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Investigation of TGF-β1 and MMP-2 expression in pancreatic ductal adenocarcinoma

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Objective: TGF-β1 is a multifunctional cytokine involved in the regulation of cell growth and differentiation. Although it inhibits the growth of epithelial cells, the growth inhibitory effect of TGF-β1 is lost in many types of tumors. Matrix metalloproteinase-2 (MMP-2) is central to matrix degradation, which is a key event in tumor invasion and metastasis.

Study Design: In an attempt to find molecular prognostic markers for pancreatic carcinoma, we studied immunohistochemical expression of TGF-β1 and MMP-2 proteins in 25 ductal pancreatic adenocarcinoma. The results were compared with grade, vascular space and perineural invasion, desmoplasia and lymph node metastasis.

Results: TGF-β1 and MMP-2 immunoreactivity were seen in 13(53%) and 19(76%) cases, respectively. TGF-β1 reactivity was seen in higher-grade tumors. Statistically significant correlation of TGF-β1 (+) immunoreactivity with grade, desmoplasia and vascular invasion was found. Although MMP-2 reactivity was seen in most of the cases, significant correlation was not found between MMP-2 and clinicopathologic parameters.

Conclusion: The results implicated that, TGF-β1 reactivity might play a role in the progression of ductal adenocarcinoma, however MMP-2 expression might not be related with the progression. Since conflicting results have been reported on this subject, TGF-β1 and MMP-2 deserve further study.

Keywords: TGF- β1, MMP-2, Pancreas, Carcinoma

Introduction

Molecular mechanisms regulating the pancreatic carcinoma have been studied in recent years. A variety of genetic alterations have been reported including mutations in tumor suppressor genes, overexpression of growth factors and disturbances in growth inhibitory pathways.[1–8] Transforming growth factor-β1 (TGF-β1) is involved mainly in the regulation of cell growth and differentiation. It arrests the cell cycle, and inhibits growth of the cells. One of the other functions of TGF-β1 is decreasing the production of enzymes that degrade extracellular matrix including collagenase.[1,2,9–12] The role of TGF-β1 in gastric, hepatocellular, breast and pancreas carcinomas has been demonstrated in a variety of molecular studies. Mutations are found in most of these cancers.[1–3,10–13]

Intense stromal reaction is a characteristic feature of pancreatic ductal adenocarcinoma. Its aggressive local invasive features suggest that matrix metalloproteinases (MMPs) may play role in the invasive phenotype of pancreatic carcinomas. The ability of malignant epithelial cells and induced desmoplastic fibroblasts to degrade extracellular

matrix (ECM) is considered as an important step in the process of invasion and metastasis.[5,6,14–16] In this regard their contribution to the invasion and metastasis has been investigated in many carcinomas such as colorectal, liver, breast and pancreas.[5–7,14,16,17–19]

In the present study, the immunohistochemical expression of TGF-β1and MMP-2, was examined in pancreatic ductal adenocarcinoma cases. Their association with clinicopathologic parameters was studied. Also correlation between TGF-β1 and MMP-2 was evaluated. Relation of TGF-β1 and MMP-2 with prognostic clinicopathologic parameters of ductal pancreas adenocarcinoma was investigated.

Material and methods

Case selection

From January 1999 to April 2004, 25 cases of pancreatic ductal adenocarcinoma were selected from the files of the Cumhuriyet University Department of Pathology. Ampullary carcinoma cases were not included the study. Of the 25 cases, 23 were male and 2 were female. H&E stained slides from the resected specimens were reviewed microscopically to determine histological subtypes, grade, perineural and vessel invasion, desmoplasia and metastasis to lymph nodes.

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens were cut 4 μm sections. Slides were deparaffinized in xylene and rehydrated in a series of ethanol. Non-enzymatic antigen retrieval was performed as previously described (20). The immunohistochemical stains were performed manually using the avidin-biotin peroxidase complex technique (Labvision, cat: TA-125-UB). Incubating slides with 3% hydrogen peroxide for 10 minutes blocked endogenous peroxidase and nonspesific background staining. After washing with phosphate-buffered saline (PBS) for 5 minutes, slides were blocked with normal serum for 20 minutes, followed by incubation with the specific primary antibody for 60 minutes. The used primary antibodies were mouse monoclonal antibody TGF-β1 (CD105, Neomarkers, MS-1290-R7), and mouse monoclonal antibody MMP-2 (Neomarkers, Ab-7, RB-1537-P). After rinsing with PBS for 5 minutes, sections were incubated with a biotinylated secondary antibody (Labvision, TP-125-BN) for 20 minutes. After washing with PBS, slides were then incubated with avidin-biotin complex (Labvision, TS-125-HR) for 30 minutes. AEC chromogen (Labvision, TA-060-HA) was applied. All slides were lightly counterstained with Mayer hematoxylin for 30 seconds before dehydration and mounting.

Positive controls and nonimmune protein negative controls were used for each section with each antibody. As positive controls, anterior pituitary was used for TGF-β1 and breast carcinoma was used for MMP-2.

Evaluation

Slides were examined under low power to identify regions containing tumor cells. All tumors were graded as well, moderately and poorly differentiated ductal adenocarcinoma. Well, moderately and poorly differentiated cases were described as Grade I, Grade II and Grade III respectively. Presence or absence of desmoplasia, vessel invasion, perineural invasion and the metastasis to peripancreatic lymph nodes were assessed.

Immunohistochemical staining was considered positive for TGF-β1 and MMP-2 only when cytoplasmic immunoreactivity was more than 30% of the tumor cells.[4] Positive staining was also graded on intensity. Tumors given score 0 were classified as negative, those given a score of 1 were classified as weakly positive; a score of 2 was considered moderately positive and a score of 3 was classified as strongly positive. The chi-square test of significance was used to test for a relationship between dichotomous variables, and the Fisher exact test was performed when expected frequencies were small. Values were considered statistically significant if $p< 0.05$.

Results

Age of the patients ranged between 37 and 80 years (mean 60.92). Twenty-three were men and 2 were women. All tumors were adenocarcinoma of the ductal type. Histological and clinicopathological results are shown in Table 1. Twelve (48%) were Grade I, 13 (52%) were Grade II. There were not any Grade III

Table 1. Clinicopathologic and histopathologic characteristics of the pancreatic adenocarcinoma cases.

Case (no/sex)	Age (y)	Grade	TGF-β1	MMP-2	Vessel invasion	Perineural invasion	Desmoplasia	Lenf node metastasis
1/M	50	I	Negative	Positive	No	No	Yes	Yes
2/M	68	I	Positive	Positive	No	Yes	Yes	No
3/M	69	II	Positive	Negative	Yes	Yes	Yes	Yes
4/M	60	II	Positive	Positive	No	No	Yes	Yes
5/M	60	II	Positive	Positive	Yes	Yes	Yes	No
6/M	70	I	Negative	Negative	No	No	No	No
7/M	64	I	Negative	Positive	No	No	No	Yes
8/M	75	I	Positive	Positive	No	No	No	No
9/M	66	II	Positive	Positive	Yes	Yes	Yes	No
10/M	70	II	Positive	Positive	No	Yes	Yes	Yes
11/M	80	I	Negative	Positive	No	No	No	No
12/M	38	I	Negative	Positive	No	Yes	No	No
13/M	37	I	Negative	Negative	No	No	Yes	No
14/M	54	II	Positive	Negative	Yes	Yes	Yes	Yes
15/M	65	II	Negative	Positive	No	Yes	Yes	Yes
16/M	53	II	Positive	Positive	Yes	Yes	Yes	No
17/M	79	I	Negative	Positive	No	No	Yes	Yes
18/F	70	II	Negative	Negative	No	Yes	No	Yes
19/M	56	II	Positive	Positive	Yes	Yes	Yes	No
20/M	60	I	Positive	Positive	No	No	No	Yes
21/M	45	I	Negative	Negative	No	Yes	Yes	Yes
22/F	50	I	Negative	Positive	No	No	No	No
23/M	60	II	Negative	Positive	No	No	No	No
24/M	70	II	Positive	Positive	No	No	Yes	Yes
25/M	54	II	Positive	Positive	No	No	Yes	Yes

cases. In 6 (24%) cases, all of which were Grade II, vascular invasion was seen. Desmoplasia was seen in 16 (%64) cases. Five (20%) of them were Grade I and 11 (44%) of them were Grade II. Perineural invasion was seen in 12 (48%) cases, 3(12%) of which were grade I, and 9 (36%) of them were Grade II. Lymph node metastasis was described in 13 (52%) cases. Five (20%) of them were grade I and 8 (32%) of them were Grade II. After surgical resections, patients referred to an oncology hospital to have chemotherapy or radiation therapy. Thus we were not able to learn their follow up and survivals.

Immunohistochemical Analysis

Immunohistochemical results are shown in Table 2. Immunohistochemically, cytoplasmic TGF-β1 immunoreactivity was detected in 13 (52%) cases. Three (12%) of them were Grade I, 10 (40%) were Grade II ductal adenocarcinoma. Staining intensity was weak, moderate, and strong in 5 (20%), 4 (16%), and 4 (16%) cases, respectively (Figure 1,2). In contrast, areas of connective tissue and fibroblasts were devoid of immunostaining. MMP-2 immunoreactivity was seen in 19 (76%) cases. Of these positive cases, 8 (32%) of them were Grade I and 11 (44%) Grade II. Staining intensity was weak, moderate and strong in 2 (8%), 7 (28%) and 10 (40%) cases, respectively (Figure 3). Stroma, between tumoral cells, has shown weak to moderately positive staining with the MMP2 antibody. Four (16%) cases have a negative staining pattern with both TGF-β1 and MMP-2 (Figure 4). Eleven (48%) cases were positively stained with both antibodies. Two (8%) of the TGF-β1 positive tumors were not immunoreactive

Table 2. Immunoreactivity of TGF-β1 and MMP-2 in Pancreatic Ductal Adenocarcinoma

	Positive cases		Intensity of staining		
	Grade-I	Grade-II	Weak	Moderate	Strong
TGF-β1	3 (12)	10 (40)	5 (20)	4 (16)	4 (16)
MMP-2	8 (32)	11 (44)	2 (8)	7 (28)	10 (40)

*Data are given as number (percentage)

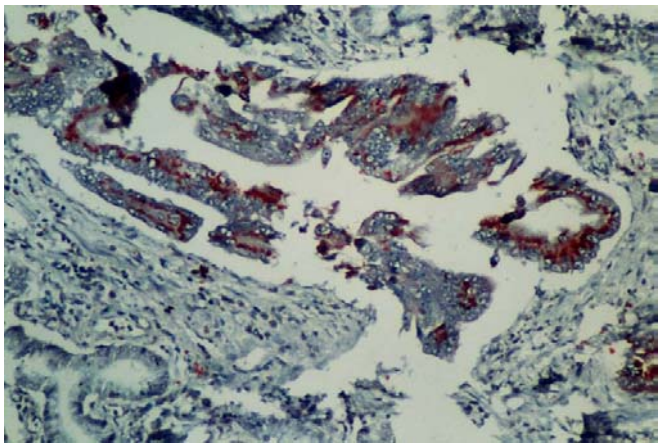

Figure 1. TGF-β1 positive cells in Grade I ductal adenocarcinoma, adjacent stroma was TGF-β1 negative (IHCx25)

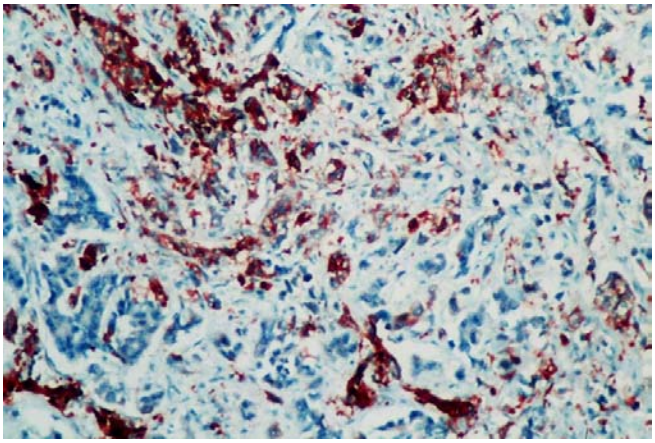

Figure 2. TGF-β1 positive Grade II ductal adenocarcinoma showing strong cytoplasmic immunoreactivity (IHCx25).

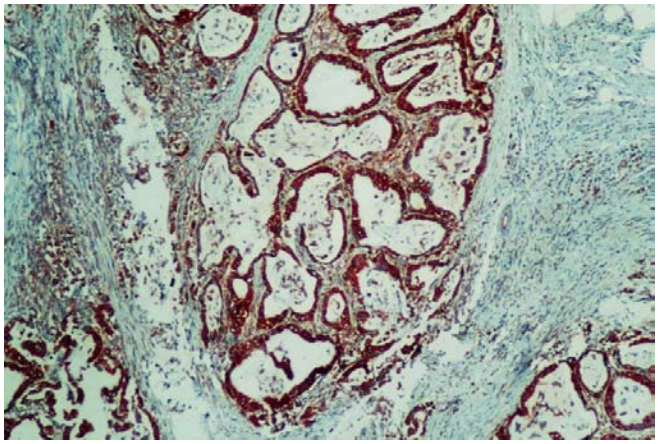

Figure 3. Strong, cytoplasmic MMP-2 positive immunoreactive cells in Grade I pancreatic ductal adenocarcinoma (IHCx25)

with MMP2. Eight (36%) of MMP-2 positive tumors were not immunoreactive with TGF-β1. No significant

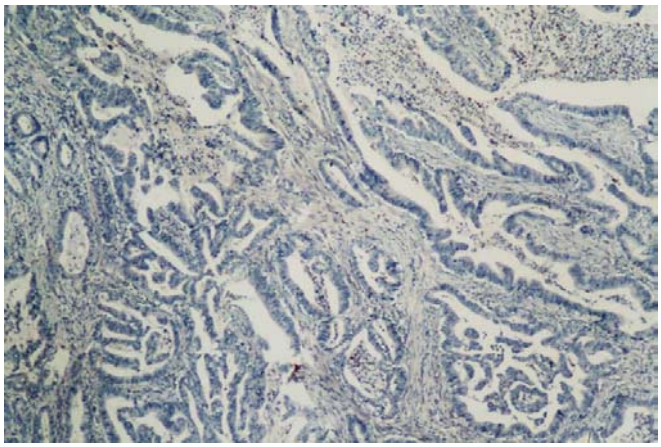

Figure 4. MMP-2 negative Grade I pancreatic ductal adenocarcinoma, prominent perineural invasion (IHCx25)

correlation was found between TGF-β1 and MMP-2 immunoreactivity ($p>0.05$).

Correlation with Clinicopathologic Characteristics

TGF-β1 immunoreactivity was mostly identified in Grade II tumors. Its correlation with Grade I tumors was statistically significant ($p<0.05$). TGF-β1 expression correlated also with vascular invasion and desmoplasia. And these correlations were statistically significant ($p<0.05$). However; TGF-β1 correlated neither with perineural invasion nor lymph node metastasis ($p>0.05$). Although most (76%) cases have shown positive immunoreactivity with MMP-2, they correlated neither with grades nor the other histological and clinicopathologic parameters ($p>0.05$).

Discussion

Since TGF-β1 inhibits cell proliferation, the disruption of its signaling pathways may play important role in the genesis of cancers. After mutations, tumor cells become resistant to growth inhibition by TGF-β1. Subsequently, the tumor cells and the stromal cells within the tumor often increase their production of TGF-β1 and tumor cells become more invasive and metastasize to distant organs.[1–3,10–13] According to studies about gastric, hepatocellular, breast and thyroid carcinoma, over expression of TGF-β1 could lead to enhance tumor cell proliferation.[11,12,13,21] Reports about TGF-β1 and its role on the pancreatic cancers have conflicting results. Although some studies have shown that TGF-β1 (+) pancreatic ductal adenocarcinoma

have a better prognosis and longer survival period or prognosis, the others have indicated that TGF-β1 (-) ductal adenocarcinoma of the pancreas have a significantly longer survival.[1–4] The reason for these contradictory results is unknown. Three different possibilities have been considered, one of which is that growth inhibitory effect of TGF- β1 is not disrupted in some of ductal pancreatic adenocarcinoma, second is that other growth factors, tumor suppressor genes or cell cycle related genes contribute the progression of disease and the last one is that the different ethical or racial background of patients may exert influences on the effect of TGF-β1.[4] In this study, we found TGF-β1 positive expression in Grade II adenocarcinoma more than in Grade I adenocarcinoma. The growth inhibition of TGF-β1 might be disturbed with increasing grades.

TGF-β1 is thought to be a potent regulator of the production and deposition of ECM.[1,3,9,11,12] It has been shown that TGF-β1 induces desmoplasia in experimental model of pancreatic adenocarcinoma.[15] Although the correlation between expression of TGF-β1 and survival of pancreatic ductal adenocarcinoma has been shown in some reports, they couldn't find the same correlation between clinicopathologic and prognostic parameters such as grade, stage, lymph node status and distant metastasis.[2,4] In this study, we found statistically significant correlation with TGF-β1 positive expression and grade, desmoplasia and vessel invasion. Although we do not have survival data of the patients, this correlation could be considered as TGF-β1's important role in the progression of pancreatic carcinomas. In contrast, we could not find any association with TGF-β1 expression and lymph node metastasis and perineural invasion. There are several and multistep process in pancreatic carcinoma. As in the other carcinomas, several factors could be responsible in the genesis of pancreatic carcinomas. And the conflicting results about TGF-β1 may be attributed to different mechanisms of pancreatic carcinogenesis such as, cell cycle related genes, oncogenes, tumor suppressor genes or other growth factors. Multiple steps, in which a lot of genetic alterations accumulate, are required for carcinogenesis. Changes with these genes might contribute to progression of pancreatic carcinomas and could be responsible for lymph node metastasis and perineural invasion.

MMPs in neoplasia have recently been attracting attention since destruction of ECM is essential for cancer invasion. MMP-2, also called gelatinase A, degrades type IV collagen and other matrix proteins and is highly correlated with the invasive metastatic phenotype.[5,6,14–18] MMP activity has been correlated with malignant potential in many kinds of tumors such as pancreas, liver and colorectal.[5,17,19] Data of the reports of ductal adenocarcinoma of pancreas support the hypothesis that the expression of MMP2 is important and it could contribute to the aggressive behavior of pancreatic carcinoma. There are contradictory results about its correlation with the clinicopathologic parameters.[5–7,14,16,17] Although some of the reports show no correlation between MMP-2 expression and clinicopathologic parameters such as grade, vessel and perineural invasion and lymph node metastasis, some of them show correlation with grade.[5,7,18] Since the conflicting results have been recorded it is not clear which MMP plays a role in progression of human pancreatic carcinoma. In the current study, we also did not find any correlation with pathologic parameters and MMP-2 expression. This result may be attributed to the small size of the sample of this study.

MMP expressions are regulated by a variety growth factors and oncogenes. TGF is one of the potent regulators of ECM and it decreases the production of enzymes including collagenase and heparinase.[9,16] Reviewing the literature, we could not find any report comparing TGF-β1 and MMP-2 in pancreas carcinoma. In this study we also evaluated the correlation between TGF-β1 and MMP-2. And there was not any correlation. Only grade I and grade II tumors were studied, since we did not have grade III tumors. Thus, this result might be attributed to their being lower grade tumors. Also in another point of view, MMP-2 expressions might not be regulated by TGF-β1 in pancreatic ductal adenocarcinoma. However, to prove this idea as well as their relation with higher grade tumors, they should be studied in large series included all of the grades as well as normal pancreatic tissue.

In conclusion, according to our results TGF-β1`s growth inhibition feature might be disturbed with increasing grades in ductal adenocarcinoma of the pancreas. However to prove this comment Grade III pancreas carcinoma cases also should be investigated. Besides, MMP-2 immunoreactivity has no importance in pancreatic carcinomas. Since conflicting results have been reported, large series including all grades and survival data of patients should be studied to conclude the prognostic value of TGF-β1 and MMP-2.

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