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Matrix Metalloproteinase-2 and -9 in Bile as a Marker of Liver Metastasis in Colorectal Cancer

Norimichi Okada,*,† Hideyuki Ishida,† Nobuo Murata,† Daijo Hashimoto,† Yousuke Seyama,*,[1] and Shunichiro Kubota*,[2]

**Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; and †Department of Surgery, Saitama Medical Center, Saitama Medical School, 1981 Kamoda-Tsujido-cho, Kawagoe City, Saitama 350-8550, Japan*

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Matrix metallproteinases (MMP)-2 and -9 are associated with cancer invasion and metastasis. MMP-2 and MMP-9 activities have never been assayed in bile. In the present study we investigated whether MMP-2 and -9 activities in the bile could be a marker for evaluation of liver metastasis in colorectal cancer. Fifty-three patients underwent colorectal resection for histologically verified adenocarcinoma. Twenty-six patients had colorectal cancer without liver metastasis and 27 patients had metastatic liver tumor. Six patients were studied as carcinoma-free control. MMP-2 and MMP-9 activities were assayed in bile using gelatin zymography and quantitated. Active MMP-2 activity of colorectal cancer with liver metastasis group (24.1 ± 2.5 pixel count) was significantly higher than that of colorectal cancer without liver metastasis group (11.4 ± 1.3 pixel count) ($P < 0.001$) or of control group (6.4 ± 1.0 pixel count) ($P < 0.001$). Active MMP-9 was not detected in bile. ProMMP-9 activity of colorectal cancer with liver metastasis group (530.3 ± 127.5 pixel count) was significantly higher than that of colorectal cancer without liver metastasis group (213.9 ± 33.2 pixel count) ($P = 0.008$). This is the first report showing that the levels of active MMP-2 and proMMP-9 in bile were significantly higher in liver metastasis of colorectal cancer than in metastasis-free colorectal cancer. The results suggest that activities of active MMP-2 and proMMP-9 in the bile may be useful markers for predicting liver metastasis in colorectal cancer.

Key Words: **MMP-2; MMP-9; bile; metastasis; colorectal cancer.**

Matrix metalloproteinases (MMPs) are a family of highly homologous enzymes, involved in matrix remodeling of both physiological processes and pathological conditions. There are currently at least 26 known human MMPs (1–5). There has been a great deal of interest in the role of MMPs in cancer invasion and metastasis (1, 2, 5). To invade and metastasize tumor cells must infiltrate blood vessels and lymphatics. As these processes involve proteolysis of the extracellular matrix, the MMPs have been implicated in tumor progression (6).

In colorectal cancer 72-kDa gelatinase A (MMP-2), 92-kDa gelatinase B (MMP-9), matrilysin (MMP-7), and stromelysin-3 (MMP-11) were demonstrated to be overexpressed (7–12). MMPs can be detected using a variety of techniques including zymography. Zymography has the advantages of measuring direct enzyme activity quantitatively and of distinguishing the active enzyme from the proform. Using zymography, enzyme-linked immunosorbent assay or one step sandwich immunoassay, levels of tissue or blood (serum or plasma) MMPs in colorectal cancer were reported (13–25), but MMP activities in bile have never been assayed and reported. We investigated whether blood MMP-2 and MMP-9 were useful for evaluation of liver metastasis in colorectal cancer and found that blood MMP-2 and MMP-9 are not good markers for liver metastasis in colorectal cancer (unpublished observation). The aim of this study was to investigate whether MMP-2 and MMP-9 activities in bile could be a marker for evaluation of liver metastasis in colorectal cancer.

MATERIALS AND METHODS

Patients. Fifty-three patients (32 men and 21 women) with a mean age of 63.0 years, ranging from 39 to 89, who underwent colorectal resection for histologically verified adenocarcinoma, or partial hepatectomy for histologically verified metastatic adenocarcinoma of colorectal cancer from April 1996 to November 2000, were included in the study. Of these patients, 26 had colorectal cancer

Abbreviation used: MMP, matrix metalloproteinase.

[1] Present address: Faculty of Human Life and Environmental Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan.

[2] To whom correspondence should be addressed. Fax: +81-3-5689-2704. E-mail: kubota@bio.m.u-tokyo.ac.jp.

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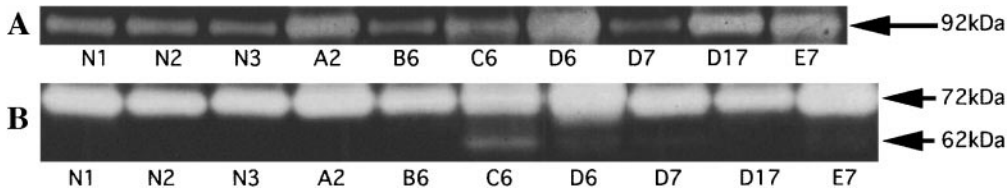

FIG. 1. Gelatin zymography in bile from colorectal cancer. Gelatin zymography for measurement of MMP-2 and MMP-9 was done using bile samples from 53 colorectal cancers and 6 controls. Typical cases [3 control (N1–3), 1 Dukes A (A2), 1 Dukes B (B6), 1 Dukes C (C6), 3 synchronous liver metastasis (D6, 7, and 17), and 1 metachronous liver metastasis (E7)] are shown in A (proMMP-9) and B (proMMP-2 and active MMP-2).

without liver metastasis (two had Dukes A tumors, 12 had Dukes B tumors, and 12 had Dukes C tumors), and 27 had metastatic liver tumor of colorectal cancer (18 had primary tumors with synchronous liver metastases, and 9 had metachronous liver metastases after resection of primary colorectal cancer). Six patients who were underwent laparoscopic cholecystectomy for cholelithiasis or cholecystic cholesterol polyps were studied as carcinoma-free control. Written informed consent was obtained from all patients and this study was approved by our institution.

Bile samples. Bile was collected by direct puncture of the gallbladder during surgical operation. No complications were observed after this procedure. No pathologic lesion in the gallbladder was noted in patients with colorectal cancer. No other gastrointestinal disease was observed in patients with laparoscopic cholecystectomy. All bile samples were immediately frozen and stored at −20°C.

Gelatin zymography. For zymography, bile samples were diluted 50-fold with phosphate-buffered saline, as preliminary experiment showed that using the 50-fold diluted samples MMP-2 and MMP-9 activities were measured quantitatively (data not shown). Ten microliters of diluted samples were mixed with 20 μl SDS–PAGE sample buffer containing SDS [8% (w/v)], glycerol [40% (w/v)], 0.25 M Tris (pH 6.8) and bromophenolblue [0.1% (W/V)]. Gelatin zymography was performed as previously described (26–28), using 10% polyacrylamide gel containing 1 mg/ml gelatin. One standard sample representing standard of the linear part of the calibration curve was included in each gel. After electrophoresis, gels were washed in 2.5% Triton-X 100 for 1 h to remove SDS. Gels were then incubated for 18 h at 37°C in 50 mM Tris–HCl (pH 7.5), 10 mM $CaCl_2$, and 1 μM $ZnCl_2$, stained with Coomassie brilliant blue R-250, and destained with 20% methanol and 7% acetic acid. Gels were scanned using an EPSON GT-7700U flat-bed scanner and an Adobe Photoshop in the Apple Macintosh computer, and quantification was done using a NIH Image (version 1.6.1) software (29). The results were expressed as pixel count.

Statistical analysis. P values for comparison of enzyme levels were calculated using the Wilcoxon paired test and the Mann–Whitney U analysis. All tests were two-sided and the P value less than 0.05 was considered as being significant.

RESULTS

Zymography of bile MMP-2 and MMP-9. MMP-2 and MMP-9 activities were measured in bile derived from 2 Dukes A, 12 Dukes B, 12 Dukes C, 18 synchronous metastasis and 9 metachronous metastasis patients. For each bile sample, active MMP-2 (M_r 62,000) and proMMP-2 (M_r 72,000), and proMMP-9 (M_r 92,000) were measured for their gelatinolytic activity (Fig. 1). Active form of MMP-9 was not detected in all bile samples in the present study.

MMP-2 activities in bile from colorectal cancer patients. ProMMP-2 and active MMP-2 activities in bile from cancer free (control) group ($n = 6$), colorectal cancer with ($n = 27$) and without ($n = 26$) liver metastasis group were measured and quantified. ProMMP-2 of carcinoma-free group (control) was 203.5 ± 22.3 pixel count (mean ± SE), while that of colorectal cancer without liver metastasis group [meta (−)] was 303.7 ± 32.0 pixel count and that of liver metastases group [meta (+)] was 286.3 ± 24.2 pixel count (Fig. 2A). There was no significant difference between all pairs of any groups ($P = 0.28$). There was no significant difference between control, Dukes A, B, and C, synchronous and metachronous liver metastasis groups ($P = 0.23$) (Fig. 2B).

On the contrary, active MMP-2 activity of colorectal cancer with liver metastasis group (24.1 ± 2.5 pixel count) was significantly higher than that of colorectal cancer without liver metastasis group (11.4 ± 1.3 pixel count) ($P < 0.001$) or of control group (6.4 ± 1.0 pixel count) ($P < 0.001$) (Fig. 3A). There was no significant difference between control group and colorectal cancer without liver metastasis group ($P = 0.06$). Active MMP-2 activity of synchronous liver metastasis group (26.9 ± 3.1 pixel count) was significantly higher than control (6.4 ± 1.0 pixel count) ($P = 0.001$), Dukes A (9.7 ± 3.7 pixel count) ($P = 0.04$), B (11.6 ± 2.6 pixel count) ($P = 0.001$), or C (11.6 ± 1.4 pixel count) ($P < 0.001$) (Fig. 3B). There was no significant difference between synchronous and metachronous liver metastasis groups (18.4 ± 3.5 pixel count) ($P = 0.09$) (Fig. 3B).

ProMMP-9 activities in bile from colorectal cancer patients. ProMMP-9 activity of colorectal cancer with liver metastasis group (530.3 ± 127.5 pixel count) was significantly higher than that of colorectal cancer without liver metastasis group (213.9 ± 33.2 pixel count) ($P = 0.008$) or that of control group (196.0 ± 47.6 pixel count) ($P = 0.05$) (Fig. 4A). There was no significant difference between control group and colorectal cancer without liver metastasis group ($P = 0.96$).

As shown in Fig. 4B, proMMP-9 activity of synchronous liver metastasis group was significantly higher (650.7 ± 182.8 pixel count) than that of control group

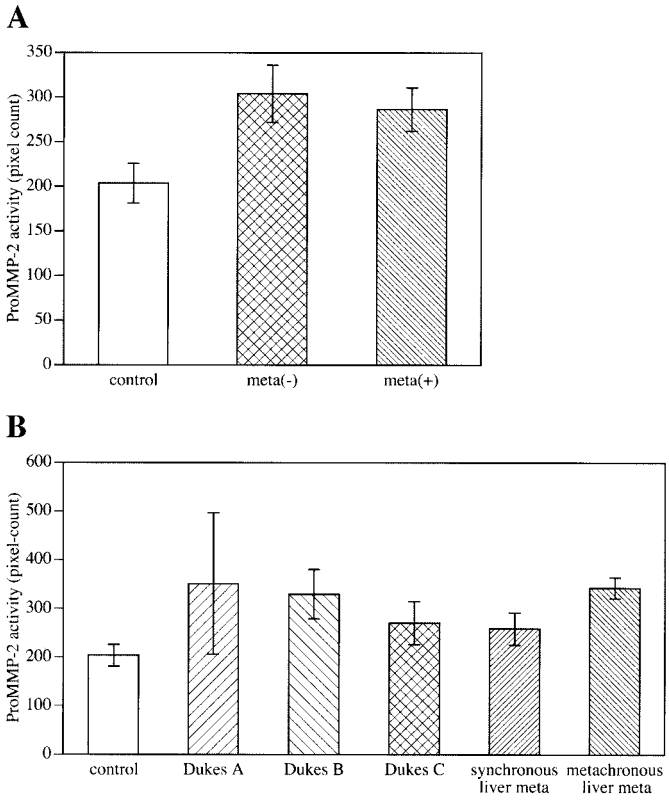

FIG. 2. ProMMP-2 activities in bile. ProMMP-2 activities in bile from control group and colorectal cancer with [meta(+)] and without [meta(−)] liver metastasis groups were measured by gelatin zymography and quantified and are shown in A. In B, the data of proMMP-2 activities in bile from control, Dukes A, B, and C, synchronous, and metachronous liver metastasis groups are shown. The data were expressed as means ± SE.

(196.0 ± 47.6 pixel count) ($P = 0.04$), Dukes B group (258.4 ± 63.3 pixel count) ($P = 0.02$) or Dukes C group (165.8 ± 30.7 pixel count) ($P = 0.006$). There was no significant difference between synchronous liver metastasis group and metachronous liver metastasis group (289.4 ± 225.0 pixel count). Although the mean level of proMMP-9 level in Dukes A group (196.0 ± 67.1 pixel count) was as low as control level, but it did not reach a statistical significance because of limited number of cases ($n = 2$).

DISCUSSION

Many studies have demonstrated an association between tumor spread and expression of MMP-2 and MMP-9 in tissue and blood, indicating that MMP-2 and MMP-9 expression may have a correlation with metastasis (13–25). However, MMP-2 and MMP-9 activities in bile have never been assayed and reported. This study is apparently the first report showing the significant increase of activated MMP-2 and proMMP-9 in bile of the patients with colorectal cancer with liver metastasis. We have shown that active MMP-2 level but not proMMP-2 was significantly higher in bile of colorectal cancer with liver metastasis. It was reported that active MMP-2 was higher in tissue or blood of colorectal cancer (19, 23). Our result is essentially consistent with these results, except that we assayed MMP-2 and -9 activities in bile. On the contrary, Mukai *et al.* demonstrated that although MMP-2 exhibited a significant expression in the primary colorectal cancer and in the synchronous liver metastasis, there was no relationship between the absence/presence of liver metastasis and serum MMP-2 levels (24). The difference between their result and ours is not clear. Our previous data also showed that there was no relationship between the absence/presence of liver metastasis and serum MMP-2 levels in colorectal cancer (unpublished observation). Although it is speculative, the MMP-2 secreted from tumor into blood may be more diluted compared to MMP-2 secreted into bile. Therefore, MMP-2 level in bile, but not MMP-2 level in blood may have reflected liver metastasis.

In the present study, we could detect proMMP-9 but not active MMP-9 in the bile. The result is essentially

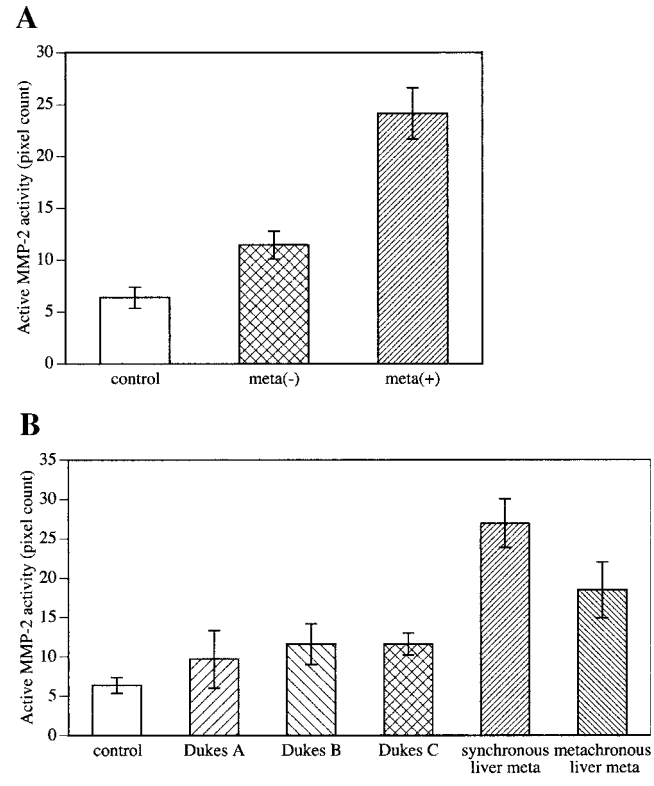

FIG. 3. Active MMP-2 activities in bile. Active MMP-2 activities in bile from control group and colorectal cancer with [meta(+)] and without [meta(−)] liver metastasis groups were measured by gelatin zymography, quantified, and are shown in A. In B, the data of active MMP-2 activities in bile from control, Dukes A, B, and C, synchronous and metachronous liver metastasis groups are shown. The data were expressed as means ± SE.

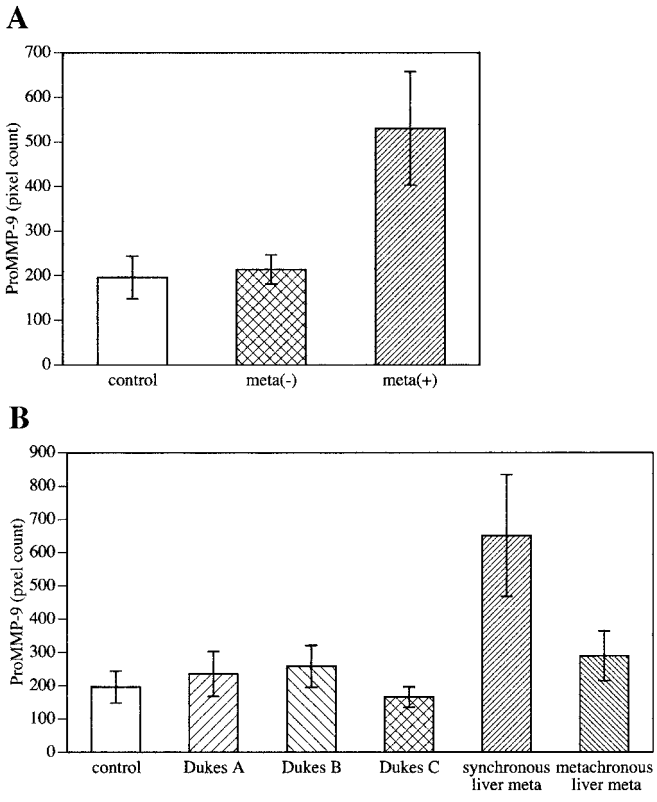

FIG. 4. ProMMP-9 activities in bile. ProMMP-9 activities in bile from control group and colorectal cancer with [meta(+)] and without [meta(−)] liver metastasis groups were measured by gelatin zymography and quantified and are shown in A. In B, the data of proMMP-9 activities in bile from control, Dukes A, B, and C, synchronous and metachronous liver metastasis groups are shown. The data were expressed as means ± SE.

consistent with previous reports (13, 21), in which the detection of only 92-kDa MMP-9 was shown in serum and tissue. Parsons *et al.* also reported that the 82-kDa (active MMP-9) band was rarely detected using zymography (16). Our result that proMMP-9 was significantly higher in metastatic group than non-metastatic one was essentially consistent with other reports that proMMP-9 was higher in metastatic liver tissue of colorectal cancer (15, 20) or in blood of colorectal cancer (21, 23). ProMMP-9 activity in bile of synchronous metastasis was higher than that of metachronous metastasis. This may be explained as follows. Metachronous liver metastasis group had the liver metastasis with curative liver resection. On the contrary, synchronous liver metastasis group had liver metastasis with noncurative liver resection or without liver resection. Therefore, the total mass of tumor of synchronous liver metastasis group was larger than that of metachronous liver metastasis group.

The elevation of MMP-2 and MMP-9 activity in bile may be more easily detected compared to plasma samples because the biliary volume is much smaller than the blood volume (30), and therefore, the MMPs may be concentrated. It is expected that measurement of MMP-2 and MMP-9 activities in bile can predict the recurrence of colorectal cancer at earlier stage compared to measurement of MMP-2 and MMP-9 in blood. To obtain bile sample is not so difficult during surgical operation and there were no complications associated with puncturing gall bladder to obtain bile sample. Measurement of MMP-2 and MMP-9 activities in bile may be more useful and accurate than measurement of those in blood.

In conclusion, we found that activity of MMP-2 and MMP-9 in bile could be assayed using gelatin zymography, and the levels of active MMP-2 and proMMP-9 in bile were significantly higher in liver metastasis of colorectal cancer than in metastasis-free colorectal cancer. The result suggests that activities of active MMP-2 and proMMP-9 in bile may be useful markers for predicting liver metastasis in colorectal cancer.

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