Expression of Matrix Metalloproteinases in Gallbladder Carcinoma and Their Significance in Carcinogenesis

Nese Karadag, MD, Hale Kirimlioglu, MD,* Burak Isik, MD,† Sezai Yilmaz, MD,† and Vedat Kirimlioglu, MD†*

Abstract: The correlation between matrix metalloproteinase (MMP)-2, MMP-9, and MMP-14 expression on the prognostic parameters of gallbladder carcinoma (GBC) and their role in carcinogenesis were evaluated. Carcinomas of the gallbladder (n = 20) and chronic cholecystitis (n = 10) were studied for the expression of MMP-2, MMP-9, and MMP-14 by immunohistochemistry. In all of the cases, metaplastic and dysplastic epithelial alterations, and (in GBC histologic type, grade of differentiation, level of infiltration, perineural and angiolymphatic invasion, liver invasion, and lymph node involvement were noted. MMP-2, MMP-9, MMP-14 were expressed in tumor epithelium in 9 (45%), 20 (100%), and 20 (100%) of the cases, respectively. MMP stromal expression including muscle layer, vascular endothelium, fibroblasts, and lymphoid cells were detected in all cases. MMP-2 was not expressed in normal, metaplastic, and dysplastic epithelia. In contrast, MMP-9 and MMP-14 immunoreactivities were present in antral-type metaplastic areas as moderate (grade 2) and strong in dysplastic epithelia (grade 3). Only in mucinous-type GBC was the expression of the MMPs lower than in the other types. No significant correlation was detected with the grade of differentiation, level of infiltration, perineural and angiolymphatic invasion, liver invasion, or lymph node involvement. These data suggest that MMP-9 and MMP-14 overexpression may have an important role in tumorigenesis. MMP-2, MMP-9, and MMP-14 were expressed in GBC epithelium but also the expression in the stromal component may be essential for the malignant potential of GBC.

Key Words: MMP, gallbladder, cancer, metaplasia, dysplasia

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Primary carcinoma of the gallbladder is characterized by strong invasion, early metastasis, and poor prognosis. Although with the increasingly widespread acceptance of laparoscopic cholecystectomy, the number of cases of incidental gallbladder carcinoma (GBC) has increased, GBC is associated with an overall 5-year survival rate that is less than 5% owing to early invasion. Many studies have demonstrated that proteolytic degradation of extracellular matrix (ECM) components by malignant epithelial cells and induced desmoplastic fibroblasts is a major step in tumor invasion.[1–3] Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent and calcium-dependent enzymes that specifically degrade ECM glycoproteins.[4,5] According to their main substrates, MMPs fall into 4 main subgroups: collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-18), and membrane-bound MMPs (MMP-14 to MMP-17).[6] The proteolytic degradation of ECM components such as type IV collagen, fibronectin, and laminin by MMPs has been connected to alterations in a broad range of cellular events including cell migration, apoptosis, and proliferation. Loss of the tight control of MMP activity in neoplasia is thought to increase destruction of the ECM, neovascularization, tumor spread, and metastases. In several human malignancies, increased expression of MMPs has been reported to have prognostic significance.[6] In carcinomas of the gastric, colorectal, renal cell, ovary, breast, prostate, lung, and pancreatic tissues, the increased MMP expression has been reported as a prognostic significance.[1,6–18] So far, there are few reports on the expression of MMPs in gallbladder. We aimed to examine the expression of MMP-2, MMP-9, and MMP-14 in GBC and to study their significance in GBC carcinogenesis by evaluating the expression in the metaplasia-dysplasia-carcinoma sequence.

MATERIALS AND METHODS

Tissue Specimens

The samples of the GBC (n = 20) and chronic cholecystitis (n = 10) were obtained from cases that had undergone surgical resection and diagnoses were confirmed histopathologically at TOTM Hospital of Inonu University, Department of Pathology from 1999 to 2004. Formalin-fixed, paraffin-embedded material available for immunohistochemistry was selected from patients for whom clinical and follow-up data were present. Clinical information and follow-up data were taken from the Department of General Surgery. Eight men and 12 women with a mean age 62.2 years (range 41 to 72) made

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From the Departments of *Pathology; and † General Surgery, Inonu University, TOTM, Malatya, Turkey.
Reprints: Hale Kirimlioglu, MD, Faculty of Medicine, Department of Pathology, Inonu University, Malatya, Turkey (e-mail: hkirimlioglu@inonu.edu.tr).

up to 20 patients with GBC cases. All of the tissues were examined by 2 independent pathologists (N.K, H.K) using hematoxylin and eosin-stained slides. Tumor type, pathologic grading, invasion depth, angiolymphatic, and perineural tumor invasion were noted. Of these, there were histologically 14 nonotherwise specified adenocarcinomas, 3 colloidal carcinomas, and 3 others (adenosquamous, clear cell and pleomorphic giant cell carcinoma); there were 5 G1 (well differentiated), 7 G2 (moderately differentiated), and 8 G3 (poorly differentiated), according to the criteria established by the World Health Organization for histologic type of tumors of the gallbladder. Clinical and pathologic details are described in Table 1.

Five male and 5 female patients with a mean age of 48.7 years (range 32 to 66) with chronic cholecystitis served as control cases. For each patient metaplastic and dysplastic epithelial changes were noted.

Immunohistochemical Procedure

Formalin-fixed and paraffin-embedded gallbladder specimens were used. Sections from each paraffin block were immunohistochemically stained for MMP-2, MMP-9, and MMP-14, using the avidin-biotin complex method. In brief, endogenous peroxidase activity was abolished by immersing the sections for 8 minutes in absolute methanol containing 3% hydrogen peroxide. Sections were stained with primary monoclonal antibodies against MMP-2 Ab-4 (Clone A-Gel VC2, 72 kDa Collagenase IV, Neomarkers, Westinghouse, USA) and MMP-9 Ab-9 (92 kDa Collagenase IV, Neomarkers, Westinghouse, USA), MMP-14 (MT1-MMP, Neomarkers, Westinghouse, USA). The sections were counterstained in Mayer hematoxylene and mounted. The sections were examined microscopically by 2 pathologists who were blind to the clinicopathologic characteristics. Cytoplasmic expression of the MMPs was defined as positive.

Evaluation of Immunostaining

Two observers scored all sections independently. The intensity of the immunostaining of the tumor epithelium, tumor stroma, and nontumoral areas were graded as negative (0), weak (1), moderate (2), and strong (3). To facilitate interpretation and comparison of heterogeneous tissue staining, whole sections were assigned a score according to the strongest intensity staining on a section, provided that $\geq 10\%$ of the specified cell type (ie, epithelium, stroma) on the section stained. Stained cells under 10% were assigned a score of 0. Differences in given scores were resolved by simultaneous reexamination by both scorers for a consensus.

RESULTS

In normal adult gallbladders, expression of MMP was infrequent. In the chronic cholecystitis and GBC, MMP-2 was not expressed in metaplastic and dysplastic epithelia. In contrast, in antral-type metaplastic areas moderate (grade 2), in dysplastic epithelia, strong

TABLE 1. Clinicopathologic Details and MMP Expression Properties of the GBC Cases

GBC	Age	Sex	Tumor Differentiation	Histologic Type	Invasion Depth	LN/Liver Metastasis	PN Invasion	AL Invasion	MMP-2	MMP-9	MMP-14
GBC1	41	F	G2	Colloidal	PML+	−/−	+	+	−	1+	1+
GBC2	63	F	G3	NOS	PML+	−/+	+	+	3+	3+	3+
GBC3	61	F	G3	NOS	PML+	+/+	+	+	2+	3+	3+
GBC4	68	F	G1	NOS	PML−	−/−	+	−	2+	3+	3+
GBC5	66	F	G2.	NOS	PML+	−/+	+	+	−	3+	3+
GBC6	60	F	G3	PGC	PML+	−/−	+	+	1+	3+	3+
GBC7	68	F	G3	NOS	PML+	−/−	+	+	3+	3+	3+
GBC8	53	F	G1	Colloidal	PML+	−/−	+	−	−	1+	1+
GBC9	60	F	G2	Clear	PML+	−/+	+	+	2+	2+	2+
GBC10	61	F	G3	NOS	PML+	−/−	+	−	−	3+	3+
GBC11	72	F	G2	Adenosq	PML+	+/+	+	+	3+	2+	2+
GBC12	64	F	G2	NOS	PML+	+/+	+	+	−	3+	3+
GBC13	67	M	G2	NOS	PML+	−/−	+	+	−	3+	3+
GBC14	52	M	G3	NOS	PML+	−/+	+	+	−	2+	3+
GBC15	71	M	G1	NOS	PML+	−/−	−	−	−	3+	3+
GBC16	64	M	G3	NOS	PML+	−/−	+	−	2+	3+	2+
GBC17	62	M	G1	Colloidal	PML+	−/−	+	−	−	1+	1+
GBC18	68	M	G1	NOS	PML−	−/−	−	−	3+	2+	3+
GBC19	65	M	G3	NOS	PML+	+/+	+	+	−	3+	3+
GBC20	57	M	G2	NOS	PML+	−/+	+	+	−	3+	3+

Adenosq indicates adenosquamous; AL, angiolymphatic; F, female; G, grade; LN, lymph node; M, male; NOS, nonotherwise specified; PN, perineural; PML, perimuscular layer.

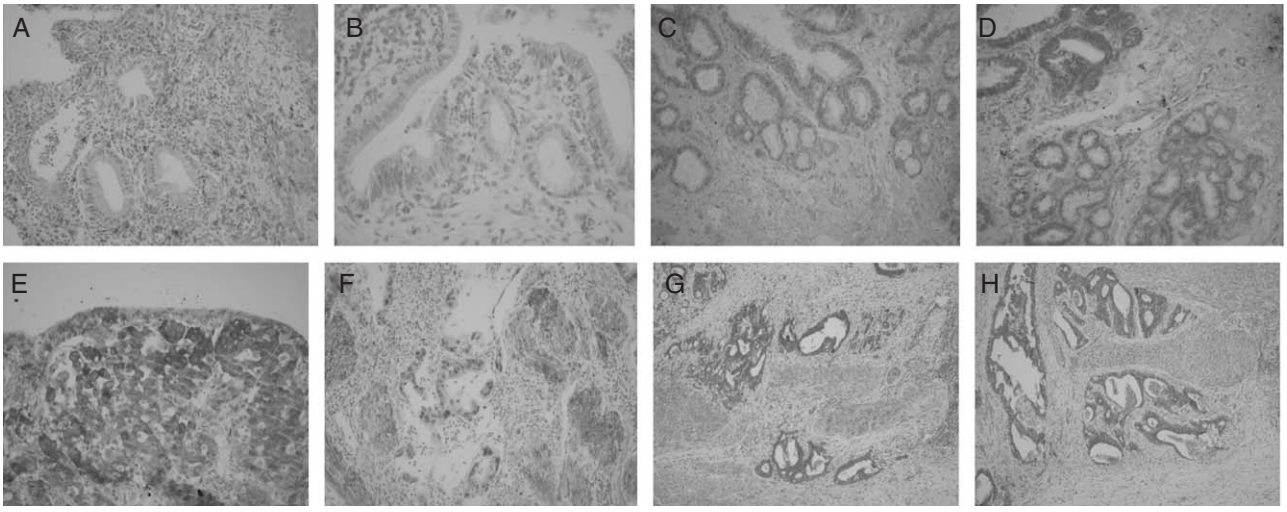

FIGURE 1. Expression of MMP was not seen in normal adult gallbladder epithelium (A). In the metaplastic and dysplastic areas there was no MMP-2 expression (B), but MMP-9 and MMP-14 overexpression were detected (C, D). MMP-2 was positive in 9 of the GBC cases in tumor epithelium. In the others, immunoreactivity was limited to the stromal component (E, F). MMP-9 (G) and MMP-14 (H) immunoreactivities were present in all of the GBC cases both in tumor epithelium and the stromal component.

(grade 3) MMP-9 and MMP-14 immunoreactivities were present (Figs. 1A–D).

Among 20 GBCs, MMP-2, MMP-9, and MMP-14 were expressed in tumor epithelium in 9 (45%), 20 (100%), and 20 (100%) cases, respectively (Figs. 1E–H) (Table 1). The expression of MMP in tumor epithelium was located in the cytoplasm, and showed a diffuse or granular pattern. All MMP immunoreactivities in the GBC stroma were situated in smooth muscle cells of the muscular layer and vascular wall, vascular endothelia, and fibroblasts. Lymphocytes and leukocytes were also stained for MMP-9 and MMP-14. Their expression was stronger in the infiltrative, less-differentiated epithelial cells than the superficial, well-differentiated part of the same tumor. There was no difference in the immunoreactivity according to either the tumor differentiation or stage. In 7 (35%) cases of GBC, there was an extracellular mucinous component and 3 of them were pure colloidal subtype. The expression of MMP-2 was not present in any cases having a mucinous component. In those 7 cases, MMP-9 and MMP-14 immunoreactivities were not present in mucinous component and at pure colloidal carcinomas the staining intensity was only weak (grade 1). Other subtypes included in the study, clear cell, adenosquamous, and pleomorphic giant cell subtypes, expressed similar immunreactivity properties to the ordinary carcinomas.

DISCUSSION

The present study showed that normal gallbladder infrequently and very faintly expressed MMP-2, MMP-9, and MMP-14, when cancer develops the tumor epithelial cells frequently expressed them. This finding suggests that gallbladder epithelia neoexpress or overexpress MMPs after malignant transformation. The present study also showed that when there were alterations of cell differentiation and atypia in the gallbladder epithelium, metaplastic and dysplastic epithelium expressed MMP-9 and MMP-14, suggesting that the expression of these MMPs is related to biliary cell carcinogenesis.

Proteolytic enzymes, collectively known as matrix proteinases, are known to play a central role in the cell migration during cancer invasion and organ development by degrading ECM proteins.[19]

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are associated with the degradation of ECM components such as type IV collagen, fibronectin, and laminin.[20] In cancer, degradation of type IV collagen, a major protein component of basement membranes MMP-2 and MMP-9, has a specific importance in tumor invasion. It has been proposed that cleavage of collagen type IV by MMP-2/MMP-9 leads to enhancement of angiogenesis, by effects on integrins.[21] Membrane-bound MMP-14 has previously been found to be exclusively localized in the tumor cells.[1] It has been suggested that MMP-14 may activate pro-MMP-2.[22]

It has been shown that MMPs in human tumors could be produced not only by cancer cells but also by stromal cells, primarily fibroblasts, and inflammatory cells.[5,23] It has been suggested that these stromal cells produce and secrete MMPs and then bind to cancer cells leading to invasion. This concept is supported by data demonstrating that MMP-2 binds to the tissue inhibitors of metalloproteases-2 (TIMP-2): MT-MMP complex on the cell surface and MMP-9 binds to CD44 and type IV collagen on the surface.[24,25] In our study, in carcinoma stromal cell MMP-2 expression was present even if no epithelial MMP-2 expression was detected. However, in our study, stromal MMP-2, MMP-9, and MMP-14

expressions were not only limited to fibroblasts and lymphoid cells, but also present in smooth muscle cells, vascular endothelial cells, suggesting that these stromal components are also important for MMP production and secretion.

In several human malignancies, there have been reports of increased expression of MMPs with prognostic significances.[1,6–18] In gastric cancer, levels of MMP-2 and MMP-9 have been found to be elevated along with MMP-7 and MMP-14.[8,10,15] The expression of MMP-2 was higher in patients with gastric cancer that had a poor prognosis, although the difference was not significant.[7] Increased expression of MMP-2 and MMP-9 and TIMPs 1 and 2 has also been shown to correlate with a poor prognosis in renal cell carcinoma.[11] Previous studies have suggested that MMP-9 expression correlates with increased metastatic potential in colorectal cancer.[26] MMP-2 and MMP-9 activities have been correlated with malignant potential, like tumor grade and stromal, vascular, and lymphatic invasion, and increased metastatic potential in a number of studies.[17,26–31] Fan et al[9] found a relationship between the stage of the GBC and MMP-2 expression, but it had no correlation with lymph node status and infiltration level. In our study, MMP expression did not correlate with any of these features.

As in the stomach and colon, it is believed that most cases of GBC are preceded by a sequence of metaplasia, dysplasia, and carcinoma in situ. Increased expression of MMP-9 and MMP-14 in gallbladder metaplasia-dysplasia-adenocarcinoma sequence as compared with normal tissue suggest their association with tumorigenesis. In previous studies, the role of MMP on the colon tumorigenesis in the adenoma-carcinoma sequence was studied.[4,32] The level of MT1-MMP expression in adenoma, carcinoma in situ, and invasive colon cancers was found to be progressively high.[4] In another study, MMP-7 was found to be significantly increased in both adenomas and carcinomas in which MMP-2 and MMP-9 were overexpressed only in carcinomas.[32] In our study, when we compare the MMP expression in metaplasia, dysplasia, and in carcinoma, we found that the progressive increase in the immunoreactivities of the MMP-9 and MMP-14, probably play an important role on the gallbladder carcinogenesis.

REFERENCES

1. Bramhall SR, Neoptolemos JP, Stamp GW, et al. Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs) in human pancreatic carcinoma. *J Pathol*. 1997;182:347–355.
2. Hart IR, Goode NT, Wilson RE. Molecular aspects of the metastatic cascade. *Biochim Biophys Acta*. 1989;989:65–84.
3. Liotta LA, Stetler-Stevenson WG. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res*. 1991;51:5054s–5059s.
4. Malhotra S, Newman E, Eisenberg D, et al. Increased membrane type 1 matrix metalloproteinase expression from adenoma to colon cancer: a possible mechanism of neoplastic progression. *Dis Colon Rectum*. 2002;45:537–543.
5. Zucker S, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev*. 2004;23:101–117.
6. Jones LE, Humphreys MJ, Campbell F, et al. Comprehensive analysis of matrix metalloproteinase and tissue inhibitor expression in pancreatic cancer: increased expression of matrix metalloproteinase-7 predicts poor survival. *Clin Cancer Res*. 2004;10:2832–2845.
7. Allgayer H, Babic R, Beyer BC, et al. Prognostic relevance of MMP-2 (72-kD collagenase IV) in gastric cancer. *Oncology*. 1998;55:152–160.
8. David L, Nesland JM, Holm R, et al. Expression of laminin, collagen IV, fibronectin, and type IV collagenase in gastric carcinoma. An immunohistochemical study of 87 patients. *Cancer*. 1994;73:518–527.
9. Fan YZ, Zhang JT, Yang HC, et al. Expression of MMP-2, TIMP-2 protein and the ratio of MMP-2/TIMP-2 in gallbladder carcinoma and their significance. *World J Gastroenterol*. 2002;8:1138–1143.
10. Honda M, Mori M, Ueo H, et al. Matrix metalloproteinase-7 expression in gastric carcinoma. *Gut*. 1996;39:444–448.
11. Kallakury BV, Karikehalli S, Haholu A, et al. Increased expression of matrix metalloproteinases 2 and 9 and tissue inhibitors of metalloproteinases 1 and 2 correlate with poor prognostic variables in renal cell carcinoma. *Clin Cancer Res*. 2001;7:3113–3119.
12. Kawano N, Osawa H, Ito T, et al. Expression of gelatinase A, tissue inhibitor of metalloproteinases-2, matrilysin, and trypsin(ogen) in lung neoplasms: an immunohistochemical study. *Hum Pathol*. 1997;28:613–622.
13. Murashige M, Miyahara M, Shiraishi N, et al. Enhanced expression of tissue inhibitors of metalloproteinases in human colorectal tumors. *Jpn J Clin Oncol*. 1996;26:303–309.
14. Murray GI, Duncan ME, O'Neil P, et al. Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer. *Nat Med*. 1996;2:461–462.
15. Nomura H, Sato H, Seiki M, et al. Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. *Cancer Res*. 1995;55:3263–3266.
16. Stearns M, Stearns ME. Evidence for increased activated metalloproteinase 2 (MMP-2a) expression associated with human prostate cancer progression. *Oncol Res*. 1996;8:69–75.
17. Talvensaari-Mattila A, Paakko P, Hoyhtya M, et al. Matrix metalloproteinase-2 immunoreactive protein: a marker of aggressiveness in breast carcinoma. *Cancer*. 1998;83:1153–1162.
18. Young TN, Rodriguez GC, Rinehart AR, et al. Characterization of gelatinases linked to extracellular matrix invasion in ovarian adenocarcinoma: purification of matrix metalloproteinase 2. *Gynecol Oncol*. 1996;62:89–99.
19. Terada T, Okada Y, Nakanuma Y. Expression of immunoreactive matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in human normal livers and primary liver tumors. *Hepatology*. 1996;23:1341–1344.
20. Sternlicht MD WZ. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol*. 2001;17:463.
21. Xu J, Rodriguez D, Petitclerc E, et al. Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol*. 2001;154:1069–1079.
22. Hernandez-Barrantes S, Toth M, Bernardo MM, et al. Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation. *J Biol Chem*. 2000;275:12080–12089.
23. Basset P, Bellocq JP, Wolf C, et al. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature*. 1990;348:699–704.
24. Olson MW, Toth M, Gervasi DC, et al. High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. *J Biol Chem*. 1998;273:10672–10681.
25. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev*. 2000;14:163–176.

26. van der Stappen JW, Hendriks T, Wobbes T. Correlation between collagenolytic activity and grade of histological differentiation in colorectal tumors. *Int J Cancer*. 1990;45:1071–1078.
27. Bernhard EJ, Muschel RJ, Hughes EN. Mr 92 000 gelatinase release correlates with the metastatic phenotype in transformed rat embryo cells. *Cancer Res*. 1990;50:3872–3877.
28. Liotta LA, Tryggvason K, Garbisa S, et al. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*. 1980;284:67–68.
29. Nakajima M, Welch DR, Belloni PN, et al. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. *Cancer Res*. 1987;47:4869–4876.
30. Wilhelm SM, Collier IE, Marmer BL, et al. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem*. 1989;264:17213–17221.
31. Wirl G, Frick J. Collagenase—a marker enzyme in human bladder cancer? *Urol Res*. 1979;7:103–108.
32. Heslin MJ, Yan J, Johnson MR, et al. Role of matrix metalloproteinases in colorectal carcinogenesis. *Ann Surg*. 2001;233:786–792.