Monoclonal antibody 16D10 to the COOH-terminal domain of the feto-acinar pancreatic protein targets pancreatic neoplastic tissues

Liliane Benkoël,[1] Jean-Paul Bernard,[1,2,5] Marie-José Payan-Defais,[3] Lydie Crescence,[1] Cécile Franceschi,[1] Mireille Delmas,[3] Mehdi Ouaissi,[1,4] Bernard Sastre,[4] José Sahel,[5] Anne-Marie Benoliel,[6] Pierre Bongrand,[6] Françoise Silvy,[1] Laurent Gauthier,[7] François Romagné,[7] Dominique Lombardo,[1,2] and Eric Mas[1,2]

[1]INSERM UMR 911-CRO2, Aix-Marseille Université, Faculté de Médecine-Timone; [2]Assistance Publique-Hôpitaux de Marseille, INSERM CIC 9502; [3]Assistance Publique-Hôpitaux de Marseille, Hôpital la Timone, Laboratoire d'Anatomo-Pathologie; [4]Assistance Publique-Hôpitaux de Marseille, Hôpital la Timone, Service de Chirurgie Digestive et Oncologique, Pôle d'Oncologie et Spécialité Médico-Chirurgicales; [5]Assistance Publique-Hôpitaux de Marseille, Hôpital La Conception, Service d'Hépato-Gastro-Entérologie; [6]INSERM UMR 600, Parc Scientifique de Luminy; [7]Innate Pharma, Marseille, France

Abstract

We have shown that the 16D10 antigen located on the mucin-like COOH-terminal domain of the feto-acinar pancreatic protein (FAPP) is expressed at the surface of human pancreatic tumor cell lines such as SOJ-6 cell line. Furthermore, an *in vivo* study indicates that targeting this cell-membrane glycopeptide by the use of the monoclonal antibody (mAb) 16D10 inhibits the growth of SOJ-6 xenografts in nude mice. To validate the potential use of the mAb16D10 in immune therapy, this study examined the expression of 16D10 antigens at the surface of human pancreatic adenocarcinomas versus control tissues. We examined the reactivity of mAb16D10 and mAb8H8 with pancreatic ductal adenocarcinomas (PDAC) compared with controls by using immunohistochemistry and confocal laser scanning microscopy. mAb8H8 does react with control or nontumoral human pancreatic tissues. mAb16D10 has a strong and specific reactivity with PDAC and does not react with other cancers of epithelia or normal tissues tested. Notable, mAb16D10 mostly recognizes membrane of tumoral cells. Furthermore, mAb8H8 and mAb16D10 recognized a protein of 110 to 120 kDa in homogenates of nontumoral and tumoral human pancreatic tissues, respectively. This size correlates with that of FAPP or with that of the normal counterpart of FAPP, the so-called bile salt-dependent lipase. The results suggest that mAb16D10 presents a unique specificity against PDAC; consequently, it could be effective in immune therapy of this cancer. Furthermore, mAb16D10 and mAb8H8 pair might be useful for diagnosis purpose in discriminating tumoral from nontumoral human pancreatic tissues. [Mol Cancer Ther 2009;8(2):282–91]

Received 5/15/08; revised 10/7/08; accepted 11/8/08;

published OnlineFirst 02/03/2009.

Grant support: Association pour la Recherche sur le Cancer grant nb 3816 (E. Mas) and Agence Nationale de la Recherche (D. Lombardo) and institutional funding from INSERM and Université de la Méditerranée. L. Crescence is a doctoral fellow of Ministère de l'Enseignement Supérieur et de la Recherche. C. Franceschi is a postdoctoral fellow of INSERM.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Eric Mas, INSERM UMR 911-CRO2, Aix-Marseille Université, Faculté de Médecine-Timone, 27 Blv Jean Moulin, 13385 Marseille cedex 05, France. Phone: 33-191324400; Fax: 33-191830187. E-mail: eric.mas@univmed.fr

doi:10.1158/1535-7163.MCT-08-0471

Introduction

The bile salt-dependent lipase (BSDL; E.C. 3.1.1.13) is found in the pancreatic secretions of all species from fish to human (1). This enzyme participates in the intestinal processing of cholesteryl esters (2–4). During its transport from the endoplasmic reticulum up to the trans-Golgi network, BSDL is associated with intracellular membranes by means of a complex that involves glycosphingolipids of rafts (5) and the 94-kDa glucose-related protein (6, 7). This Grp 94 controls the late folding steps and the sorting of the active enzyme toward secretion (8). Once in the trans-Golgi network and after completion of glycosylation (9, 10), the complex is released from membranes on the phosphorylation of the threonine residue at position 340 (11) by a protein kinase CKII (12, 13). Once released from intracellular membranes, BSDL enters into the secretion route until the duodenum where it accomplishes its physiologic role.

The feto-acinar pancreatic protein (FAPP) is a specific component of acinar cells of the human pancreas, which is associated with the ontogenesis and the development of the gland (14). Maximum synthesis of FAPP, determined as the emergence of the J28 epitope recognized by the monoclonal antibody (mAb) J28 (15), occurs when acinar cell proliferation is maximal between 20 and 22 weeks of gestation; thereafter, it declines to parturition (16). In adult, FAPP defined by the expression of the J28 epitope behaves as an oncodevelopment-associated antigen (14). FAPP (a 100-120 kDa protein) presents strong homologies with BSDL (a 100 kDa protein; ref. 17) and its cloning from human pancreatic tumoral cells (18) indicates that the NH_2-terminal domain encoded by exons 1 to 10 is identical to that of BSDL. However, the sequence corresponding to

exon 11, which encodes the mucin-like COOH-terminal domain of BSDL, is shortened by 330 bp (18). FAPP is poorly secreted by pancreatic tumoral cells (14, 19, 20); its low rate of secretion might not result from inherent properties of the protein, which is a priori normally *N*- and *O*-glycosylated (17, 18) and phosphorylated (11). The retention of FAPP in human pancreatic tumoral SOJ-6 cells could result from the expression of a defective Rab-GDIβ (21). The localization of FAPP in tumoral cells indicates that the protein is mainly membrane-associated like its normal counterpart BSDL and distributes within the endoplasmic reticulum and the Golgi (19). FAPP is then degraded (22) and the COOH-terminal domain of FAPP that carries out oncofetal epitopes is presented at the surface of tumoral cells such as SOJ-6 cells (23). This 32-kDa glycopeptide issued from the FAPP degradation is specifically recognized by mAbJ28 and mAb16D10, two mAbs directed against the *O*-glycosylated COOH-terminal domain of FAPP. The formation of the J28 glycotope requires the core 2 β1,6-*N*-acetylglucosaminyltransferase and the α1,3/4-fucosyltransferase (24, 25), two glycosyltransferases whose expression is up-regulated in pancreatic cancer (26). Furthermore, we have shown that the growth of xenografted SOJ-6 cells in nude mice was significantly decreased by preventative injections of mAb16D10 (23). To validate the potential use of this mAb16D10 in immune therapy, it was important to determine the expression of 16D10 antigen on human pancreatic adenocarcinomas. In this study, we have shown that mAb16D10 is capable to discriminate specifically the human pancreatic tumoral tissue from nontumoral tissue. This study was carried out on both frozen tissues and formalin-fixed, paraffin-embedded archival tissues.

Materials and Methods

Human Tissues

Seven fresh frozen tissue sections of primary malignant pancreatic ductal adenocarcinomas (PDAC) were collected from the Department of Pathology (Ste Marguerite Hospital). Frozen tissue slides of a PDAC provided by BioChain were also studied. Adjacent nontumoral areas from three cases of pancreas neoplasia, one benign pancreatic tumor, and one biopsy obtained during transplantation process were used as nontumoral controls. Samples were collected from recently diagnosed adenocarcinomas. Median age of patients was 66 years (range, 42-75). Table 1 gives histopathologic data of each patient. A lung adenocarcinoma case was provided by the Department of Thoracic Surgery (Ste Marguerite Hospital). Carcinomas of colon, esophagus, liver, stomach, and thyroid were studied as negative tissue controls (BioChain).

Formalin-fixed, paraffin-embedded tissue sections from 10 patients with histologic nontumoral pancreas were studied as controls. Four cases were provided by the Department of Pathology. Three cases were previously studied on frozen sections and six other cases were provided by BioChain (Table 1). Twenty-one archival PDAC cases were studied. Six cases were previously studied on frozen sections. Median age of patients was 66 years (range, 42-82). Histopathologic data of each patient are also provided in Table 1. Samples of PDAC were collected from patients without prior chemotherapy or radiotherapeutic treatments. One sample from bladder metastasis of pancreatic cancer and one sample from a patient with a preoperative gemcitabine treatment before the tumor resection were provided by the Department of Pathology.

Table 1. Clinicopathologic data of nontumoral pancreas and PDAC

Cases	Age	Gender	Histopathology
C1*	78	F	Nontumoral tissue obtained in a case of carcinoma of the ampulla of Vater
C2*	25	M	Normal tissue obtained during the collection of organ for transplantation after an accidental death
C3*†	30	F	Nontumoral tissue obtained in a case of mucinous cystadenoma
C4*†	52	M	Nontumoral tissue obtained in a case of mucinous adenocarcinoma
C5*†	39	F	Nontumoral tissue obtained in a case of mucinous cystadenoma
C6†	82	F	Normal tissue obtained in a case of kidney tumor resection
C7‡	72	F	Chronic obstructive pulmonary disease
C8†‡	83	F	Congestive heart failure
C9†‡	86	F	End-stage heart disease
C10†‡	88	F	Dementia
C11†‡	62	M	Congestive heart failure
C12†‡	76	M	Chronic obstructive pulmonary disease
PDAC1*†	42	M	Ductal adenocarcinoma
PDAC2*†	73	F	Ductal adenocarcinoma
PDAC3*†	75	F	Ductal adenocarcinoma
PDAC4*†	54	F	Ductal adenocarcinoma
PDAC5*†	73	F	Ductal adenocarcinoma
PDAC6*†	63	M	Ductal adenocarcinoma
PDAC7*‡	66	M	Ductal adenocarcinoma
PDAC8†	78	F	Ductal adenocarcinoma
PDAC9†	71	M	Ductal adenocarcinoma
PDAC10†	56	F	Ductal adenocarcinoma
PDAC11†	82	M	Ductal adenocarcinoma
PDAC12†	66	M	Ductal adenocarcinoma
PDAC13†	77	F	Ductal adenocarcinoma
PDAC14†	64	F	Ductal adenocarcinoma
PDAC15†	41	F	Ductal adenocarcinoma
PDAC16†	64	F	Ductal adenocarcinoma
PDAC17†	65	F	Ductal adenocarcinoma
PDAC18†	75	M	Ductal adenocarcinoma
PDAC19†	67	M	Ductal adenocarcinoma
PDAC20†	53	M	Ductal adenocarcinoma
PDAC21†	57	F	Ductal adenocarcinoma
PDAC22†	74	M	Ductal adenocarcinoma
M†	61	F	Bladder metastasis of pancreatic cancer
T†	73	F	Ductal adenocarcinoma obtained after a preoperative gemcitabine treatment

*Frozen sections.
†Formalin-fixed, paraffin-embedded sections.
‡Tissue provided by BioChain. All controls and patients were Caucasians, except PDAC7, which was Asiatic.

The 16D10 antigen expression on human normal tissues has been determined by MDS Pharma Services. The human normal tissue bank was prepared in accordance to the French Bioethical Law nb. 2004-800 of August 2004 and under the control of the Institut de Médecine Légale. Placenta was obtained just after parturition and came from the appropriate medical service of the Centre Hospitalier Universitaire de Lyon with the same safety and scientific precautions.

Tissue Treatment and Histologic Study

All tissues from the human normal tissue bank and freshly collected pancreatic and lung tissues were frozen in liquid nitrogen, stored at -80°C, and further cut into 5 μm thick cryotome sections at -20°C. The frozen sections were dried, fixed at 4°C in acetone, and stained with hematoxylin-phloxine-saffron. Formalin-fixed, paraffin-embedded tissue specimens were routinely fixed in 10% formalin, embedded in paraffin, and further cut into 5 μm sections stored at 4°C or stained with hematoxylin-phloxine-saffron. Each sample was processed for histopathologic diagnosis, which was done by a senior anatomopathologist investigator (M-J.P-D.) specialized in pancreatic histology. All the examined patients were in stage 0 or I.

Antibodies

The patented IgM mAb16D10 directed against *O*-glycosylated repeated COOH-terminal mucin domain of FAPP (23), the patented IgG mAb8H8 against BSDL,[8] and the polyclonal antibodies (pAb) L64 against BSDL/FAPP (17) were homemade. mAbJ28 specific for the fucosylated J28 glycotope carried by repeated COOH-terminal sequences of FAPP was a generous gift from Dr. M-J. Escribano. BSDL and FAPP were isolated as already described (2, 14, 17). Anti-β-actin antibody and irrelevant mouse IgM, IgG, and rabbit IgG were from Sigma.

Immunohistochemistry

Cryotome tissue sections were dried, treated for 10 min with acetone at 4°C, and rehydrated in TBS (pH 7.6). Unspecific binding sites were blocked 30 min at room temperature with a nonimmune goat serum. The sections were then incubated (1 h, room temperature) with mAb8H8 (20 μg/mL) or pAbL64 (20 μg/mL) overnight at 4°C with mAb16D10 (20 μg/mL). The sections were washed in TBS and processed with EnVision system (DAKOCytomation) containing alkaline phosphatase-conjugated antibodies to rabbit and to mouse and Fast Red substrate-chromogen. Sections were washed in distilled water, counterstained in Mayer's hematoxylin solution, and mounted in aqueous permanent mounting medium.

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized with Clearify (MM France) and gradually rehydrated with decreasing concentrations of ethanol and washed with water. Antigen retrieval was done on paraffin sections with DAKOCytomation Target Retrieval Solution (pH 9) for 40 min at 98°C and 20 min at room temperature. After rinsing with water and PBS, slides were incubated (2 h, room temperature) with mAb8H8 (20 μg/mL), pAbL64 (20 μg/mL), or mAb16D10 (100 μg/mL), washed in PBS, and incubated with 0.3% hydrogen peroxide for 5 min to block endogenous peroxidase. Thus, slides were processed with Vector Vectastain ABC kits containing biotinylated secondary antibodies and a preformed avidin and biotinylated horseradish peroxidase complex. Sections were washed in distilled water, counterstained in Mayer's hematoxylin solution, and mounted in aqueous permanent mounting medium.

For all controls, slides were incubated with irrelevant IgM or IgG primary antibodies or adequate secondary antibodies.

Confocal Laser Scanning Microscopy

Dry cryotome tissue sections were fixed for 10 min with acetone at 4°C and rehydrated in PBS (pH 7.2). Sections were incubated (1 h, room temperature) with mAb8H8 (20 μg/mL), pAbL64 (20 μg/mL), or mAb16D10 (20 μg/mL), washed in PBS, and incubated (1 h, room temperature) with secondary antibodies diluted to 1:50 [biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG or biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgM; Immunotech]. The sections were washed in PBS and treated (1 h, room temperature) with 1:50 dilution of streptavidin-fluorescein (Immunotech). Sections were washed in PBS, counterstained in Mayer's hematoxylin solution allowing the correct determination of the studied cellular zones, and then washed with water and mounted in aqueous permanent mounting medium. For all controls, slides were incubated with irrelevant IgM or IgG primary antibodies or adequate secondary antibodies.

Antigen retrieval was done on formalin-fixed, paraffin-embedded sections as described previously for immunohistochemistry. After rinsing with water and PBS, the slides were incubated (2 h, room temperature) with mAb8H8 (20 μg/mL), pAbL64 (20 μg/mL) or mAb16D10 (100 μg/mL), washed in PBS, and incubated (1 h, room temperature) with secondary antibodies diluted to 1:50 biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgG or biotin-conjugated $F(ab')_2$ fragment goat anti-mouse IgM (Immunotech). The sections were washed, treated with streptavidin-fluorescein, and processed as for frozen sections. Sections were observed by means of a confocal laser scanning microscope (Leica) with a ×25 objective.

Image Processing

Images were processed as described previously (27). For each primary antibody, the staining was calculated as the ratio between the total fluorescence of the area (total specific fluorescence) and the surface of this area [mean specific fluorescence (MSF)]. The mean values of six stained areas for each biopsy were then calculated.

SDS-PAGE

Frozen tissues were collected from glass slides using a razor blade, homogenized in Laemmli's loading buffer (28), centrifuged (2 min, 1,000 rpm), and analyzed on 10%

[8] Unpublished observation.

polyacrylamide gel in the presence of 1% SDS. Proteins were electrotransferred onto nitrocellulose membranes and the immunodetection was done using mAb and alkaline phosphatase-labeled secondary antibodies as required.

Statistical Analysis

All results are expressed as mean ± SE. Differences between controls and biopsies were tested using ANOVA. The significance was calculated with Fisher's post hoc least significant difference test and/or the Scheffe's F test. Differences were considered significant when $P < 0.05$.

Results

Determination of 8H8 and 16D10 Antigen Expression by Immunohistochemistry

On frozen sections, the plasma membrane and cytoplasm of acinar cells of all control cases were stained with mAb8H8 (Fig. 1). Endocrine cells of the islet of Langherans (Fig. 1, *arrowhead*) were always negative to mAb8H8. Tumor cells of PDAC were not reactive to mAb8H8 (Fig. 1) or very slightly stained for few cases (Fig. 1). No staining was found in nontumoral tissue with mAb16D10, whereas PDAC sections were all positive to mAb16D10 (Fig. 1). A strong staining was found at the plasma membrane and around the tumoral cells or in cytoplasm.

Results obtained with formalin-fixed, paraffin-embedded sections were quite similar to those found with frozen sections. With mAb8H8, the staining was mainly found in cytoplasm of acinar cells. Pancreatic ducts were slightly stained (Fig. 1, *arrow*). Any or slight staining was observed in PDAC cells (Fig. 1). No staining was found in nontumoral tissue with mAb16D10, whereas all PDAC cases were stained with mAb16D10. The staining was mainly found in cytoplasm of tumor cells and at the plasma membrane (Fig. 1).

We also used pAbL64, a pAb directed against BSDL and FAPP, on frozen and formalin-fixed, paraffin-embedded sections. As expected, pictures showed a strong staining localized at the plasma membrane and in the cytoplasm of tumoral cells. The cytoplasm of acinar cells of control cases was also decorated by pAbL64 (Fig. 1).

Determination of 8H8 and 16D10 Antigen Expression by Confocal Laser Scanning Microscopy

To determine the precise localization of 16D10 antigen in the tumoral cells, we examined the reactivity of mAb8H8 and mAb16D10 with PDAC and control cases by using confocal laser scanning microscopy (CLSM). Using mAb8H8, a strong fluorescence was found on frozen control sections at the plasma membrane and in the cytoplasm, whereas PDAC cases showed a negative or slight staining (Fig. 2A). With mAb16D10, the staining was negative or extremely faint in control cases (Fig. 2A). The staining of all cases of ductal adenocarcinomas was very strong at the plasma membrane. This labeling of cell plasma membranes by mAb16D10 is clearly visible on enlarged Fig. 2B showing the same area counterstained with Mayer's hematoxylin. CLSM of six stained areas from each image were quantified by measuring the MSF

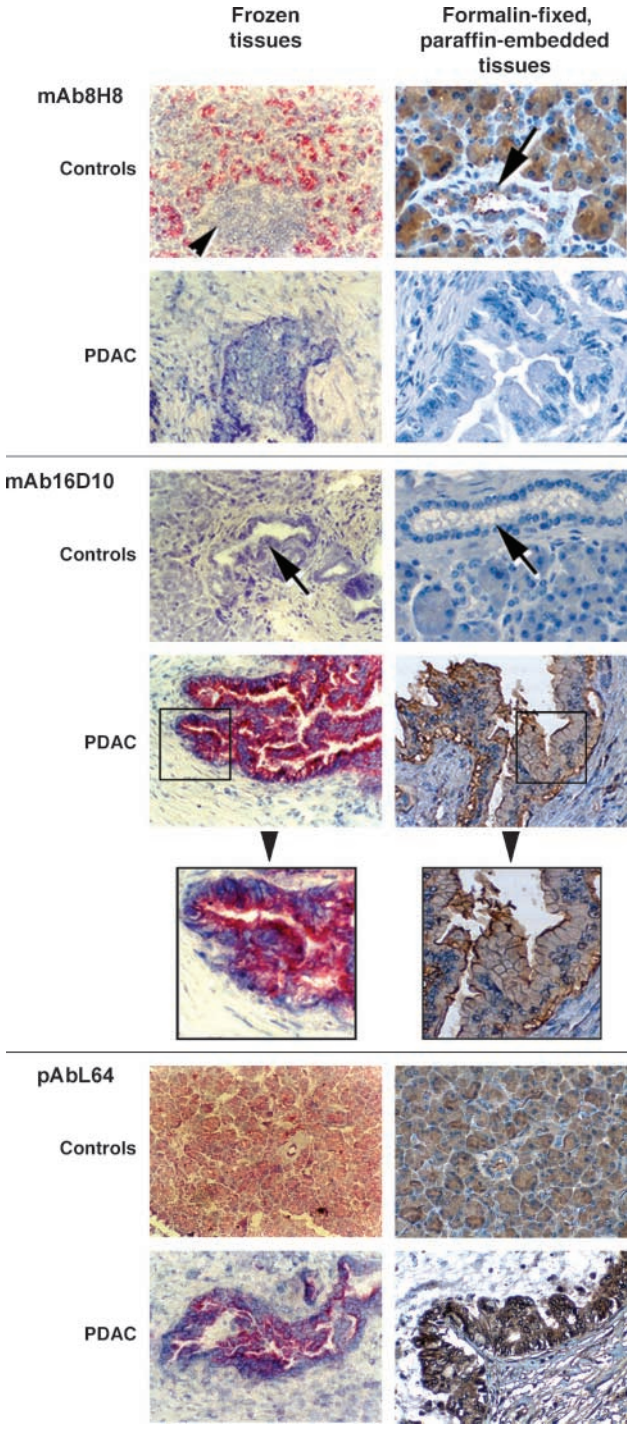

Figure 1. Representative views of immunohistochemical staining with mAb8H8 and mAb16D10. Cytoplasm of acinar cells are stained with mAb8H8 and pAbL64 in frozen and formalin-fixed, paraffin-embedded sections from nontumoral pancreas. *Arrowheads,* endocrine tissue of the Islet of Langherans is not stained; *arrows,* intralobular ducts are slightly stained. Tumoral cells are not reactive to mAb8H8 in sections from PDAC. Acinar cells and pancreatic ducts were unreactive to mAb16D10 in all sections from nontumoral pancreas. All PDAC cases are strongly stained on plasma membrane and/or cytoplasm with mAb16D10 and pAbL64. *Right,* enlargement of the boxed portion in the micrographs. Original magnification, ×250.

intensity of stained areas. The average of the MSF intensity found with mAb8H8 was significantly decreased ($P < 0.001$) in PDAC (mean = 16 ± 6) compared with controls (mean, 113 ± 7; Fig. 3A). On the other hand, a significant increase in MSF ($P < 0.001$) was found with mAb16D10 (Fig. 3B) in each PDAC (mean, 131 ± 2) compared with controls (mean, 8 ± 2).

Labeling obtained with formalin-fixed, paraffin-embedded sections was stronger than those found with frozen sections. A strong fluorescence was found in cytoplasm in all control cases using mAb8H8 (Fig. 2A). This difference observed between frozen section and formalin-fixed, paraffin-embedded sections could be due to heat-induced antigen retrieval increasing the accessibility of epitopes to the antibodies. Another possibility was that soluble FAPP in the cytoplasm could be partially fixed and extracted during immunohistochemical staining of fresh frozen tissue sections (29).

On the contrary, this antibody did not or slightly react with PDAC. With mAb16D10, the staining was negative or extremely faint in control cases (Fig. 2A). In all cases of PDAC, the staining was very strong on plasma membranes and the cytoplasm was sporadically decorated. This labeling of cell plasma membranes by mAb16D10 is clearly visible on enlarged Fig. 2B associated to the same areas counterstained with Mayer's hematoxylin.

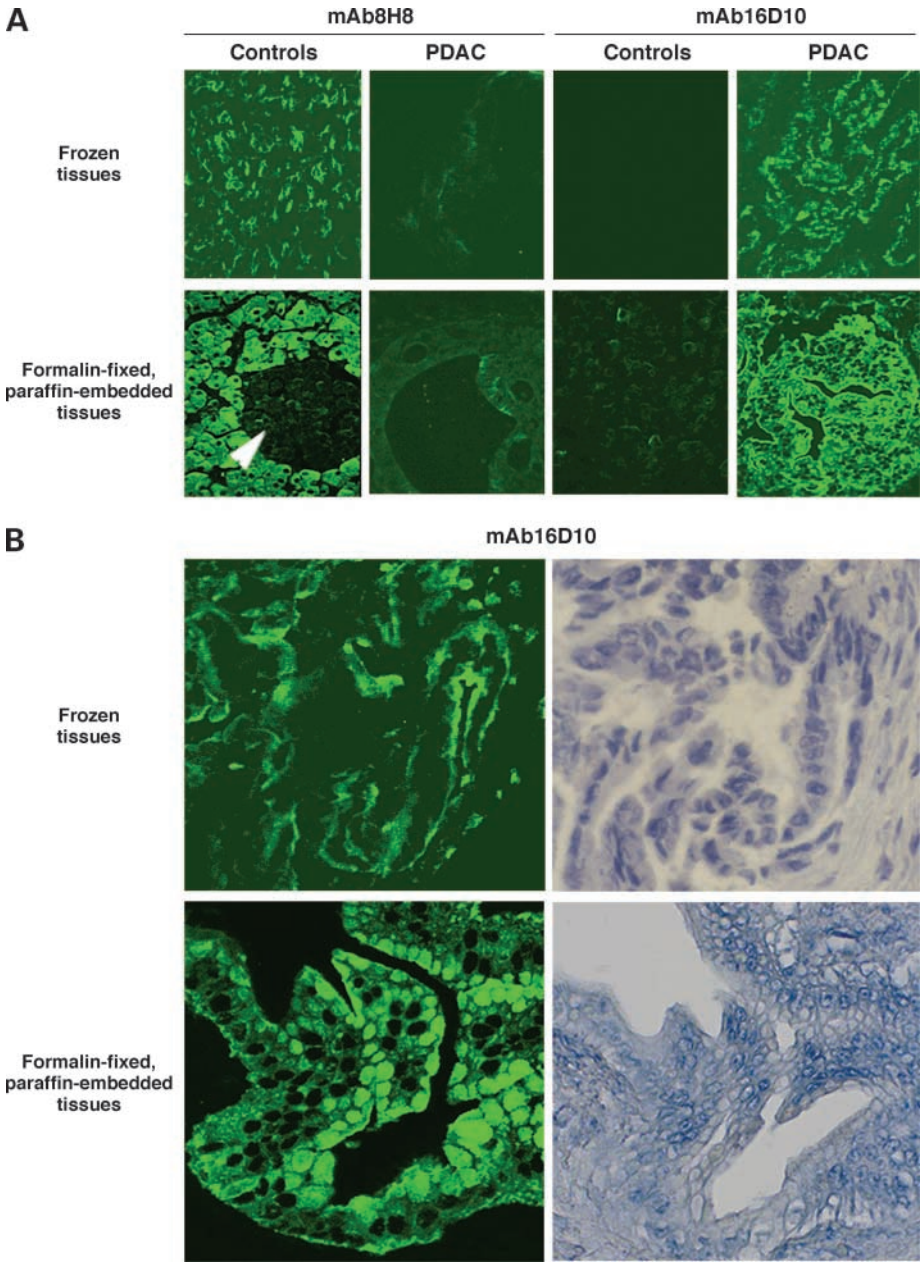

Figure 2. Representative results of CLSM staining with mAb8H8 and mAb16D10 in frozen and formalin-fixed, paraffin-embedded sections. **A,** all nontumoral pancreatic tissue are stained with mAb8H8. *Arrowhead,* endocrine tissue of the Islet of Langherans is not stained. In PDAC, no detectable or very slight staining is found with mAb8H8. With mAb16D10, no staining or slight staining is found in nontumoral pancreas. In PDAC, a strong staining is found in plasma membrane and cytoplasm. **B,** higher magnification of images stained with mAb16D10 with Mayer's hematoxylin counterstaining before CLSM observation. Original magnification, ×300.

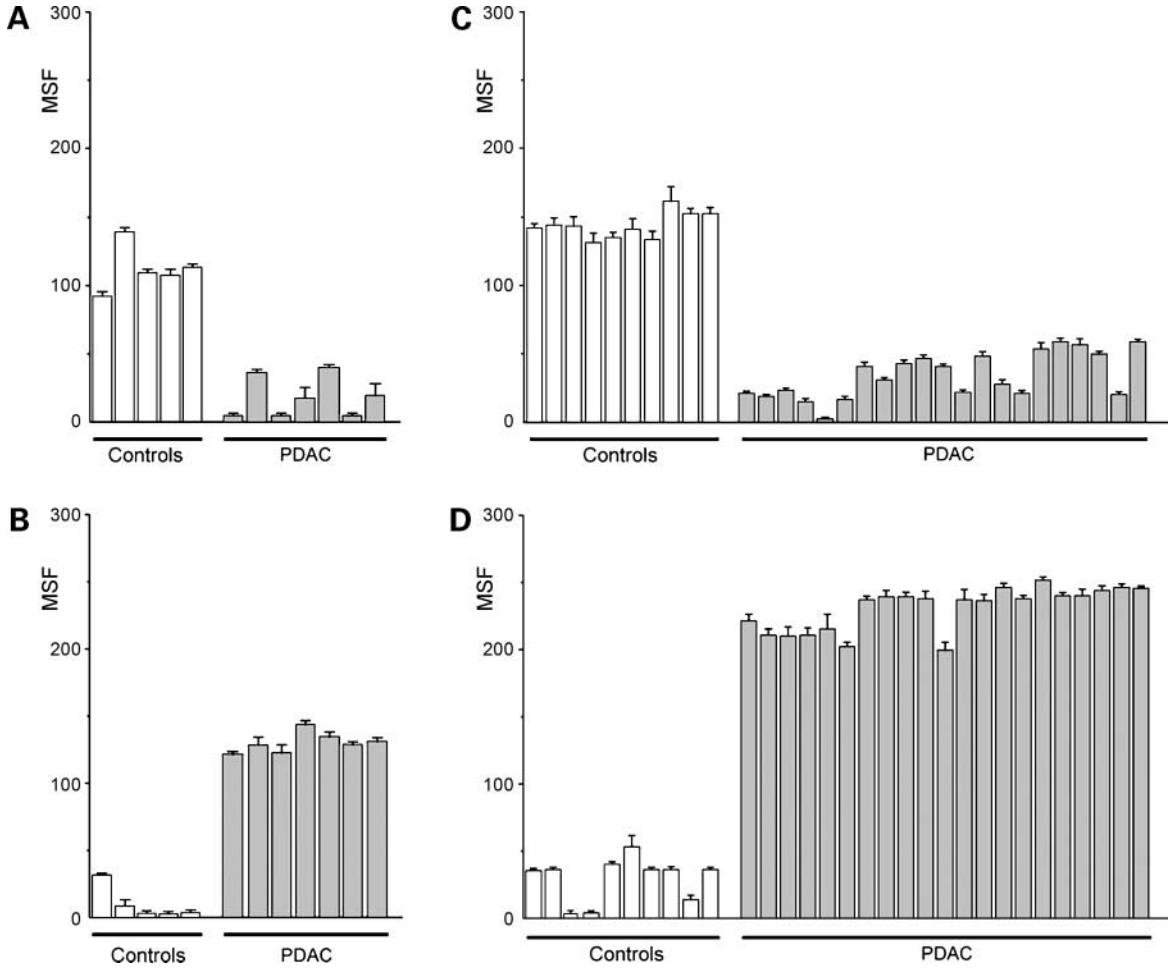

Figure 3. Quantitative determination of MSF in frozen sections (**A** and **B**) and formalin-fixed, paraffin-embedded sections (**C** and **D**). mAb8H8 (**A** and **C**) and mAb16D10 (**B** and **D**). Six areas in each case were measured. Data obtained with one case were compared to each other. Mean ± SE. Comparison were made by ANOVA followed by Fisher's and Scheffe's tests. $P < 0.001$.

As for frozen sections, CLSM of six stained areas from each image was quantified by measuring the MSF intensity of stained areas. With mAb8H8, the average of the MSF intensity was significantly decreased ($P < 0.001$) in PDAC (mean, 34 ± 3) compared with controls (mean, 144 ± 3; Fig. 3C). On the other hand, a significant increase in MSF ($P < 0.001$) was found with mAb16D10 (Fig. 3D) in each PDAC (mean, 226 ± 3) compared with controls (mean, 31 ± 5).

16D10 Antigen Expression on Human Cancer and Normal Tissues

The expression of the 16D10 antigen was recorded on six human cancer tissues. mAb16D10 did not react with other epithelial neoplastic cells such as colon, esophagus, liver, stomach, thyroid, and lung (Fig. 4).

The expression of the 16D10 antigen was also recorded on 18 human normal tissues including epithelium such as colon, duodenum, skin + breast, ileum, jejunum, liver, lung, pancreas, thyroid, placenta, stomach, endothelium such as artery and vein endothelial tissues, and cerebellum, cerebrum, pituitary gland, and striated muscle. No staining was found in all tissues treated with mAb16D10, irrelevant mouse IgM, or labeled secondary antibodies (data not shown).

16D10 Antigen Expression in Metastasis of Pancreatic Cancer and in Tumor from Patient with a Preoperative Chemotherapy

To determine whether the mAb16D10 could be used in advanced cancers and in patients after standard therapies, the expression of the 16D10 and 8H8 antigens was recorded on human bladder metastasis of pancreatic cancer and cancer tissue from a patient with a preoperative gemcitabine treatment. A slight staining was observed with mAb8H8, whereas these two tumors were strongly stained with mAb16D10 (Supplementary Fig. S1A and B).[9] CLSM

[9] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

of six stained areas from each image was quantified by measuring the MSF intensity of stained areas. With mAb8H8, the average of the MSF intensity in pancreatic bladder metastasis (mean, 36 ± 1) and tumor from patient with a preoperative chemotherapy (mean, 34 ± 2) was similar to that found with PDAC (mean, 34 ± 3) and was significantly decreased ($P < 0.001$) compared with controls (mean, 144 ± 3; Supplementary Fig. S1C).[9] On the other hand, a significant increase in MSF ($P < 0.001$) was found with mAb16D10 in pancreatic bladder metastasis (mean, 123 ± 6) and tumor from patient with a preoperative chemotherapy (mean, 189 ± 5) compared with controls (mean, 31 ± 5), although CLSM was less important in these tissues compared with that of PDAC (mean, 226 ± 3; Supplementary Fig. S1C).[9]

SDS-PAGE Analysis of Pancreatic Tissues

We next checked that mAbs used in the present study reacted with BSDL or FAPP in pancreatic tissues. For this purpose, nontumoral and tumoral tissues corresponding to sections of nontumoral control tissue C3 and tumoral tissue PDAC3, respectively, were removed from slide and analyzed by SDS-PAGE and immunodetection. As shown on Fig. 5A, mAb8H8 specifically and strongly reacted with a protein, the M_r of which (~110-120 kDa) corresponds to that of BSDL also recognized by pAbL64. mAb16D10 reacted very slightly with a protein present in nontumoral control tissue. In that concerning tumoral tissue, no mAb8H8-immunoreactive protein can be detected; however, proteic material present in lysate reacted with mAb16D10. In particular, this mAb, as well as mAbJ28 and pAbL64, recognized a 120-kDa protein, the M_r of this material corresponds to that of FAPP (Fig. 5B). Interestingly, this tumor presents two isoforms of FAPP due to a polymorphism located in the exon 11 encoding the COOH-terminal domain of the protein (30). mAb16D10 also recognized peptides presenting higher migration; in part, it reacted with 40- to 60- and 25- to 35-kDa peptides. This material could be representative of different proteolysis products of the COOH-terminal domain of FAPP, which has been recently detected at the level of the plasma membrane of pancreatic tumoral cells (23). Furthermore, mAb16D10 also binds to material with a very low migration; this material could be aggregated molecules of FAPP or FAPP associated with membrane lipid in cell lysate (5). These data strengthen immunohistochemical results and show that mAb8H8 recognizes the BSDL in nontumoral pancreatic tissue and does not react with the tumoral tissue. Contrarily, mAb16D10 reacts with FAPP in tumoral tissue and has no reactivity with protein in normal tissue.

Discussion

During the last decade, no significant progress has been made against the cancer of the exocrine pancreas, although chemotherapy associated with radiotherapy has some positive effect on the survival of patients (31). The curative resectability rate is very low because the cancer is asymptomatic and its diagnosis in most patients occurs at a late stage and had already formed distant metastases (32). At this stage, the prognosis is extremely poor and efforts are urging to identify and evaluate diagnostic and therapeutic markers. Specific markers to target pancreatic tumoral cells are spare, albeit many mAbs were generated against malignant pancreatic epithelial cells. These include Span-1 (33), Du-Pan-2 (34), CA 19-9 (35), CA50 (36), CAR-3 (37), and CA242 (38). These antibodies displayed distinct patterns of tissue staining, being more highly expressed in certain tissues and tumors than others. Furthermore, the reactivity of these antibodies depends on the genotype of patients (39). Consequently, the sensitivity and the

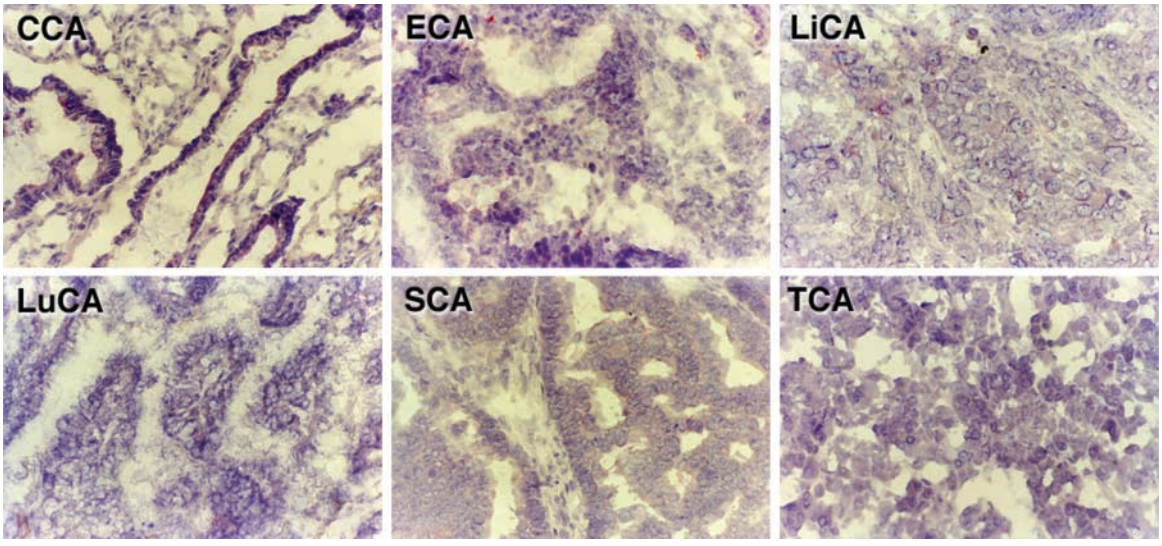

Figure 4. Immunohistochemical results in nonpancreatic carcinoma cases with mAb16D10. *CCA,* colon adenocarcinoma; *ECA,* esophagus adenocarcinoma; *LiCA,* liver adenocarcinoma; *LuCA,* lung adenocarcinoma; *SCA,* stomach adenocarcinoma; *TCA,* thyroid adenocarcinoma. No staining using mAb16D10 is found in all epithelial tissues examined. Original magnification, ×250.

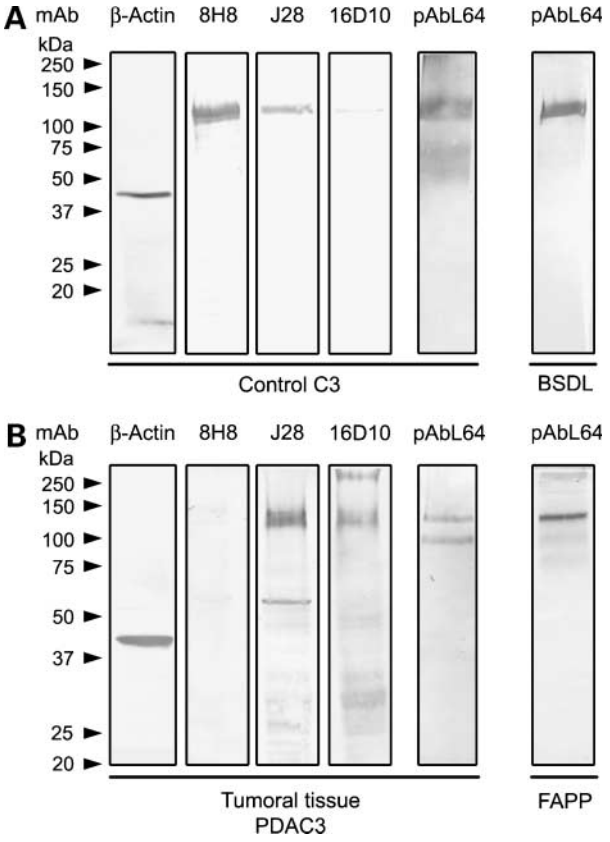

Figure 5. Immunoreactivity of tissue homogenates with antibodies. Tissue sections were removed from slide C3 (control tissue; **A**) and slide PDAC3 (tumoral tissue; **B**). Tissues were homogenized before SDS-PAGE analysis and immunodetection with anti-β-actin antibody and antibodies mAb8H8, mAbJ28, mAb16D10, and pAbL64. BSDL and FAPP (50 ng of each protein were loaded on the top of the gel) were also analyzed on SDS-PAGE and immunodetected using pAbL64, pAbs that reacted with these two proteins.

specificity of these markers are not ideal. The combined use of some of these antigens expression may potentiate the specificity for pancreatic diagnosis (40). Mucin expression pattern may be interesting as overexpression of MUC1 and MUC6 and *de novo* expression of MUC5AC occurred early in the development of pancreatic adenocarcinoma, whereas sialyl Tn antigen expression occurs later, at the stage of invasive adenocarcinoma (41). Unfortunately, mucins are expressed by normal epithelia (MUC1) or by various normal gastric cells (MUC5AC and MUC6; refs. 42, 43). Although interesting progresses have been done with mesothelin and the prostate stem cell antigen (44), physicians are still awaiting for a specific marker of the pancreatic malignancy with interest both in diagnostic and eventually in targeting the pancreatic neoplastic cell.

FAPP, an oncofetal glycovariant of the BSDL (16), is a specific constituent of the pancreatic acinar cells, which is associated with the ontogenesis and development of the human pancreas. FAPP was characterized in human pancreas with the mAbJ28 (14). This mAb recognized a structure found in human tumoral pancreas, in human pancreatic tumor cell lines SOJ-6 and BxPC-3 (18, 45), and in inflamed pancreas or pancreatic juice consecutive to a pancreatitis (16, 46). This antibody had no reactivity with normal tissue (14). Furthermore, it has been shown that mAbJ28 recognizes a carbohydrate antigenic structure (14, 24) located within *O*-glycosylated mucin-like COOH-terminal domain of FAPP (24, 25). This oncofetal carbohydrate antigenic structure is dependent on the β6-*N*-acetylglucosaminyltransferase and α3/4-fucosyltransferase activities, two glycosyltransferases overexpressed by the tumoral pancreas (26).

Many works suggested that epitopic structures recognized by mAbs to FAPP are present at the surface of tumoral pancreatic cells. These studies included immunocytologic localization of FAPP within human pancreatic tumoral tissue compared with normal tissue (19) and the targeting of nitrosamine-induced pancreatic tumor in hamster (which expressed the J28 epitope) with the radiolabeled mAbJ28 (47). A recent study showed that the mucin-like COOH-terminal domain of FAPP is actually presented at the surface of human pancreatic tumoral SOJ-6 cells (23). This glycopeptide is recognized by mAbJ28 and mAb16D10. A prospective study suggested that this latter mAb has an *in vivo* preventative antitumor activity against SOJ-6 cells. I.p. injections of mAb16D10 in mice xenotransplanted with SOJ-6 cells decreased the growth rate of the established tumor (23). Opposite to mAb16D10, the mAbJ28 recognizes pancreatic tumor, but it has no effect on tumor growth.[8] Therefore, mAb16D10 could be of interest as therapeutic agents for passive immunization as described for breast cancers (48), provided that these antibodies specifically recognize human pancreatic tumoral tissues *in vivo*. Moreover, we have shown that the 16D10 antigen was expressed in metastatic pancreatic tumor and in tumor from patient after a preoperative chemotherapy. These results suggested that mAb16D10 could be used as a first-line therapeutic strategy in advanced, unresectable pancreatic cancer in combination with chemotherapies as described for cetuximab (49) or bevacizumab (50).

In this article, we showed that the mAb16D10 directed against the *O*-glycosylated COOH-terminal domain of FAPP recognized structures present at the cell surface of human pancreatic tumoral tissues. This mAb recognized 22 tumoral tissues over the 22 tested independently of the tissue pretreatment (frozen sections or formalin-fixed, paraffin-embedded sections) and presented a unique specificity for membranes of neoplastic cells. Furthermore, the mAb16D10 had no reactivity with all nontumoral tissues examined and with all nonpancreatic tumoral and normal tissues tested. Another mAb, the mAb8H8, seemed to be the counterreactive antibody of mAb16D10 as it recognized nontumoral pancreatic tissue but had very poor or even no affinity for tumoral tissue. The same pattern of antibodies reactivity was obtained with pancreatic tissue homogenates in which a protein of the same M_r than that of BSDL or of FAPP was detected in nontumoral tissue using mAb8H8 and in tumoral tissues using mAb16D10, respectively.

Although the size of the cohort used here is still limited, this study confirmed the unique specificity of mAb16D10 for the tumoral human pancreatic tissues. Furthermore, the mAb16D10 and mAb8H8 pair of mAbs might be useful for diagnosis purpose in discriminating nontumoral from tumoral human pancreatic tissues. The potential use of mAb16D10 in the identification of precancerous lesions is actually under investigation. Overall, mAb16D10, which does not react with normal human tissues examined here, could be effective in passive immunologic therapy of exocrine pancreatic tumors as suggested by recent results obtained with model animal (23).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Lombardo D. Bile salt-dependent lipase: its pathophysiological implications. Biochim Biophys Acta 2001;1533:1 – 28.

2. Lombardo D, Guy O. Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters. Biochim Biophys Acta 1980;611: 147 – 55.

3. Shamir R, Johnson WJ, Zolfaghari R, et al. Role of bile salt-dependent cholesteryl ester hydrolase in the uptake of micellar cholesterol by intestinal cells. Biochemistry 1995;34:6351 – 8.

4. Howles P, Carter C, Hui DY. Dietary free and esterified cholesterol absorption in cholesterol esterase (bile salt-stimulated lipase) gene-targeted mice. J Biol Chem 1996;271:7196 – 202.

5. Aubert-Jousset E, Garmy N, Sbarra V, et al. The combinatorial extension method reveals a sphingolipid binding domain on pancreatic bile salt-dependent lipase: role in secretion. Structure (Camb) 2004;12: 1437 – 47.

6. Bruneau N, Lombardo D. Chaperone function of a Grp 94-related protein for folding and transport of the pancreatic bile salt-dependent lipase. J Biol Chem 1995;270:13524 – 33.

7. Bruneau N, Lechene de la Porte P, Sbarra V, et al. Association of bile salt-dependent lipase with membranes of human pancreatic microsomes. Eur J Biochem 1995;233:209 – 18.

8. Nganga A, Bruneau N, Sbarra V, et al. Control of the pancreatic bile salt-dependent lipase secretion by Grp94. Biochem J 2000;352:865 – 74.

9. Abouakil N, Mas E, Bruneau N, et al. Bile salt-dependent lipase biosynthesis in rat pancreatic AR 4-2 J cells. Essential requirement of N-linked oligosaccharide for secretion and expression of a fully active enzyme. J Biol Chem 1993;268:25755 – 63.

10. Bruneau N, Nganga A, Fisher EA, et al. *O*-glycosylation of C-terminal tandem-repeated sequences regulates the secretion of rat pancreatic bile salt-dependent lipase. J Biol Chem 1997;272:27353 – 61.

11. Vérine A, Le Petit-Thévenin J, Panicot-Dubois L, et al. Phosphorylation of human bile salt dependent lipase: identification of phosphorylation site and relation with secretion process. J Biol Chem 2000;276: 12356 – 61.

12. Pasqualini E, Caillol N, Mas E, et al. Association of bile salt-dependent lipase with membranes of human pancreatic microsomes is under the control of ATP and phosphorylation. Biochem J 1997;327:527 – 37.

13. Pasqualini E, Caillol N, Valette A, et al. Phosphorylation of the rat pancreatic bile salt-dependent lipase by casein-kinase II is essential for secretion. Biochem J 2000;345:121 – 8.

14. Escribano MJ, Imperial S. Purification and molecular characterization of FAP, a feto-acinar protein associated with the differentiation of human pancreas. J Biol Chem 1989;264:21865 – 71.

15. Escribano MJ, Cordier J, Nap M, et al. Differentiation antigens in fetal human pancreas. Int J Cancer 1986;38:155 – 60.

16. Albers GHR, Escribano MJ, Gonzalez M, et al. Fetoacinar pancreatic protein in the developing human pancreas. Differentiation 1987;34:210 – 5.

17. Mas E, Abouakil N, Roudani S, et al. Human fetoacinar pancreatic protein: an oncofetal glycoform of the normally secreted pancreatic bile salt-dependent lipase. Biochem J 1993;269:609 – 15.

18. Pasqualini E, Caillol N, Panicot L, et al. Molecular cloning of the oncofetal isoform of the human pancreatic bile salt-dependent lipase. J Biol Chem 1998;273:28208 – 18.

19. Miralles F, Langa F, Mazo A, et al. Retention of the fetoacinar pancreatic (FAP) protein to the endoplasmic reticulum of tumor cells. Eur J Cell Biol 1993;60:115 – 21.

20. Roudani S, Pasqualini E, Margotat A, et al. Expression of a 46 kDa protein in human pancreatic tumors and its possible relationship with the bile salt-dependent lipase. Eur J Cell Biol 1994;64:132 – 44.

21. Caillol N, Pasqualini E, Lloubes R, et al. Impairment of bile salt-dependent lipase secretion in human pancreatic tumoral SOJ-6 cells. J Cell Biochem 2000;79:628 – 47.

22. Le Petit-Thévenin J, Verine A, Nganga A, et al. Impairment of bile salt-dependent lipase secretion in AR4-2J rat pancreatic cells induces its degradation by the proteasome. Biochim Biophys Acta 2001;1530: 184 – 98.

23. Panicot-Dubois L, Aubert M, Franceschi C, et al. Monoclonal antibody 16D10 to the C-terminal domain of the feto-acinar pancreatic protein binds to membrane of human pancreatic tumoral SOJ-6 cells and inhibits the growth of tumor xenografts. Neoplasia 2004;6:713 – 24.

24. Mas E, Crotte C, Lecestre D, et al. The oncofetal J28 epitope involves fucosylated *O*-linked oligosaccharide structures of the fetoacinar pancreatic protein. Glycobiology 1997;7:745 – 55.

25. Panicot L, Mas E, Pasqualini E, et al. The formation of the oncofetal J28 glycotope involves core-2 β6-*N*-acetylglucosaminyltransferase and α3/4- fucosyltransferase activities. Glycobiology 1999;9:935 – 46.

26. Mas E, Pasqualini E, Caillol N, et al. The fucosyltransferase activities in human pancreatic tissues: comparative study between cancer tissues and established cell lines. Glycobiology 1998;8:605 – 13.

27. Benkoel L, Dodero F, Hardwigsen J, et al. Effect of ischemia-reperfusion on bile canalicular F-actin microfilaments in hepatocytes of human liver allograft: image analysis by confocal laser scanning microscopy. Dig Dis Sci 2001;46:1663 – 7.

28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680 – 5.

29. Kakimoto K, Takekoshi S, Miyajima K, Osamura RY. Hypothesis for the mechanism for heat-induced antigen retrieval occurring on fresh frozen sections without formalin-fixation in immunohistochemistry. J Mol Histol 2008;39:389 – 99.

30. Raeder H, Johansson S, Holm PI, et al. Mutations in the CEL VNTR cause a syndrome of diabetes and pancreatic exocrine dysfunction. Nat Genet 2006;38:54 – 62.

31. Yang GY, Wagner TD, Fuss M, Thomas CR. Multimodality approaches for pancreatic cancer. CA Cancer J Clin 2005;55:352 – 67.

32. Ellenrieder V, Adler G, Gress TM. Invasion and metastasis in pancreatic cancer. Ann Oncol 1999;10:46 – 50.

33. Kobayashi T, Kawa S, Tokoo M, et al. Comparative study of CA-50 (time-resolved fluoroimmunoassay), Span-1, and CA 19-9 in the diagnosis of pancreatic cancer. Scand J Gastroenterol 1991;26:787 – 97.

34. Lan MS, Khorrani A, Kaufman B, et al. Molecular characterization of a mucin-type antigen associated with human pancreatic cancers: the Du-Pan 2 antigen. J Biol Chem 1987;262:12863 – 70.

35. Magnani JL, Steplewski Z, Koprowski H, et al. Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patient as mucin. Cancer Res 1983; 43:5489 – 92.

36. Masson P, Palsson B, Andren-Sandberg A. Cancer-associated tumour markers CA 19-9 and CA-50 in patients with pancreatic cancer with special reference to the Lewis blood cell status. Br J Cancer 1990;62: 118 – 21.

37. Prat M, Medico E, Rossino P, et al. Biochemical and immunological properties of the human carcinoma-associated CAR-3 epitope defined by the monoclonal antibody AR-3. Cancer Res 1989;49:1415 – 21.

38. Ozkan H, Kaya M, Cengiz A. Comparison of tumor marker CA 242 with CA 19-9 and carcinoembryonic antigen (CEA) in pancreatic cancer. Hepatogastroenterology 2003;50:1669 – 74.

39. Narimatsu H, Iwasaki H, Nakayama F, et al. Lewis and secretor gene dosages affect CA 19-9 and DU-PAN-2 serum levels in normal individuals and colorectal cancer patients. Cancer Res 1998;58:512 – 8.

40. Jiang JT, Wu CP, Deng HF, et al. Serum level of TSGF, CA242 and CA19-9 in pancreatic cancer. World J Gastroenterol 2004;10:1675 – 7.

41. Kim GE, Bae HI, Park HU, et al. Aberrant expression of MUC5AC and MUC6 gastric mucins and sialyl Tn antigen in intraepithelial neoplasms of the pancreas. Gastroenterology 2002;123:1052 – 60.

42. Kim YS, Gum J, Brockhausen I. Mucin glycoproteins in neoplasia. Glycoconj J 1996;13:693 – 707.

43. Audie JP, Janin A, Porchet N, et al. Expression of human mucin genes in respiratory, digestive, and reproductive tracts ascertained by *in situ* hybridization. J Histochem Cytochem 1993;41:1479 – 85.

44. McCarthy DM, Maitra A, Argani P, et al. Novel markers of pancreatic adenocarcinoma in fine-needle aspiration: mesothelin and prostate stem cell antigen labelling increases accuracy in cytologically borderline cases. Appl Immunohistochem Mol Morphol 2003;11:238 – 43.

45. Mazo A, Fujii Y, Shimotake J, et al. Expression of fetoacinar pancreatic (FAP) protein in the pancreatic human tumor cell line BxPC-3. Pancreas 1991;6:37 – 45.

46. Imperial S, Escribano MJ. Concanavalin A variants of the fetoacinar pancreatic protein in the developing human pancreas and other biological sources. Biochim Biophys Acta 1988;967:25 – 33.

47. Takeda Y, Miralles F, Daher N, et al. Radioimmunolocalization of the monoclonal antibody J28 in early transformation stages in *N*-nitrosobis(2-hydroxypropyl)amine-induced pancreatic tumors in the Syrian golden hamster. J Cancer Res Clin Oncol 1992;118:377 – 85.

48. Walker RA. The significance of histological determination of HER-2 status in breast cancer. Breast 2000;9:130 – 3.

49. Xiong HQ, Rosenberg A, LoBuglio A, et al. Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II trial. J Clin Oncol 2004;22:2610 – 6.

50. Crane CH, Ellis LM, Abbruzzese JL, et al. Phase I trial evaluating the safety of bevacizumab with concurrent radiotherapy and capecitabine in locally advanced pancreatic cancer. J Clin Oncol 2006;24:1145 – 51.