma was higher than that in the non-cancerous region (Fig. 3, Table II).

The normal liver does not express the MK gene, as described previously.[14] This was confirmed here (cases 36, 37, 38 and 47). MK mRNA was weakly expressed in 2 of the 4 cases in which liver cirrhosis was observed (cases 30, 31, 34 and 35) and also in one of 2 cases in which the non-cancerous region exhibited chronic hepatitis (cases 32 and 33) (Table I). In all the cases, the cancerous region expressed more MK mRNA than the non-cancerous region (Table II). Fig. 3 shows a representative case (case 35).

Non-cancerous pancreatic tissue expressed MK in 2 cases, but not in two other cases (Table II). The reason for this difference is unknown. In all 4 cases, the pancreatic carcinoma expressed MK, and the level was higher than the value in the non-cancerous tissue (Table II). Two examples of actual hybridization patterns are shown (Fig. 3, No. 41 and 43).

The non-cancerous portion of esophagus did not express MK. MK mRNA was expressed in both cases of esophageal squamous cell carcinoma (Table II).

All these findings strongly indicate that the increased expression of MK during tumorigenesis and/or tumor

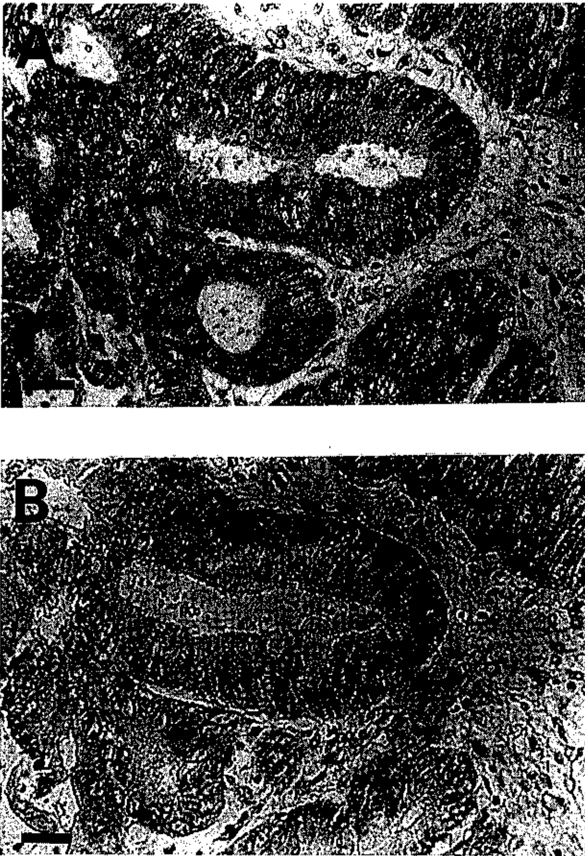

Fig. 2. Immunohistochemical staining of human rectal carcinoma (case 23, Table I) with rabbit anti-MK antibodies. A, Cancer cells were scarcely stained in the cytoplasm. B, Negative control. A serial section was treated only with the second antibody. Bar: 50 μm.

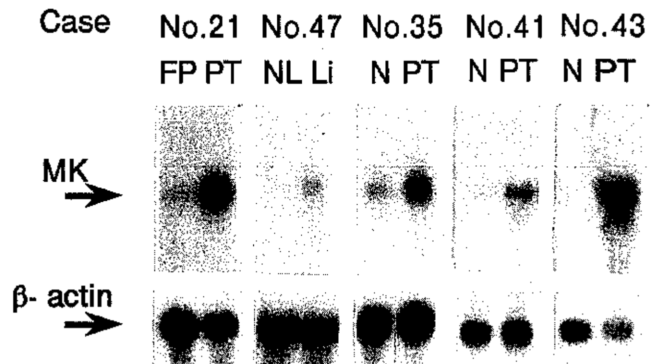

Fig. 3. MK mRNA expression in colon cancer with familial polyposis (case 21, Table I), liver metastasis from extrahepatic bile duct cancer (case 47), hepatocellular carcinoma with liver cirrhosis (case 35), pancreatic duct carcinoma (case 41) and solid and cystic tumor in the pancreas (case 43). The results of Northern blotting are shown. FP, corresponding mucosa with familial polyposis; PT, primary tumor, NL; normal liver tissue close to the tumor; Li, metastatic tumor in the liver; N, non-cancerous corresponding tissue.

progression is a phenomenon occurring in many carcinomas of the gastrointestinal organs.

Influence of histopathological types of stomach carcinomas on MK expression We examined the factors that influence the degree of increase in MK gene expression in stomach carcinoma. Factors in the TMN classification (pT, pN, pM) had no clear influence on MK gene expression. However, the histopathological type of the carcinoma was found significantly to influence MK gene expression. Fig. 4A shows the MK hybridization signal of gastric carcinomas of 4 different histological types. The increase of MK gene expression was high in well differentiated adenocarcinoma (case 7) and moderately differentiated adenocarcinoma (case 6) as compared to poorly differentiated adenocarcinoma (case 5) and signet ring cell carcinoma (case 15).

As Fig. 4B shows, MK gene expression in cancer tissue was 4.5 to 9.4 fold higher than that in the normal mucosa in 6 cases of differentiated-type carcinomas (well differentiated and moderately differentiated adenocarcinomas), and 0.1 to 2.7 fold higher in the undifferentiated type (poorly differentiated adenocarcinoma and signet ring cell carcinoma). The difference in the increase of MK gene expression between the differentiated type and the undifferentiated type is statistically significant ($P<0.01$).

DISCUSSION

The present findings clearly indicate that the MK gene expression is increased, in most cases, in a variety of human gastrointestinal carcinomas. The liver and the esophagus did not express MK; thus, cancer in these organs newly expressed MK. In the stomach and the colon, MK RNA was expressed in most of the normal mucosa, but the cancerous region usually expressed more MK RNA. In the pancreas, the cancerous region expressed more MK RNA in all 4 cases. The present findings suggest that MK plays a role in tumorigenesis and/or tumor progression in cancers of the gastrointestinal organs. We previously found that MK was intensely expressed in all of 6 cases of Wilms' tumor[14] and that the growth of cultured Wilms' tumor cells was partly inhibited by anti-MK antibody.[10] Furthermore, transfection with cDNA of pleiotrophin, which has about 50% sequence identity with MK at the amino acid level, makes 3T3 cells tumorigenic.[20] It is necessary to examine whether inhibition of MK activity results in retarded growth of the cancer cells of digestive organs. During the preparation of this manuscript, increased MK expression

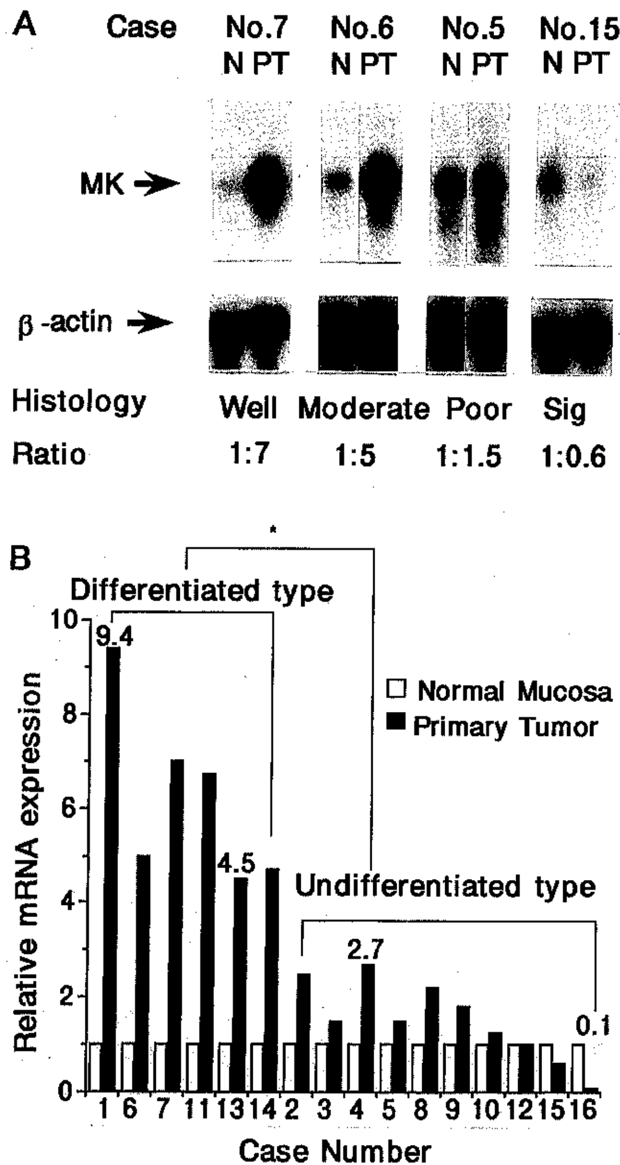

Fig. 4. MK mRNA expression in gastric cancers. A, MK mRNA expression in 4 representative gastric cancers. The results of Northern blotting are shown. PT stands for primary tumor tissue and N, corresponding normal mucosa. Well, well differentiated adenocarcinoma; Moderate, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma; Sig, signet ring cell carcinoma. Ratio stands for MK expression relative to the non-cancerous region. B, Relative MK mRNA expression. The intensity of the signals in cancer is shown by the ratio to that in the noncancerous region, which was taken as 1.0. Case numbers are identical to those shown in Table I. MK gene expression was significantly more activated in differentiated (Well or Moderate) type gastric cancers than in undifferentiated (Poor or Sig) type (* $P<0.01$, Student's t test).

was also reported in human lung cancers[21] and breast cancers.[22] Broad expression of MK in human cancers suggests that MK can be used as a tumor marker, especially in organs where MK is not expressed in the normal tissue, namely the liver and esophagus.

In gastric carcinoma, MK mRNA was more intensely expressed in differentiated-type (well differentiated and moderately differentiated) carcinomas than undifferentiated-type (poorly differentiated and signet ring cell) carcinomas. In mouse embryos, epithelial cells of the digestive tract intensely express MK on days 11–13 of gestation.[23] These embryonic epithelial cells actively multiply, but still retain epithelial morphology with tight intercellular junctions. These properties of the embryonic epithelial cells are shared by differentiated-type gastric carcinomas, and both intensely express MK. This consideration does not necessarily mean the existence of a causal relation between the histological type and the extent of MK expression, and further studies are needed to clarify this point.

Although the normal liver does not express MK, a small amount of MK was detected in one of the 2 cases of chronic hepatitis, and 2 of the 4 cases of liver cirrhosis. In all cases, hepatocellular carcinomas expressed a higher level of MK mRNA than the non-cancerous regions mentioned above. The expression of MK mRNA in inflammatory or degenerative regions is of special interest. We have recently found that in the early stages of experimental cerebral infarction, MK becomes intensely expressed in the brain.[24] MK may be a factor required not only during embryogenesis, but also during tissue repair in adulthood. The reason for the high level of MK expression in cancer may be an abnormality in tissue repair, leading to tumorigenesis.

MK is a retinoic acid-responsive gene and a functional retinoic acid-responsive element has been identified in its upstream region.[25] However, overexpression of MK gene in tumor cells may not involve retinoic acid receptors; it is quite possible that other regulatory factors such as oncogene products or tumor suppressor gene products bind to other regulatory elements in MK promoter and are involved in regulation of MK expression in relation to tumorigenesis.

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