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The expression of matrix metalloproteinases in intrahepatic cholangiocarcinoma, hilar (Klatskin tumor), middle and distal extrahepatic cholangiocarcinoma, gallbladder cancer, and ampullary carcinoma: Role of matrix metalloproteinases in tumor progression and prognosis

İntrahepatik kolanjiokarsinom, hilar (Klatskin tümörü), orta ve distal ekstrahepatik kolanjiokarsinomlar, safra kesesi karsinomları, ampuller karsinomlarda matriks metalloproteinaz ekspresyonu; matriks metalloproteinazların tümör progresyonu ve prognozuna etkisi

Hale KIRIMLIOĞLU[1], İlknur TÜRKMEN[1], Nuray BAŞSÜLLÜ[1], Abuzer DİRİCAN[2], Neşe KARADAĞ[1], Vedat KIRIMLIOĞLU[2]

Departments of [1]Pathology and [2]General Surgery, İnönü University, School of Medicine, Malatya

Background/aims: *Carcinomas of the biliary tree are rare tumors of the gastrointestinal tract, with an increasing incidence in recent years. Biliary neoplasms are classified into intra- and extrahepatic cholangiocarcinoma (Klatskin tumor, middle and distal extrahepatic tumors), gallbladder adenocarcinoma, and ampullary carcinoma. We aimed to determine the expression profile of matrix metalloproteinase (MMP)-2, MMP-9 and MMP-14 in the biliary neoplasms classified according to their localization and the relation with the prognosis.* ***Methods:*** *Ten gallbladder adenocarcinoma, 8 distal bile duct carcinomas (distal cholangiocarcinoma), 8 Klatskin tumors, 8 intrahepatic cholangiocarcinomas and 10 ampullary carcinomas were included in the study. The immunohistochemical expression of MMP-2, MMP-9 and MMP-14 was detected in the nontumoral, metaplastic, dysplastic and tumoral epithelia. The tumor differentiation, angiolymphatic and perineural invasion of the tumor, and presence of lymph node and distant metastasis were determined. Survey of the patients was noted from the patient follow-up data.* ***Results:*** *The nontumoral epithelia of the gallbladder, intrahepatic ducts, and Klatskin tumor did not express MMP-2. MMP-2 expression was detected in the distal part of the biliary ducts, in 75% (6/18) of cases and in the nontumoral epithelia of the ampullary region in 50% (5/10) of cases. The metaplastic and dysplastic epithelia were positively stained in all of the gallbladder adenocarcinoma, distal cholangiocarcinoma and ampullary tumors. In the intrahepatic cholangiocarcinoma, the hepatocytes were positively stained but the infiltrative tumors were spared. Klatskin tumors were also not stained with MMP-2. The gallbladder adenocarcinoma, distal cholangiocarcinoma and ampullary carcinomas expressed MMP-2 in 30%, 37% and 40% of the cases, respectively. MMP-9 and MMP-14 were expressed in normal, metaplastic, and dysplastic epithelium and tumoral cells in all of the cases of the groups. Expressions of MMPs were higher in subjects with neural invasion, but there was no correlation between MMP expression and tumor differentiation or angiolymphatic invasion.* ***Conclusions:*** *When tumors of the biliary system are divided as intrahepatic and extrahepatic cholangiocarcinomas, MMP-2 expression was present in the extrahepatic cholangiocarcinomas including gallbladder carcinomas. Like the intrahepatic cholangiocarcinoma, Klatskin tumors also did not express MMP-2. This can be related with its characteristic growth pattern. MMP-9 and MMP-14 were present in metaplasia, dysplasia carcinoma sequence in all of the bile tract tumors, suggesting that MMPs play an important role in carcinogenesis. The higher expression of the MMPs with neural invasion suggests the significant role of those tumors in the invasion activity.*

Key words: Matrix metalloproteinases, biliary neoplasms, immunohistochemistry

Amaç: *Safra yolu karsinomları son yıllarda insidansı artan gastrointestinal sistemin nadir tümörleridir. Bilier neoplaziler intra ve ekstrahepatik kolanjiokarsinomlar (Klatskin tümörü, orta ve distal ekstrahepatik tümörler), safra kesesi karsinomları, ve ampüller karsinomlar olarak sınıflandırılır. Çalışmamızda lokalizasyonlarına gore sınıflandırılan bilier neoplazilerde matriks metalloproteinaz (MMP)- 2, MMP-9 ve MMP-14 ekspresyonlarını ve prognozla ilişkilerini değerlendirmeyi amaçladık.* ***Yöntem:*** *On safra kesesi karsinomu, 8 distal safra yolu karsinomu, 8 Klatskin tümörü, 8 intrahepatik kolanjiokarsinom, 10 ampuller karsinom çalışmaya dahil edildi. Nontumoral, metaplastik, displastik ve tümoral epitelde immunohistokimyasal olarak MMP-2, MMP-9 ve MMP-14 ekspresyonu incelendi. Tümör diferansiyasyonu, anjiolenfatik ve perinöral tumor invazyonları, lenf nodu ve uzak metastazlar kaydedildi. Hasta izlemlerinde hasta yaşam süreleri belirlendi.* ***Bulgular:*** *Safra kesesi, intrahepatik safra duktuslarında ve Klatskin tümörlerinde nontumoral epitelde MMP-2 ekspresyonu yoktu. MMP-2 ekspresyonu safra yollarının distal bölümlerinde %75 (6/8) ve ampuller bölgede nontumoral epitelde %50 (5/10) olguda saptandı. Tüm safra kesesi karsinomu, distal kolanjiokarsinom ve ampüller tümörlerde metaplastik ve displastik epitel MMP-2 ile pozitif boyandı. Intrahepatik kolanjiokarsinom'da hepatositler MMP-2 ile boyanırken infiltratif tümör hücreleri negatifdi. Klatskin tümörlerinde de MMP-2 ekspresyonu görülmedi. Safra kesesi karsinomları, distal kolanjiokarsinom ve ampüller karsinomlarda sırası ile %30, %37, %40 MMP-2 ekspresyou saptandı. MMP-9 ve MMP-14 tüm gruplarda olguların tamamında normal, metaplastik, displastik epitel ve tümöral hücrelerde saptandı. MMP'lerin ekspresyonları nöral invazyon olan olgularda daha yüksekti ancak MMP ekspresyonu ile tumor dferansiyasyonu, anjiolenfatik invazyon arasında korelasyon saptanmadı.* ***Sonuç:*** *Biliyer sistem tümörlerini intrahepatik ve ekstrahepatik kolanjiokarsinom'lar olarak ayırdığımızda, safra kesesi de dahil olmak üzere ekstrahepatik kolanjiokarsinomda MMP-2 ekspresyonu saptandı. Intrahepatik kolanjiokarsinomlar gibi Klatskin tümörlerinde de MMP-2 ekspresyonu görülmedi. Bunun büyüme karakteri ile ilişkili olduğu düşünülebilir. MMP-9 ve MMP-14 metaplazi, displazi ve karsinom alanlarında ekspresyonlarının olması MMP'lerin karsinogenezde rol oynayabileceğini düşündürmektedir. Nöral invazyon olan olgularda MMP'lerin artmış ekspresyonu tümörlerin invaziv aktivitesinde etkin olabileceğini düşündürmektedir.*

Anahtar kelimeler: Matriks metalloproteinazlar, biliyer neoplaziler, immunohistokimya

Address for correspondence: Vedat KIRIMLIOĞLU
İnönü Üniversitesi Tıp Fakültesi
Turgut Özal Tıp Merkezi Genel Cerrahi A.D. Malatya, Turkey
Phone: + 90 422 341 02 29
E-mail: vkirimlioglu@inonu.edu.tr

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INTRODUCTION

Biliary neoplasm continues to have an unfavorable prognosis, despite an improved rate of curative resection and the development of surgical techniques. Carcinomas of the biliary tree originate from the gallbladder, intra- and extrahepatic bile ducts and are classified as intra- and extrahepatic cholangiocarcinoma (CC) (Klatskin tumor, middle and distal extrahepatic tumors), gallbladder adenocarcinoma, and ampullary carcinoma. Most cases are found at an advanced stage, accompanied by invasion to the peripheral tissues, metastases to the lymph nodes and distant organs, and peritoneal dissemination.

Matrix metalloproteinases (MMPs) play an important role in cancer cell invasion by degrading extracellular matrix (ECM) proteins (1). In the present study, we aimed to examine the immunohistochemical expression of the MMPs in the biliary neoplasms and differences between those subgroups determined according to the localization. We also evaluated the expression of MMPs in cancer, dysplastic lesions, metaplastic epithelia and normal (non-atypical) epithelia (glandulous ducts) in patients with biliary neoplasia. The tumor differentiation, angiolymphatic and perineural invasion, and the stage of the tumor were determined and the relationship between MMP expression and those clinicopathologic factors was examined.

MATERIALS AND METHODS

Tissue samples from surgical resections of 44 cases were included in the study. One or two blocks that contained the tumoral and nontumoral peripheral tissues were used for the study. The immunohistochemical expression of MMP-2, MMP-9 and MMP-14 was evaluated in patients with gallbladder adenocarcinoma (n=10), distal CCs (n=8), Klatskin tumors (n=8), intrahepatic CCs (n=8), and ampullary carcinomas (n=10). The nontumoral, peritumoral metaplastic, dysplastic and tumoral epithelia were examined. The tumor differentiation, angiolymphatic and perineural invasion of the tumor and the presence of lymph node and distant metastasis were noted. The survey of the patients was obtained from the clinical follow-up data.

Immunohistochemistry

Formalin-fixed and paraffin-embedded liver specimens were cut into 4 Ìm sections, deparaffinized in xylene, and rehydrated in phosphate-buffered saline. The sections were stained with immunohistochemistry based on standard streptavidin-biotin peroxidase method (Labvision, Anti-polyvalant, HRP, Westinghouse, USA). Endogenous peroxidase activity was suppressed by a solution of 3% hydrogen peroxide for 8 min. Sections were stained with primary monoclonal antibodies against MMP-2 Ab-4 (Clone A-Gel VC2, 72kDa Collagenase IV, Neomarkers, Westinghouse, USA), MMP-9 Ab-9 (92kDa Collagenase IV, Neomarkers, Westinghouse, USA), and MMP-14/MT1-MMP Ab-1 (Neomarkers, Westinghouse, USA). The sections were counterstained in Mayer's hematoxylin and mounted. Cytoplasmic expression of the MMPs was defined as positive.

Grading of immunostained slides

Two pathologists blinded to the source of the tissue viewed each slide independently. Tumor on the entire slide was assessed, and staining for each MMP was graded for both intensity and distribution. A score of 0 to 3+ was assigned for each, according to the strongest staining intensity in more than 20% of the cells.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 13.0 program. Kruskal Wallis analysis of variance and Bonferroni test were used for comparison of variables among the studied groups. A value of $p<0.05$ was considered significant.

RESULTS

The nontumoral epithelia of the gallbladder, intrahepatic ducts and the cases with Klatskin tumor did not express MMP-2. MMP-2 expression was detected in 75% (6/8) of the distal part of the biliary ducts, and in 50% (5/10) of the nontumoral epithelia of the ampullary carcinoma. The metaplastic and dysplastic epithelia were positively stained in all of the gallbladder, distal CC and ampullary tumors. The gallbladder carcinoma, distal CC and ampullary carcinomas expressed MMP-2 in 30% (3/10), 37% (3/8) and 40% (4/10) of the cases, respectively. In the intrahepatic CC, the hepatocytes were positively stained but the infiltrative tumors were spared. Klatskin tumors were also not stained with MMP-2. MMP-9 and MMP-14 were expressed in normal, metaplastic, and dysplastic epithelium and tumoral cells in all of the cases of the groups. Expressions of MMP-2 and MMP-9 were higher in subjects with neural invasion ($p=0.009$ and $p=0.001$, respectively), but there was no correlation between MMP expression and tu-

mor differentiation, angiolymphatic invasion, lymph node status or distant metastasis at the time of operation, according to the survey of the patients ($p>0.05$). The MMP expressions of the cases are demonstrated in Table 1 and Figure 1.

DISCUSSION

Biliary neoplasms originating from the biliary tree and the gallbladder have different clinical presentations according to their localization. Although recent data have shown an increasing incidence of intrahepatic CC, adenocarcinoma of the gallbladder and the extrahepatic bile ducts (cholangiocarcinoma) are the most common biliary neoplasms (2). It is uncertain whether suggested location-related differences in pathogenesis influence clinical behavior (3-5). Although a common molecular pathogenesis has been suggested (6), clinical data suggest that gallbladder adenocarcinoma and intrahepatic CC are distinct entities with different clinical behavior (7, 8). After resection, recurrent gallbladder adenocarcinoma is much more likely than recurrent intrahepatic CC to involve a distant site. Gallbladder adenocarcinoma is also

Table 1. The relation between MMP expression intensity and tumor type, differentiation, angiolymphatic and perineural invasion, and lymph node and distant metastasis: survey of the patients as prognostic indicators

	Tumor	MMP-2				MMP-9				MMP-14				Tumor	AL	PN	LN	Distant	Survey
Case	type	NT	M	D	T	NT	M	D	T	NT	M	D	T	diff	inv	inv	met	met	
1	Klatskin	0	0	0	0	1	2	1	1	2	2	2	2	well	-	-	-	-	6y
2	Klatskin	0	0	0	0	2	2	2	2	1	2	1	1	mod	+	-	-	+	Ex, 3m
3	Klatskin	0	0	0	0	2	2	2	2	2	2	2	2	mod	-	-	-	-	4y, 2m
4	Klatskin	0	0	0	0	3	3	3	3	2	2	2	2	poor	-	+	-	-	Ex, 2y
5	Klatskin	0	0	0	0	1	1	1	1	2	2	2	2	well	-	-	-	-	Ex, 7 y
6	Klatskin	0	0	0	0	2	2	2	2	2	2	3	2	mod	-	-	-	-	9y
7	Klatskin	0	0	0	0	3	3	3	3	3	3	3	3	well	+	+	+	+	Ex, 1m
8	Klatskin	0	0	0	0	3	3	3	3	3	3	3	3	well	+	+	-	+	Ex, 0m
9	IHCC	0	0	0	0	3	3	3	3	2	3	2	2	mod	-	+	-	-	Ex, 6m
10	IHCC	0	0	0	0	3	3	3	3	3	3	3	3	well	-	+	-	-	8y
11	IHCC	0	0	0	0	3	3	3	3	2	2	2	2	well	+	+	-	+	Ex, 3y
12	IHCC	0	0	0	0	2	2	2	2	3	3	3	3	poor	+	-	+	+	Ex, 1m
13	IHCC	0	0	0	0	2	2	2	2	3	3	3	3	mod	-	+	-	-	3y, 2m
14	IHCC	0	0	0	0	3	3	3	3	3	3	3	3	mod	-	+	-	+	5y, 3m
15	IHCC	0	0	0	0	3	3	3	3	3	3	3	3	poor	-	+	-	-	Ex, 2m
16	IHCC	0	0	0	0	3	3	3	3	2	2	2	2	well	-	+	-	+	Ex, 4y
17	GBAC	0	2	2	2	3	3	3	3	3	3	3	3	mod	-	+	-	+	Ex, 1m
18	GBAC	0	2	2	0	3	3	3	3	2	2	2	2	well	-	+	-	-	3y
19	GBAC	0	1	1	0	3	3	3	3	3	3	3	3	well	+	+	+	+	Ex, 1m
20	GBAC	0	3	3	3	2	3	2	2	3	3	3	3	poor	+	+	-	+	Ex, 3m
21	GBAC	0	2	2	0	2	2	2	2	3	3	3	3	mod	-	-	-	-	7y, 3m
22	GBAC	0	1	1	0	3	3	3	3	3	3	3	3	mod	+	+	-	+	Ex, 4m
23	GBAC	0	2	2	0	3	3	3	3	3	3	3	3	poor	-	+	-	-	Ex, 1y
24	GBAC	0	2	2	2	3	3	3	3	2	2	2	2	poor	-	+	+	+	Ex, 3m
25	GBAC	0	3	3	0	2	2	2	2	2	2	2	2	well	-	-	-	+	Ex, 4m
26	GBAC	0	3	3	0	3	3	3	3	2	2	2	2	mod	-	+	+	+	Ex, 0m
27	DBCC	2	3	3	0	1	1	2	1	3	3	3	3	well	+	-	+	+	2y, 3m
28	DBCC	2	2	2	2	3	3	3	3	1	1	1	1	well	+	+	-	-	8y, 2m
29	DBCC	2	2	3	0	3	3	3	3	2	2	2	2	mod	-	-	-	-	4y, 7m
30	DBCC	2	2	2	2	3	3	3	3	2	2	2	2	mod	+	+	+	+	Ex, 2m
31	DBCC	2	2	2	0	1	1	1	1	3	3	3	3	mod	+	-	-	-	6y
32	DBCC	0	0	0	0	3	3	3	3	3	3	3	3	poor	-	+	-	-	Ex,1m
33	DBCC	3	3	3	3	3	3	3	3	3	3	3	3	poor	+	+	-	+	Ex,1m
34	DBCC	2	2	2	0	2	2	2	2	3	3	3	3	mod	-	-	-	-	Ex, 1y
35	AC	2	2	2	2	3	3	3	3	2	2	2	2	well	-	+	-	-	Ex, 0m
36	AC	3	2	2	3	3	3	3	3	2	2	2	2	well	-	+	-	-	6y, 1m
37	AC	0	2	2	0	3	3	3	3	2	2	2	2	poor	-	-	+	+	Ex, 4m
38	AC	0	3	3	0	2	2	2	2	3	3	3	3	mod	-	+	-	-	Ex, 3y
39	AC	0	2	2	0	2	2	2	2	1	1	1	1	mod	+	-	+	+	Ex, 2m
40	AC	2	2	2	2	3	3	3	3	2	2	2	2	poor	+	+	+	+	Ex, 2m
41	AC	2	2	2	2	3	3	3	3	2	2	2	2	poor	-	+	+	+	Ex, 0m
42	AC	0	2	2	0	3	3	3	3	3	3	3	3	well	-	+	-	-	8y, 6m
43	AC	3	3	3	0	2	2	2	2	2	2	2	2	mod	-	-	-	-	2y, 4m
44	AC	0	2	1	0	2	2	2	2	3	3	3	3	well	-	+	-	-	4y, 5m

IHCC: Intrahepatic cholangiocarcinoma. GB: Gallbladder carcinoma. DBCC: Distal bile duct carcinoma. AC: Ampullary carcinoma. NT: Nontumoral. M: Metaplasia. D: Dysplasia. T: Tumor. AL inv: Angiolymphatic invasion. PN inv: Perineural invasion. LN met: Lymph node metastasis.

associated with a much shorter time to recurrence and a shorter survival period after recurrence (9). Because of a common characteristic feature of biliary neoplasms - the early metastasis and strong invasion - it is very important to estimate the malignant degree and invasion tendency in order to guide clinical diagnosis and treatment of the carcinoma. Breakage of the ECM and basement

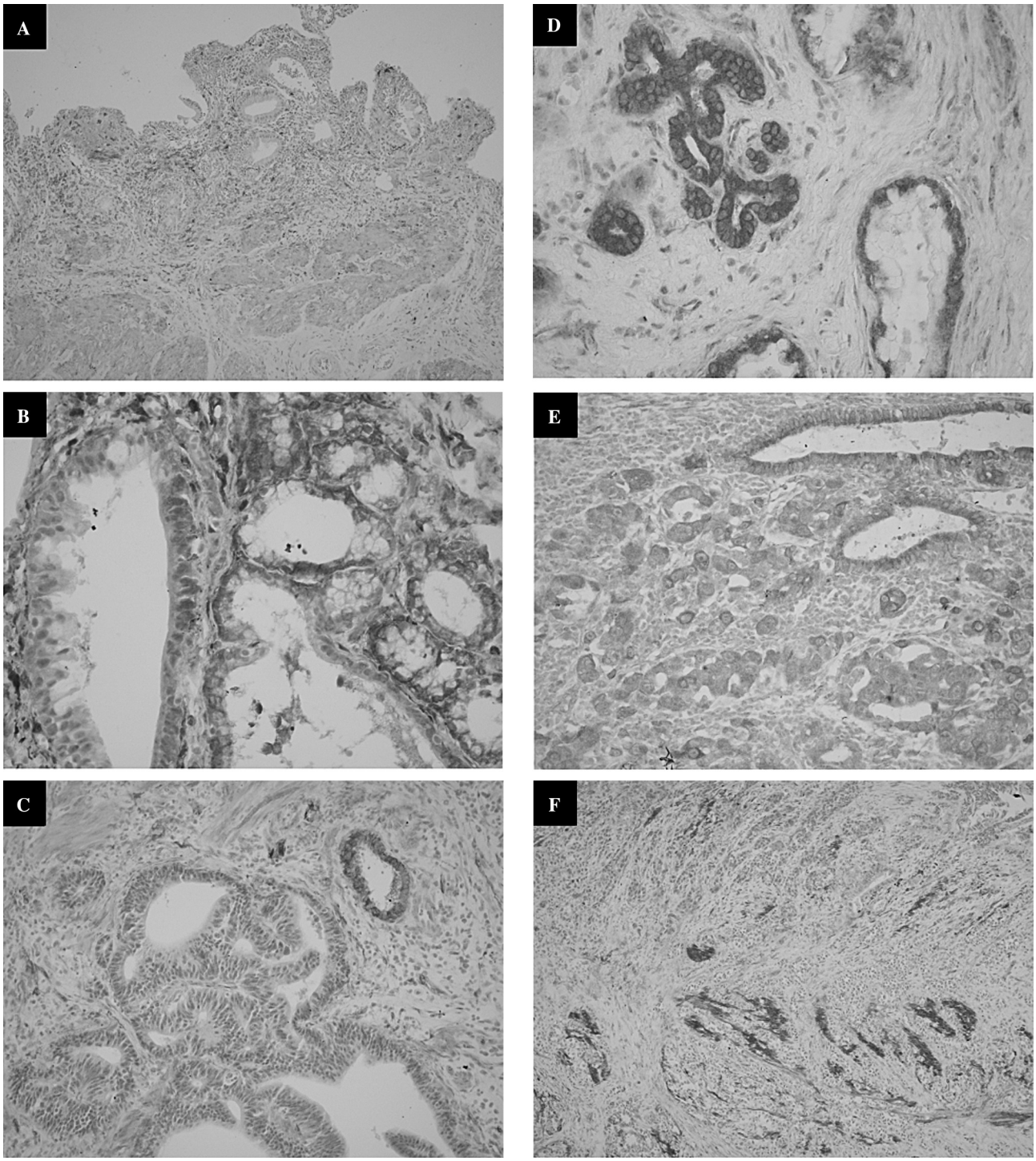

Figure 1. MMP-2 expression was not detected in gallbladder epithelia, although the smooth muscle cells were positively stained as internal control **(A)** in Case 27, distal CC metaplastic and dysplastic epithelia exhibit strong MMP-2 staining but the carcinoma did not **(B)** similarly, in a case of ampullary carcinoma (Case 38), dysplastic epithelia versus non-stained carcinoma area with MMP-2 was demonstrated **(C)**. MMP-9 **(D)** and MMP-14 (MT-1MMP) **(E)** expression was detected in the nontumoral and tumoral areas in all biliary neoplasms in our study. MMP-2 expression was negative in 7/10 of the gallbladder adenocarcinoma **(F)**.

membrane is necessary in the process of tumor invasion. MMPs are a family of ECM-modifying enzymes associated with tumor progression and metastasis by degradation of all components of the ECM. The gelatinases MMP-2 and MMP-9 have been studied extensively because of their specific ability to degrade collagen IV, the major structural component of the basement membrane. The transmembrane collagenase MT1-MMP (membrane-type 1 MMP), also known as MMP-14, has a critical function both in normal development and in cancer progression, and is subject to extensive controls at the post-translational level that affect proteinase activity (10). MMP-2, MT1-MMP, and tissue inhibitor of MMP (TIMP)-2 form a trimolecular complex on the cell membrane and decompose ECMs, including collagen. TIMP-1 and TIMP-2 increase the proliferative activity of the normal and tumor cells of humans, bovine and rabbit. MMP-related factors thus augment the infiltration and proliferation of neoplastic cells by decomposing the ECM and promoting mitosis (11). MMP-9 also had a key role in the regulation of angiogenesis in a model of carcinogenesis (12). Previous studies about MMP expression in gallbladder carcinoma and bile duct carcinoma demonstrated the presence of MMP in carcinoma and a relation with the tumor progression (13-17). In our study, we aimed to define the differential expression of MMP-2, MMP-9 and MMP-14 based on the anatomic site of origin and to determine whether such differences have a role in tumor invasion. As the metaplasia-dysplasia-carcinoma sequence has been proposed to explain the development of bile duct carcinoma and gallbladder carcinoma, we also evaluated the expression of the MMPs in the benign, metaplastic and dysplastic lesions. MMP-9 and MMP-14 expression were detected in nontumoral epithelia, metaplastic and dysplastic lesions and tumor cells in all groups in 100% of cases, exceeding the levels of expression in nontumoral and dysplastic epithelia in the study of Chiba et al. (11). However, our results were similar in CC areas with this study. When the relation between tumor differentiation, angiolymphatic invasion and MMP expression intensity was studied, we could not find any statistical significance.

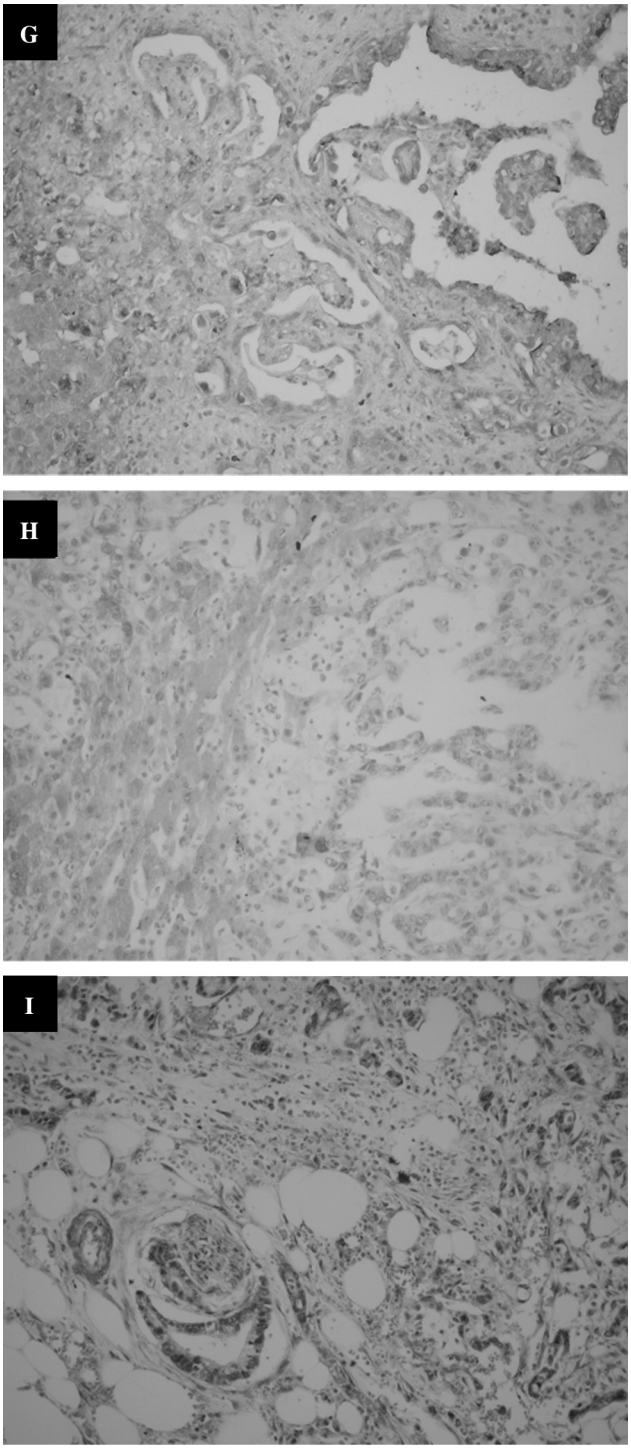

Figure 1. MMP-9 positivity was present in intrahepatic CC cases **(G)**. MMP-2 expression was seen in hepatocytes but not in the CC areas in intrahepatic CC cases **(H)**. MMP-2 positivity in a distal CC case (Case 33): perineural tumor invasion was noted in the case **(I)**.

On the other hand, the nontumoral epithelia of the gallbladder, intrahepatic bile ducts, proximal bile duct and bile duct epithelia in the distal bile duct and periampullary region demonstrated different MMP-2 expression. The benign, metaplastic and dysplastic epithelia did not stain with MMP-2 in the intrahepatic bile ducts and proximal bile duct. In the same cases, tumor cells also did not express MMP-2. In the distal bile duct and ampullary

carcinoma cases, the nontumoral epithelia, including the benign, metaplastic and dysplastic epithelia, expressed MMP-2 in 75% of the cases; when the carcinoma developed, some of the cases lost their MMP-2 expression in the tumoral cells. In the study of the Chiba et al. (11), MMP-2 was present in 4/18 cases, but the localization of the CC was not mentioned. This ratio is similar with our result in CC localized in the distal part of the biliary tree in our study. In other studies that examined MMP-2 and MMP-9, higher ratios of MMP-2 and lower ratios of MMP-9 were found (16-18). In our study, in the gallbladder carcinoma, similar to our distal bile duct and ampullary carcinoma cases, MMP-2 expression loss was detected in the tumoral cells, except in the gallbladder benign epithelia, where there was no MMP-2 expression, although MMP-2 expression was determined in some of the cases in the metaplastic and dysplastic epithelia. This observation is of particular interest given the association between localization and MMP expression.

In a recent study, survival differences according to tumor localization of the biliary neoplasms have been suggested to be related to differences in p27 expression. A major finding in this regard was the differential expression of the cycle-dependent kinase inhibitor p27, which was more common in proximal biliary tumors (intrahepatic and hilar) compared to that of gallbladder and distal tumors. Biliary neoplasms exhibit differential expression of cell cycle–regulatory proteins according to tumor site of origin and morphology. The expression profiles of the gallbladder and hilar were similar, suggesting the possibility of overlap in pathogenesis. In our study, there was no difference between groups in MMP-9 and MMP-14 in the nontumoral and tumoral tissue. However, the MMP-2 expression was similar in hilar carcinoma, gallbladder and intrahepatic CC in the benign epithelia. When the carcinoma develops, the gallbladder carcinoma exhibits a MMP-2 expression profile more similar to the distal and ampullary carcinomas. This was correlated with the p27 expression in the previous study.

Although linked anatomically and histopathologically, it is unclear whether biliary neoplasms from different sites share common pathogenetic features. MMP-2 expression profiles in our results correlated with the previous studies that suggested location-related differences in pathogenesis (4). MMP-9 and MMP-14 were present in metaplasia and dysplasia carcinoma sequence in all of the bile tract tumors, suggesting that MMPs played an important role in carcinogenesis. The higher expression of the MMPs with neural invasion suggested the significant role of MMPs in the invasion activity in those tumors.

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