The expression of the carboxyl ester lipase gene in pancreas and pancreatic adenocarcinomas

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Abstract. Since pancreatic cancer is an aggressive and often incurable malignancy, we investigated if the carboxyl ester lipase gene (CEL) is specifically expressed in pancreatic tissues and its promoter can be used for a specific suicide gene approach. Twenty-five tumor samples, 24 samples of normal pancreatic tissue and control tissues from other organs were examined by radioactive *in situ* hybridization (ISH) to localize CEL mRNA. Two carcinoma samples and 6 permanent cell lines were examined by reverse transcriptase-polymerase chain reaction (RT-PCR). By ISH, we verified a strong CEL gene expression in acinar cells of the normal pancreas. A minor expression was noted in a single sample of acinar cell carcinoma and adenocarcinomas did not show any expression. By RT-PCR, no specific expression in both tested adenocarcinomas was observed. In summary, these results show that, contrary to notable expression of carboxyl ester lipase in acinar cells of normal pancreatic tissue, this lipase is not significantly active in pancreatic adenocarcinomas and thus not an apt genetic marker for diagnostic or therapeutic approaches.

Introduction

Applying a gene therapeutic approach using, for example, a suicide gene driven by a heterodimeric transcription factor (1) to carcinomas of the pancreas requires a specific promoter for pancreatic tissue. Such a promoter would regulate the expression of one component of the transcription factor responsible for the activity of a suicide gene, such as cytosine deaminase. The second promoter, driving the expression of the other part of the transcription machinery, should preferably be tumor-specific or, if possible, markedly active in adenocarcinomas of the pancreas. At present, the cellular origin of ductal carcinoma of the pancreas has not been clearly determined (2,3): despite the similar morphology of ductal adenocarcinomas of the pancreas to ductal structures, an origin from a stem cell (4) or a transdifferentiation process from other cell types (2,5,6) seems possible. Several studies demonstrated an expression of acinar enzymes in adenocarcinomas of the pancreas (4,7,8). Additionally, in the AR42J cell line derived from a rat tumor and in the human carcinoma cell line MIA PaCa-2 the expression of carboxyl ester lipase (CEL) (in the latter an isoform) was demonstrated (9,10). CEL is an important lipolytic enzyme with a broad substrate specifity (E.C. 3.1.1.1, EC 3.1.1.2, E.C. 3.1.1.3, E.C. 3.1.1.5, E.C. 3.1.1.13, E.C. 3.1.1.23, E.C. 3.1.1.32; ECx.x.x.x.: four-digit enzyme commission number) (11). However, to our knowledge, no comprehensive study exists on the expression of CEL in human adenocarcinomas of the pancreas. Therefore, we investigated the pattern of CEL gene expression in human adenocarcinomas and normal tissues of the pancreas as well as in control tissues. This was done by applying reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridization (ISH), thus accounting for the extensive induction of desmoplastic stroma in this type of carcinoma. In the case of marked transcription in pancreatic adenocarcinoma the CEL promoter could be used within a gene therapeutic approach as outlined above.

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Materials and methods

Cell lines and tissues. Normal tissue from pancreases (n=24), 3 cases of pancreatitis, 25 adenocarcinomas of the pancreas, 5 other malignomas and several control tissues were examined. Tissue samples were obtained from archived paraffin blocks that had been prepared for diagnostic reasons by histologic standard methods after fixation in 4% paraformaldehyde overnight or from dissected tissue immediately frozen after pathological examination following surgery and routine

biopsy. In the latter case, patients gave their informed consent prior to the study (Ethical vote #148/2001, Ethics Commission of the Medical Faculty, Eberhard-Karls University Tübingen). This study used the following cell lines: BxPC-3 (ATCC# CRL-1687), Capan-2 (DKFZ, ATCC# HTB-80), DAN-G (DKFZ, TZB 610006), IMIM-PC1 (12), IMIM-PC2 (12), PANC-1 (ATCC# CRL-1469), HT-29 (ATCC# HTB-38), MKN-45 (Machado, Porto, Portugal), GLC-4 (SCLC), and K-562 (ATCC# CCL-243). Adherend cells were maintained in an optimised medium (Life Technologies GmbH, Karlsruhe, Germany and Sigma Chemical Co., St. Louis, USA) for each cell type at 37˚C, 6.9% CO_2 concentration and 95% air moisture. At confluence, RNA was isolated.

RNA and cDNA preparation. Cells were harvested and RNA was isolated using peqGOLD RNAPure™ (Peqlab, Erlangen, Germany) according to the supplier's instructions. Following denaturation, reverse transcription was performed on total cellular RNA at 37˚C for 2 h using a $(dT)_{15}$ primer.

PCR. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis using an intron-spanning primer pair specific for carboxyl ester lipase (161 bp) was performed. GAPDH served as an internal control. Information on sequence was obtained from NCBI (GenBank: http:// www.ncbi.nlm.nih.gov/; accession numbers for CEL M94579 (13) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) AY340484 (14). For CEL, the forward primer had the sequence 5'-GTC TGC TGG AGG TGC CAG C-3' and the reverse primer had the sequence 5'-GGC AAC CCA CCT TCT CAG C-3'. They spanned two introns to allow for detection of DNA contamination. For GAPDH, the forward primer had the sequence 5'-GGG CTC TCC AGA ACA TCA TC-3' and the reverse primer had the sequence 5'-GAA GGC CAT GCC AGT GAG-3'. All primers were obtained from MWG-Biotech AG, Ebersberg, Germany. The PCR was performed in a total volume of 25 μl hydrous solution containing 2.5 μl 10X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 0.8 μM (each) primers and 2.5 U Taq DNA polymerase. Denaturation was performed at 95˚C for 10 sec, annealing at 69˚C for 30 sec and extension at 72˚C for 1 min. Forty cycles were run. Amplified complementary DNA fragments (CEL 161, GAPDH 98 base pairs) were detected by electrophoresis in a 0.7% agarose gel and ethidium bromide staining. The identity of the isolated PCR product was verified by sequencing obtaining 100% conformity with sequence data. For each sample, two independent runs were performed.

In situ hybridization. *In situ* hybridization was performed on 5 μm paraffin or 10 μm frozen sections mounted on SuperFrostPlus slides® Langenbrinck, Emmendingen, Germany). The latter were fixed in 4% paraformaldehyde for 10 min. To obtain hybridization probes, a PCR product obtained using the CEL primers listed above was ligated into the phagemid pBK-CMV (Stratagene, Amsterdam, The Netherlands). After linearization, antisense CEL riboprobes or sense control riboprobes were generated by *in vitro* transcription using ^{35}S-rUTP (ICN Biomedicals GmbH, Eschwege, Germany). Thick tissue sections (5 μm) were dewaxed and hybridized basically as described (15,16). The hybridization mixture contained either the ^{35}S-labeled RNA antisense or sense control CEL probe (500 ng/ml) in 10 mM Tris-HCl, pH 7.4/50% (vol/vol) deionized formamide/600 mM NaCl/ 1 mM EDTA/0.02% polyvinylpyrrolidone/0.02% Ficoll/ 0.05% bovine serum albumin/10% dextrane sulfate/ 10 mM dithiothreitol/denatured sonicated salmon sperm DNA at 200 μg/ml/rabbit liver tRNA at 100 μg/ml. Hybridization with RNA probes proceeded at 42˚C for 18 h. Slides were then washed as described (15,16) followed by 1 h at 55˚C in 2X standard saline citrate. Non-hybridized single-stranded RNA probes were digested by RNase A (20 μg/ml) in 10 mM Tris-HCl, pH 8.0/0.5 M NaCl for 30 min at 37˚C. Tissue slide preparations were autoradiographed (16) for 5 weeks and stained with hematoxylin/eosin. Photographs were taken with the Axioplan 2 imaging microscope and Axiovision 3.0 software (Zeiss, Oberkochen, Germany). For each sample, generally two independent runs were performed.

Results

Hybridization results using the antisense RNA probes displayed variable levels of CEL transcription in different samples. All 24 samples of normal pancreas showed a strong signal in their acinar cell part. As expected, the mRNA exhibited a cytoplasmic distribution pattern. Fig. 1a-d illustrates a typical hybridization pattern. Three inflammatory tissue samples showed a considerably reduced level of transcription (data not shown). Ductal cells or islet cells did not show hybridization signals that exceeded background. As expected, *in situ* hybridization with the sense RNA probes revealed no specific signals.

Different tissues with reportedly low CEL expression were tested as additional controls using the CEL riboprobe (Fig. 2). The expression pattern is summarized in Table I. None of the 25 examined adenocarcinomas of the pancreas showed significant transcription of CEL. Fig. 1e-f illustrates such a typical result. Histology and tumor grading had no influence on the gene's activity (Table II). In other malignomas including one adenocarcinoma of ductus choledochus and one neuroendocrine carcinoma of the pancreas no or very low levels of CEL mRNA were detected. In a single case of acinar cell carcinoma of the pancreas only a very weak signal was observed (Table III).

In summary, ISH signals of CEL in 25 adenocarcinomas of the pancreas were comparable to negative control tissue and significantly below levels of transcription in normal pancreatic tissue.

By amplification and detection of the expected RT-PCR product of 161 bp all of 6 examined permanent pancreatic cell lines (BxPC-3, Capan-2, DAN-G, IMIM-PC-1, IMIM-PC-2, PANC-1) were positive. Transcription in BxPC-3 was highly significant but less pronounced in the remaining pancreatic cell lines. In a stomach carcinoma cell line (MKN-45) and a colon carcinoma cell line (HT-29) the transcription was also detectable. Cell lines derived from myeloid leukaemia (K-562) or small cell lung cancer (GLC-4) showed hardly detectable or absent CEL signals (Fig. 3a, not all results are depicted).

RT-PCR results confirmed the *in situ* hybridization results obtained from various tissues: while in two pancreatic adeno-

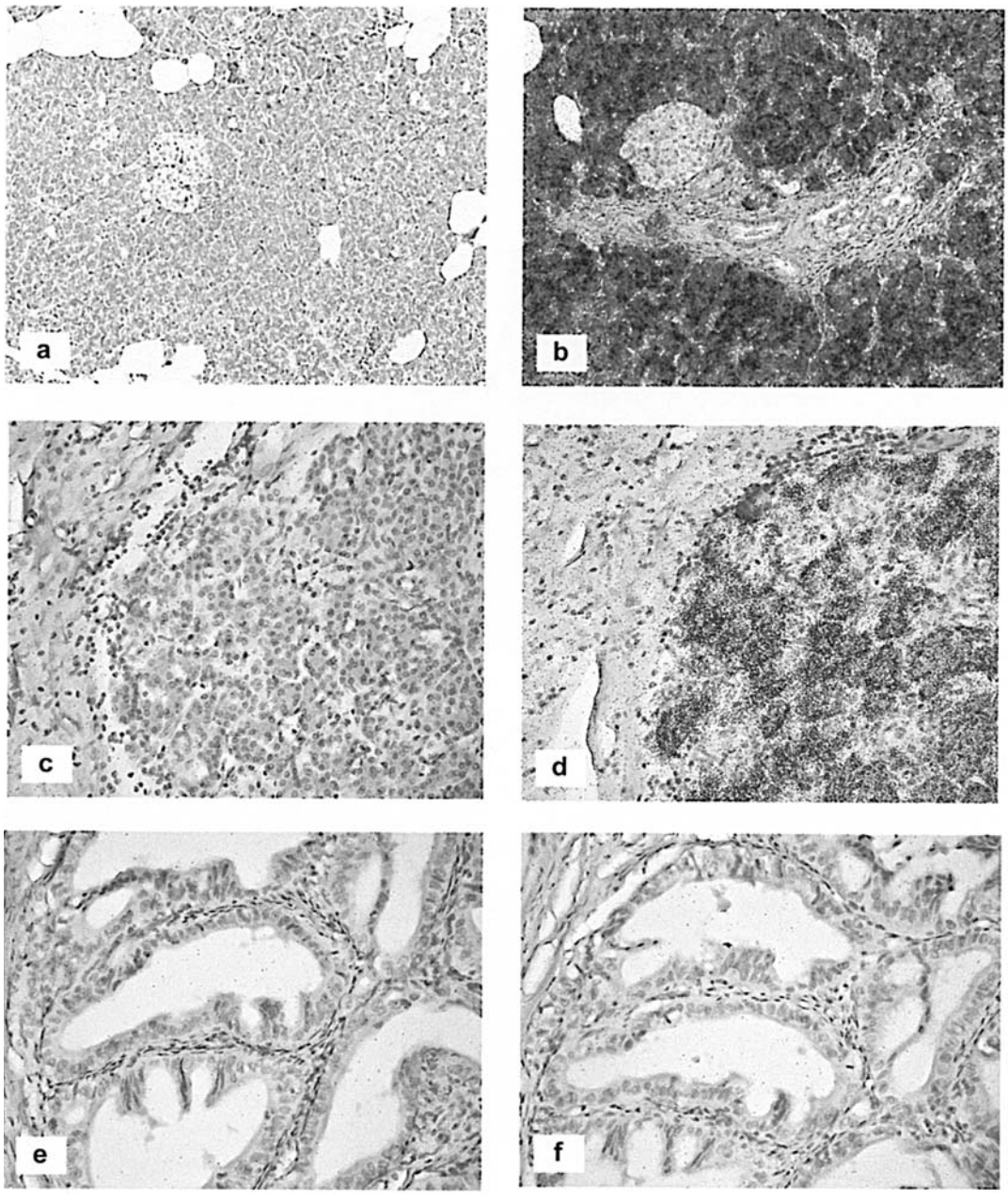

Figure 1. Expression of CEL in normal pancreas and ductal adenocarcinoma of the pancreas. Radioactive *in situ* hybridization in normal pancreas with the sense (a and c), and the antisense riboprobe, islets of Langerhans and ducts are not marked (b and d). (a and b) Magnification x100; (c and d) Magnification x200. Sense (e) or antisense riboprobe (f) in a higher differentiated part of a ductal adenocarcinoma of the pancreas. (e and f) Magnification x200.

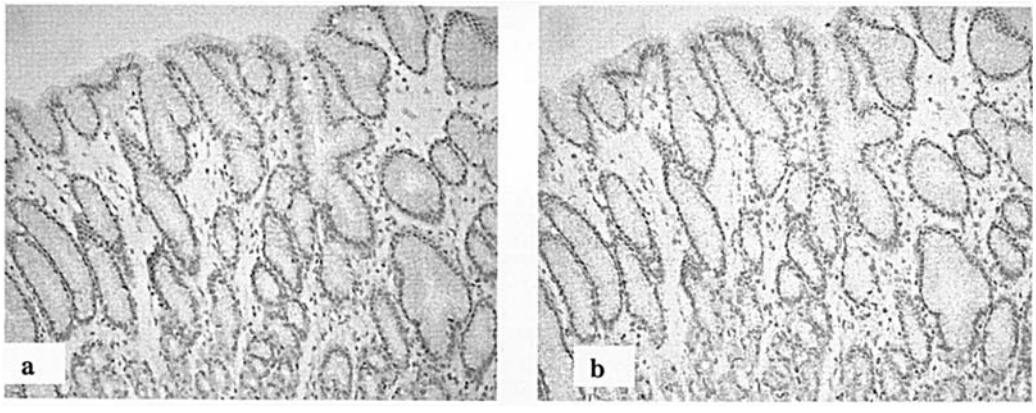

Figure 2. Expression of CEL in the stomach. Radioactive *in situ* hybridization with the sense (a) or antisense probe (b) shows no relevant difference in signals in the foveolar epithelium. Magnification x200.

Table I. *In situ* hybridization in control tissues with the antisense CEL riboprobe.

Tissue	No.	Fraction of positive case and signal intensity
Stomach epithelium	5	1/5 (+)
Bronchial epithelium	2	1/2 (+)
Goblet cells of conjunctiva	2	1/2 -/+
Duodenum	8	1/8 (+)
Ductus hepaticus communis	1	0/1 -
Lymph nodes	1	0/1 -
Striated muscle	1	0/1 -
Lymphocytes	4	0/4 -
Granulocytes	2	0/2 -
Erythrocytes	several	0 -
Colon epithelium	1	1/1 (+)
Endothelium	Aorta: 1	0/1 -
Liver epithelium	3	1/3 (+)
Macrophages	2	1/2 -/(+)
Glandula submandibularis, pleomorphic adenoma	1	0/1 -
Spleen	2	0/2 -
Glandula parotis	1	0/1 -
Lymphoid cells from bone marrow, CLL (B-type)	1	0/1 -
Pancreas (acinar cells)	24	24/24 ++/+++

Signal intensity: -, none; (+), very weak; +, weak; ++, middle; +++, strong. Generally, at least two slides per sample were examined.

Table II. *In situ* hybridization with the antisense CEL riboprobe in pancreatic adenocarcinomas.

No.	Localisation	Histology	Grading	CEL *in situ* signal
1	Liver metastasis	Mucinous	G3	0
2	Head	Ductal	G2/3	0
3	Tail	Ductal	G3	0
4	Head	Mucinous	G2/3	0
5	Head	Ductular-papillar	G2	0
6	Unknown	Ductal	G2	0
7	Tail	Ductal	G3	0
8	Head	Ductal	G2/3	0
9	Unknown	Ductal	G2	0-(+)
10	Tail	Mucinous	G2	0-(+)
11	Head	Mucinous	G3	0
12	Corpus/tail	Ductal	G2/3	0
13	Infiltrated duodenum	Ductal	G2/3	0-(+)
14	Unknown	Ductal	G2	0
15	Head	Mucinous	G2	0
16	Unknown	Ductal	G2/3	0-(+)
17	Unknown	Ductal	G3	0
18	Head	Ductal	G2	0
19	Head	Ductal	G2	0
20	Head	Ductal	G2	0
21	Unknown	Ductal	G2	0-(+)
22	Head	Ductal	G2/3	0
23	Unknown	Ductal	G2	0
24	Head	Mucinous	G2	0
25	Head	Ductal	G2	0

Signal intensity: 0, none; (+), very weak.

Table III. *In situ* hybridization with the CEL riboprobe in further carcinomas.

No.	Localisation	Histology	Grading	CEL *in situ* signal
26	dc	Ductal	G2	0
27	pt	Acinar cell	G2	(+)
28	gs	Acinar cell	G3	0
29	gp	Acinar cell	G1	0
30	ph	Neuroendocrine	G2/3	0-(+)

dc, ductus choledochus; pt, pancreatic tail; gs, Gl. submandibularis; gp, Glandula parotis; ph, pancreatic head. Signal intensity: 0, none; (+), very weak; +, weak.

carcinomas and tissue samples from stomach, spleen, colon, and duodenum the CEL mRNA was essentially absent, normal pancreas revealed strong PCR signals (Fig. 3b).

In summary, all 25 examined adenocarcinomas of the pancreas failed to demonstrate a significant transcription of the CEL gene. ISH and RT-PCR revealed comparable results for the same carcinoma samples. Results in control cell lines equal the control tissues results and indicate a notably reduced CEL expression in gastrointestinal tissue as compared to acinar cells in the pancreas with their highly active CEL gene.

Discussion

Acinar enzymes are expressed in adenocarcinomas of the pancreas (4,7,8) and pancreatic cell lines (9,10). Therefore, pancreatic adenocarcinoma-specific CEL expression is feasible but has not been verified. In normal pancreas, CEL is mainly produced in acinar cells (17). Ductal adenocarcinomas that account for approximately 85% of pancreatic tumors resemble ductal structures (18) which might derive from ductal cell types (4). Yet, the cell type that undergoes the neoplastic transformation has not been established (2,3). Similarly, an origin from a stem cell (4) or a transdifferentiation process from other cell types (2,5,6) might be possible.

We used semiquantitative RT-PCR and ISH to examine CEL transcription. Both methods are complementary: PCR is very sensitive, while ISH allows the localization of mRNA in context with histological findings. Thus, transcription could be assessed at the level of specific cell types. Tumor cell lines are easily accessible and well defined, but initial differentiation can be lost during *in vitro* culture. Tissue samples are more difficult to collect but they represent the native situation more exactly. Archived paraffin blocks are often reduced in quality as fixation processes cause degeneration of macro-molecules. As a consequence we used a combination of methods and samples.

Both methods revealed that while in acinar cells of normal pancreatic tissue CEL is expressed at high levels, islets of Langerhans and ducts within the same tissue remain negative.

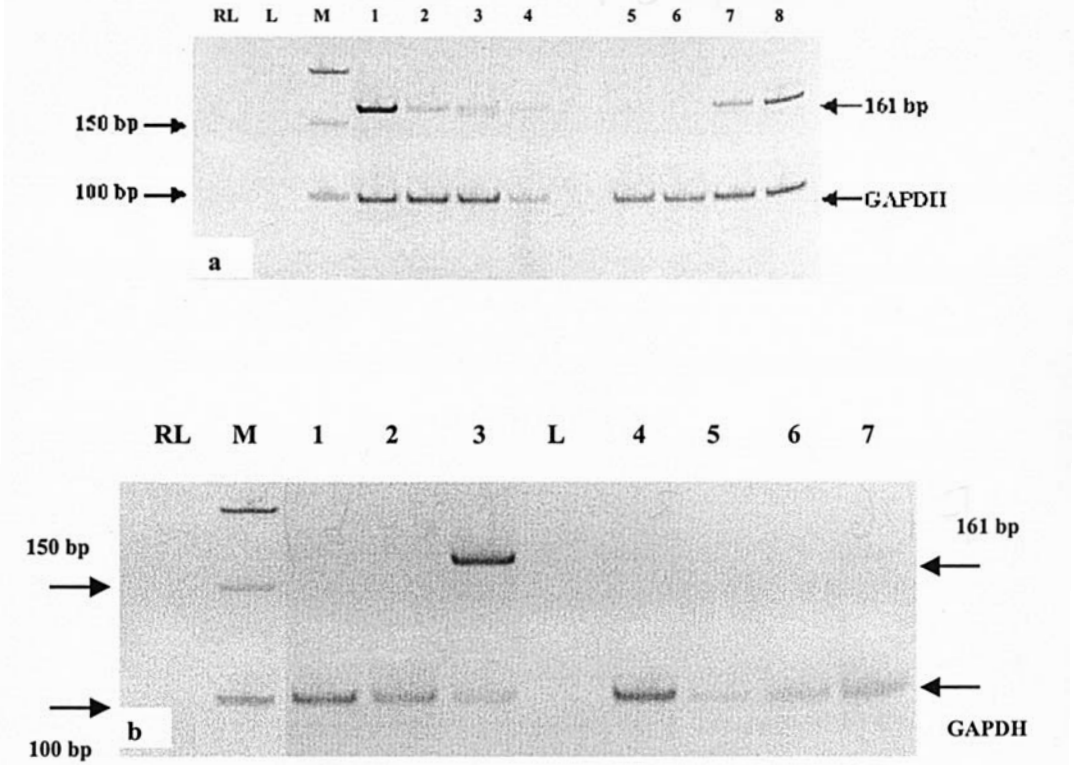

Figure 3. Expression of CEL in various cell lines and tissues. RL and L: negative controls for combined RT-PCR or PCR, respectively; M, molecular marker. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (PCR fragments: CEL 161, GAPDH 98 base pairs). (a) In one of four permanent cell lines derived from pancreatic cancers (1, BxPC-3) the expression was strong. In three others (2, IMIM-PC2; 3, PANC-1; 4, Capan-2), a reduced expression was seen. Cell lines derived from various tumors also showed a variable expression: none in 5, GLC-4 and 6, K562 and a more pronounced in 7: HT-29 and 8: MKN-45. (b) In samples of two adenocarcinomas of the pancreas no cDNA fragment was noted (1 and 2). In normal pancreatic tissue strong expression was found (3). In other gastrointestinal control tissues the expression level was not significant (4, stomach; 5, duodenum; 6, large intestine; 7, spleen).

This finding corresponds to well-established results reported by others (19). For the first time, we demonstrated that none of the investigated pancreatic adenocarcinomas displayed significant levels of CEL. Kim *et al* (4) reported trypsin activity in 6/29 human ductal adenocarcinomas, yet active pancreatic lipase only in 1/29. Terada *et al* (8) reported pancreatic lipase in half of their cases but none of the two groups examined CEL expression. In line with our data other investigators (9,10) noted CEL transcription in 6 examined pancreatic tumor cell lines.

Pancreatic lipase and other acinar markers were demonstrated immunocytochemically in a collection of 22 acinar cell carcinomas of the pancreas (20,21). Moreover, in three cases CEL showed an immunohistochemical reaction (22). Due to our single case of acinar cell carcinoma, our study does not allow a generalization of CEL's activity in this subtype of pancreatic cancer. Lack of CEL expression in the ductal and mucinous 'standard types' of pancreas cancer renders this gene an inappropriate target for strategies in gene therapeutic approaches as applied for gastric cancer cell lines (23). The promise for successful use of the CEL promoter in acinar cell carcinoma needs further evaluation.

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