Mucin (*MUC*) Gene Expression in Human Pancreatic Adenocarcinoma and Chronic Pancreatitis: A Potential Role of *MUC4* as a Tumor Marker of Diagnostic Significance[1]

Mahefatiana Andrianifahanana,[2] Nicolas Moniaux,[2] Bruno M. Schmied, Jörg Ringel, Helmut Friess, Michael A. Hollingsworth, Markus W. Büchler, Jean-Pierre Aubert, and Surinder K. Batra[3]

Department of Biochemistry and Molecular Biology [M. A., N. M., J. R., S. K. B.], Eppley Institute for Research in Cancer and Allied Diseases [M. A. H., S. K. B], University of Nebraska Medical Center, Omaha, Nebraska 68198; Unité 377 INSERM, Place de Verdun, 59045 Lille Cedex, France [J-P. A.]; and Department of Visceral and Transplantation Surgery, University of Bern, Inselhospital, Bern, Switzerland 3010 [B. M. S., H. F., M. W. B.]

ABSTRACT

Purpose: **Mucins are important biomolecules that frequently display an altered expression under pathological conditions. In a search for a unique and reliable marker(s) specific for pancreatic adenocarcinoma, we investigated the expression of different *MUC* genes in pancreatic tumors and tumor cell lines, in chronic pancreatitis, and in the normal pancreas.**

Experimental Design: **Total RNA from 16 pancreatic tumors, 10 chronic pancreatitis tissues, 7 normal pancreas tissues, and 15 pancreatic tumor cell lines were analyzed by reverse transcription-PCR with primers specific for *MUC1*, *MUC2*, *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC7* genes and by RNA slot blot analyses.**

Results: **Our results revealed that of all of the mucins examined, only *MUC4* displayed a differential expression that was specific for pancreatic adenocarcinoma. Indeed, a substantial number of tumor tissue samples (12 of 16) and tumor cell lines (11 of 15) expressed *MUC4* mRNA, whereas samples from chronic pancreatitis (0 of 10) and the normal pancreas (0 of 7) tissues failed to exhibit any detectable level of this mucin. In contrast, no significant alteration was observed in the expression of the other mucins relative to that in the normal pancreas samples.**

Conclusions: **Overall, this work demonstrates that pancreatic mucin *MUC4* is a tumor-associated mucin. Furthermore, the present study introduces a novel avenue to discriminate between pancreatic adenocarcinoma and pancreatitis. Future investigations of the role played by *MUC4* in pancreatic adenocarcinoma may prove to be useful in the formulation of strategies for the diagnosis and therapeutic treatment of this malignancy.**

INTRODUCTION

Adenocarcinoma of pancreatic ducts is the fifth leading cause of cancer-related deaths in the United States (1, 2). This type of neoplasia is characterized by a poor prognosis, which can be attributed to a delayed detection of the disease onset and a lack of effective treatment. Despite the efforts made to unveil the principles governing the initiation and progression of this devastating malignancy and to identify the factors that confer its particular aggressiveness, the exact succession of molecular events underlying the development of this devastating malignancy has remained unsolved (3, 4). The management of pancreatic cancer is therefore an ongoing challenge.

From a clinical standpoint, the development of methods that are sensitive and specific enough to permit an early diagnosis of pancreatic adenocarcinoma may greatly facilitate the detection and subsequent treatment of this disease. However, an important factor adds another level of complexity to this already demanding task. In fact, numerous overlapping symptomatological features are known to link pancreatic adenocarcinoma to the inflammatory disease, chronic pancreatitis, thereby, often obscuring the distinction between both pathological cases (3). Furthermore, although these two types of disorders are not necessarily interconnected, several lines of evidence suggest that chronic pancreatitis may precede pancreatic adenocarcinoma and may even constitute a predisposing condition to malignancy (5). A variety of biochemical, genetic, and imaging techniques have been developed for diagnostic purposes and/or for monitoring the outcome of pancreatic adenocarcinoma treatment. Yet, a correct differential diagnosis has not been successfully achieved through the use of these reagents alone (6–9). Likewise, inconclusive results were obtained from alternative methods targeting autoantibodies specific for the tumor suppressor gene *p53*, which is frequently overexpressed and mutated in pancreatic adenocarcinoma (10, 11). On the other hand, the combined implementation of imaging techniques and other biomolecular tools may provide enhanced sensitivity and specificity to the diagnostic procedure (12). Nonetheless, the accuracy of these methods can be compromised by the presence of

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[1] This work was supported in part by the NIH Grant CA 78590.
[2] These authors contributed equally to this work.
[3] To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE 68198-4525. Phone: (402) 559-4555; Fax: (402) 559-6650; E-mail: sbatra@unmc.edu.

lesions of different etiology, which may result in misdiagnoses. In consideration of the potentials and limitations inherent in each of these methods, the need for efficient differential diagnostic markers that specifically discriminate pancreatic adenocarcinoma cannot be overemphasized. In that regard, MUCs[4] may represent potential candidates for such a purpose, with respect to their biochemical properties relating to malignant conditions.

MUCs are high molecular weight glycoproteins that are widely expressed in epithelial cells. Fourteen MUC genes have been identified thus far (13–20). Under normal physiological conditions, MUCs are known to play a protective role for the adjoining epithelial tissues. The involvement of MUCs in the renewal and differentiation of the epithelium, as well as in modulation of cell adhesion and in cell signaling, has also been proposed (15, 21–25). However, in various instances, changes in the biochemical characteristics of MUCs were shown to occur in both preneoplastic and neoplastic lesions (26, 27). Malignant transformation of epithelial cells was generally associated with the abnormal glycosylation of MUCs (28).

In general, MUCs follow a defined spatial and temporal pattern of expression throughout the development of an organism. However, recent studies have demonstrated the association of a deregulated expression of MUCs with various types of malignancies. In pancreatic adenocarcinoma, tumors and tumor cell lines can overexpress *MUC1* (29–31). Additionally, an aberrant expression of *MUC4* in pancreatic tumors and cell lines has been reported, a MUC that is usually undetectable in normal pancreas (32–34).

In the present study, we examined the expression of the well-characterized MUCs (*MUC1–7*) in pancreatic adenocarcinoma and compared the expression profiles with those observed in chronic pancreatitis and normal pancreas samples. The overall goal was to identify a MUC(s) that is differentially expressed in pancreatic adenocarcinoma and that potentially may represent a specific marker of carcinoma. We performed analyses by semiquantitative RT-PCR experiments using primers contained within nonrepetitive sequences of the MUC genes and by RNA slot blotting using probes generated from repetitive MUC sequences. Our data indicate that, among all MUCs tested, only *MUC4* showed an aberrant expression in pancreatic tumors and tumor cell lines. This dysregulation was specific for adenocarcinoma because *MUC4* transcripts remained undetectable in chronic pancreatitis samples. Furthermore, the *MUC4* expression profile observed in pancreatic tumor cell lines may suggest a link between the level of *MUC4* mRNA and the tumor cell differentiation stage. In contrast, no significant alteration was found in the expression patterns of the other MUC genes with respect to normal *versus* diseased states.

MATERIALS AND METHODS

Tissue Specimens and Cell Lines. Sixteen pancreatic adenocarcinoma and 10 chronic pancreatitis tissue samples were obtained at the time of primary surgery from consenting patients. Seven normal pancreatic tissue samples obtained from previously healthy organ donors were also used in this study. Samples were collected under approved protocol by the Institutional Review Board at the University of Nebraska Medical Center, and the Department of Visceral and Transplantation Surgery, University of Bern. Informed consent was obtained from all subjects. Tissue specimens were frozen in liquid nitrogen and stored at −80°C until processing for RNA extraction.

Fifteen pancreatic adenocarcinoma cell lines with different levels of differentiation (poorly, moderately, and well-differentiated) were also examined for MUC expression. These included ASPC-1, BxPC3, CaPan-1, CaPan-2, Colo357, HCG25, HPAC, HPAF, HS766T, MiaPaCa, Panc89, Panc-1, QGP1, SC2P9, and T3M4.

RNA Isolation. Total RNA was isolated from tissue samples and cell lines by the guanidinium isothiocyanate-cesium chloride ultracentrifugation method (35). Tissue samples were manually ground to a fine powder in liquid nitrogen with mortar and pestle and resuspended in guanidinium isothiocyanate buffer. Cultured adenocarcinoma cells at subconfluence were rinsed twice in ice-cold PBS and solubilized in guanidinium isothiocyanate buffer. Total RNAs from tissue and cell homogenates were extracted as described previously (36).

RT-PCR Analysis. Total RNA (1 μg) was reverse transcribed by the SUPERSCRIPT II RNase H^- Reverse Transcriptase system (Life Technologies, Inc.). Samples were subjected to PCR amplification in a final reaction volume of 50 μl containing 5 μl of 10× buffer (Life Technologies, Inc.), 1.5 μl of 50 mM $MgCl_2$ (Life Technologies, Inc.), 5 μl of 10 mM deoxynucleotide triphosphates, 10 pmol of each primer, and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Unless stated otherwise, PCR parameters were as follows: hot start and first denaturation at 94°C for 4 min, followed by 30 cycles (25 cycles for *MUC1*) of denaturation at 94°C for 30 s; primer annealing was carried out at 60°C for 30 s, and elongation at 72°C for 45 s. Final elongation was conducted at 72°C for 15 min. For *MUC3A*, *MUC5AC*, and *MUC6*, amplification was performed with AmpliTaq Gold (1.25 units/50-μl reaction; Perkin-Elmer) in the presence of 10% DMSO (final concentration). PCR conditions were as follows: hot start and first denaturation at 96°C (enzyme activation) for 4 min, followed by 30 cycles of denaturation at 96°C for 30 s; primer annealing was carried out at 60°C for 1 min;, and elongation at 72°C for 45 s. PCR products were electrophoretically resolved on 1% agarose gel stained with ethidium bromide. After exposure to UV light, the density of DNA bands was determined using the GelExpert software system (Nucleotech). Density values for MUC-specific amplification products were normalized against those for the internal control, *GAPDH*. The primers used in this study were generated from regions contained within nonrepetitive sequences of MUC genes (33).

RNA Slot Blot Analysis. Total RNA (10 μg) per sample was denatured in loading buffer containing formamide/formaldehyde and applied to nylon membrane (GeneScreenPlus Hybridization Transfer Membrane; NEN Life Sciences) by vacuum suction with a 48-well slot filtration manifold system (Life Technologies, Inc.). Membranes were prehybridized in 6× saline/sodium phosphate/EDTA, 5× Denhardt's reagent, 0.5% SDS, 50% formamide, and 100 μg/ml sheared salmon sperm

[4] The abbreviations used are: MUC, mucin; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

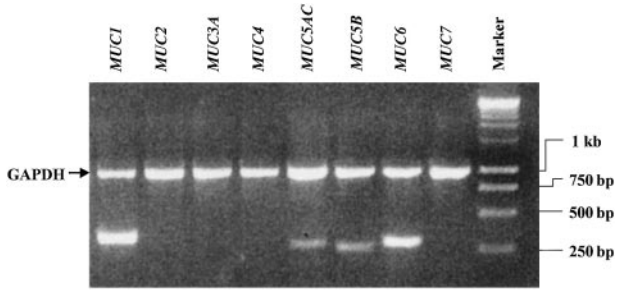

Fig. 1 Expression of MUC genes in normal pancreas. We analyzed total RNA from normal pancreatic tissue by RT-PCR using primers specific for *MUC1*, *MUC2*, *MUC3A*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC7*. *GAPDH* was used as an internal control. PCR products were electrophoresed on 1% agarose gel containing ethidium bromide. Data shown are representative of seven normal pancreatic samples from different individuals.

DNA for 12 h at 42°C. Hybridization was performed under the same conditions with ^{32}P-labeled cDNA probes specific for each of the MUCs tested (37–39). Probes consisted of DNA fragments spanning repetitive MUC sequences. Membranes were washed in 0.1× saline/sodium citrate and 0.1% SDS at 65°C for 30 min and exposed to a PhosphorImager (Molecular Dynamics) screen. We performed subsequent analyses using ImageQuant software (Molecular Dynamics). Membranes were stripped and rehybridized with cDNA probes specific for *GAPDH* to account for any differences in the amount of RNA loaded.

RESULTS

Expression of MUC Genes in Normal Pancreatic Samples. To establish the basis for subsequent experiments and to challenge our system, we first examined the expression of MUC genes in normal pancreatic samples. Total RNAs from tissue samples were subjected to RT-PCR and Northern slot blot analyses. Various tissues of known MUC expression profiles were included as controls. In agreement with data published in earlier reports, we have consistently detected *MUC1*, *MUC5AC*, *MUC5B*, and *MUC6* in normal pancreatic samples (32, 34, 37). In contrast, *MUC2*, *MUC3A*, *MUC4*, and *MUC7* were not expressed in any of the normal pancreatic tissue samples tested (Fig. 1). The authenticity of the PCR amplification products was confirmed by DNA sequencing and comparison with MUC sequences published previously (33). Additionally, the results from RT-PCR analyses paralleled those from slot blotting with the exception of *MUC1* expression in the cell line Panc-1, for which the more sensitive RT-PCR proved more useful in detecting lower levels of MUC transcripts (Fig. 2).

***MUC4* Is Aberrantly Expressed in Pancreatic Adenocarcinoma but not in Chronic Pancreatitis.** Dysregulation of MUC genes is of frequent occurrence in malignancies of epithelial origin. This observation, combined with the biomolecular properties of MUCs, suggests that these molecules have a potential role as tumor markers. We and others have reported previously (32–34) the aberrant expression of *MUC4* in pancreatic adenocarcinoma. We intended to further investigate whether *MUC4* deregulation was specific for pancreatic adenocarcinoma and whether this abnormal expression could facilitate the distinction between pancreatic adenocarcinoma and chronic pancreatitis.

We examined the expression of MUC genes in a panel of 15 pancreatic tumor cell lines, 16 pancreatic tumor tissue samples, and 10 chronic pancreatitis tissue samples. As shown in Tables 1 and 2, results from RT-PCR analyses revealed an aberrant expression of *MUC4* in a large portion of the pancreatic tumors [12 of 16 (75%)] and tumor cell lines [11 of 15 (73%)]. In contrast, none of the chronic pancreatitis samples tested expressed this MUC (Table 3), suggesting that *MUC4* up-regulation was specific for and restricted to pancreatic adenocarcinoma. Furthermore, using the semiquantitative RT-PCR method, we found that the profile of *MUC4* expression in pancreatic tumor cell lines seemed to indicate a correlation between *MUC4* mRNA levels and the differentiation stage of the cell lines. *MUC4* transcripts were not detected in the poorly differentiated cell lines HCG-25 and Panc-1. In contrast, a substantial up-regulation of *MUC4* was observed in moderately (BxPC3, Panc89, and T3M4) and well-differentiated (CaPan-1, CaPan-2, Colo357, HPAF, and S2CP9) cell lines (Table 2). A more pronounced fluctuation of *MUC4* level was recorded in pancreatic tumor samples (Table 1). This was probably attributable to the heterogeneous nature of the tissues under study. Moreover, a close examination of the clinical data on patients did not reveal any obvious relationship between *MUC4* levels and tumor differentiation stage or Tumor-Node-Metastasis scores (Table 2). Although such lack of correlation appears to be in conflict with the observations made in cell lines (Table 1), this disparity may be connected, in part, to the variation in the amount of stromal tissues present in the tumors and to the heterogeneity associated with the sampling procedures. A more systematic and comprehensive analysis of an entire pancreas may be necessary to resolve this issue.

Expression Profiles of other MUC Genes. We further aimed to determine whether any additional information could be obtained from the expression profiles of the other well-characterized MUCs. We subjected the same set of samples analyzed for *MUC4* expression to RT-PCR using primers specific for *MUC1*, *MUC2*, *MUC3A*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC7*. As expected, relatively high levels of *MUC1* transcripts were detected in most pancreatic tumors and tumor cell lines (Tables 1 and 2). This MUC was detected at lower levels in the majority of the chronic pancreatitis samples tested, as compared with the normal pancreatic samples (Table 3). On the other hand, *MUC2* appeared to be up-regulated in 7 of 15 (47%) of the tumor cell lines. However, this is likely to represent an *in vitro* artifact because no *MUC2* transcript could be detected in any of the tumor tissue samples tested. Furthermore, a down-regulation of the secreted MUCs *MUC5AC* [7 of 15 (47%)], *MUC5B* [6 of 15 (40%)], and *MUC6* [12 of 15 (80%)] was observed in tumor cell lines. *MUC3* and *MUC7* remained undetectable in all samples under study. This was identical to the pattern observed in the normal pancreatic samples. In summary, these data demonstrate that, unlike *MUC4*, none of the other MUCs included in our study displayed a distinguishing feature that was specific for pancreatic adenocarcinoma or that may discriminate pancreatic adenocarcinoma from chronic pancreatitis.

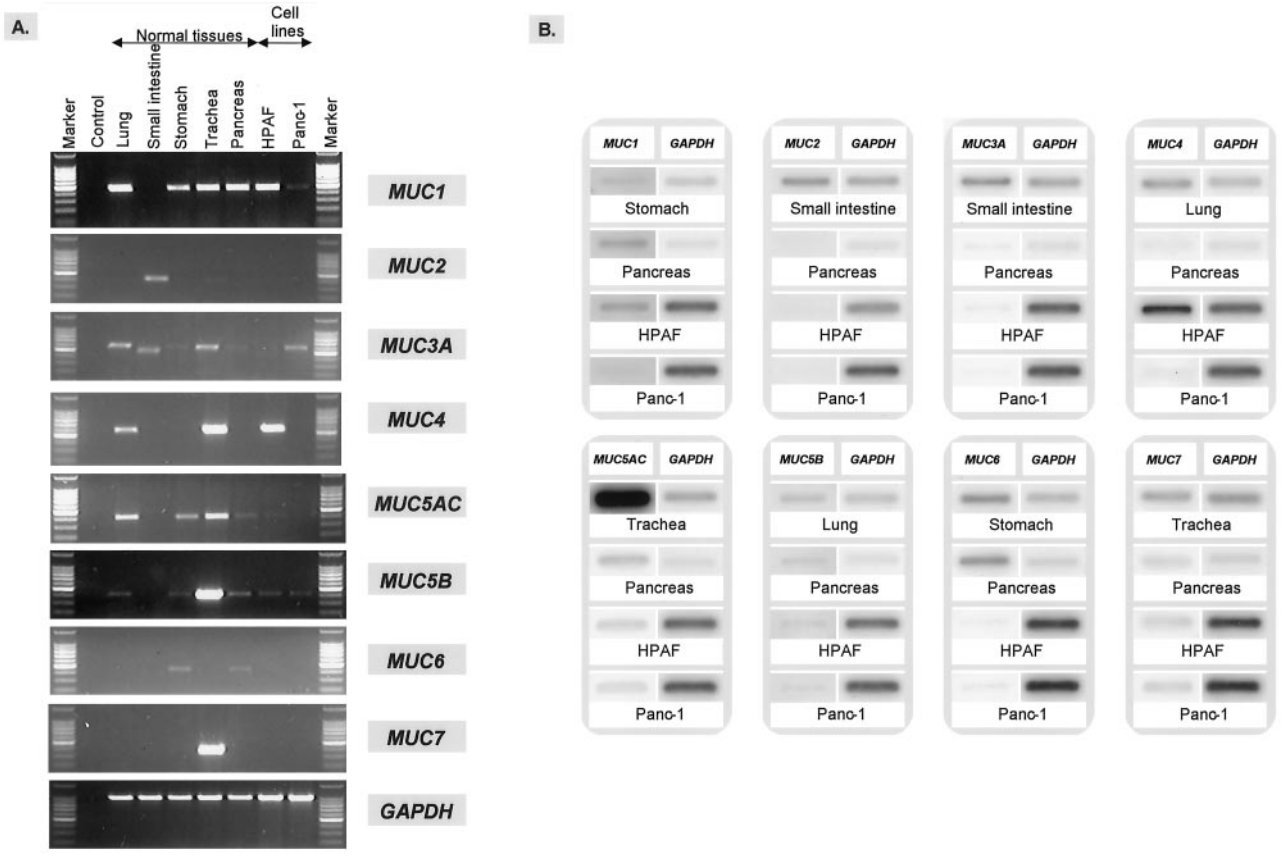

Fig. 2 MUC gene expression by RT-PCR and RNA slot blot analyses. *A*, total RNA from normal tissue samples (lung, pancreas, small intestine, stomach, and trachea) and pancreatic tumor cell lines (HPAF and Panc-1) was analyzed by RT-PCR using primers specific for *MUC1*, *MUC2*, *MUC3A*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, and *MUC7*. *GAPDH* was used as an internal control. PCR products were electrophoresed on 1% agarose gel containing ethidium bromide. The various tissues of known MUC expression profiles were included as controls. *B*, RNA slot blot analysis of MUC gene expression in normal pancreas and pancreatic adenocarcinoma cell lines. Blots were generated and processed as described in "Materials and Methods" with total RNA from normal tissue samples (lung, pancreas, small intestine, stomach, and trachea) and pancreatic adenocarcinoma cell lines (HPAF and Panc-1). All samples were tested in duplicate. Membranes were hybridized with ^{32}P-labeled, MUC-specific cDNA probes, stripped, and rehybridized with a probe specific for *GAPDH* (internal control). For each panel, blots are displayed in pairs with those reacted with MUC- and *GAPDH*-specific probes shown to the *left* and *right* of the panel, respectively. The tissue sources are indicated *below* each pair of blots.

DISCUSSION

Management of pancreatic cancer in humans is one the most puzzling challenges facing clinicians and researchers. In general, the majority of treatment options for pancreatic cancer patients have proven inefficient because of the common refractory response of this malignancy to the widely used treatment methods, leaving surgery as the only "curative" treatment option for eligible patients (40, 41).

However, because of the recent advances in the field of cancer research (42–44), a wealth of information has been made available that can be explored to design strategies aimed at the improvement of pancreatic cancer management. Nonetheless, most of the treatment methodologies that can be envisaged rely on a timely and accurate diagnosis of the disease. Most importantly, the particular aggressiveness and high rate of metastases associated with pancreatic adenocarcinoma represent a considerable impediment to the performance of proper treatment. Furthermore, the development of chronic pancreatitis alone or in the peritumoral regions constitutes an additional complication to the problem. A legitimate approach to circumvent these quandaries would emphasize the identification of specific and reliable tumor-associated molecular markers, which may greatly help achieve an early diagnosis. In the present study, we point out the potential role of MUC4 as a marker for pancreatic adenocarcinoma. Our data clearly demonstrated that MUC4 was aberrantly expressed in the majority of the pancreatic tumors and tumor cell lines investigated. This was in stark contrast to the normal pancreatic and chronic pancreatitis samples, which did not express *MUC4* transcripts at a detectable level. These findings bear a practical significance to the extent that this MUC can be considered as a tumor-associated molecule. Interestingly enough, the inclusion of MUC4 in diagnostic procedures may also eliminate any concerns posed by the potential interference by chronic pancreatitis. Various investigators have proposed the combined use of newer molecular techniques and imaging procedures as an improved alternative to the currently available diagnostic methods (12). The use of MUC4 as a differential diagnostic marker in this context would seem appropriate. On

Table 1 Expression of MUC genes in pancreatic tumor cell lines by RT-PCR analysis

Total RNA from 15 pancreatic tumor cell lines was isolated and subjected to semiquantitative RT-PCR as described in "Materials and Methods." Amplification products were resolved on 1% agarose gel containing ethidium bromide. After exposure to UV light, the density of DNA bands was determined using the GelExpert software system (Nucleotech). Density values for MUC-specific amplification products were normalized against those for the internal control, *GAPDH*.

		Expression level[b]							
Cell lines	D.S.[a]	*MUC1*	*MUC2*	*MUC3A*	*MUC4*	*MUC5AC*	*MUC5B*	*MUC6*	*MUC7*
SC2P9	WD	+	++	−	+++	++	−	−	−
Capan 1	WD	+++	+++	−	++++	+++	+++	+++	−
Capan 2	WD	++	−	−	++++	−	−	−	−
HPAF	WD	+++	+	−	++++	+++	++	−	−
Panc1	PD	+	−	−	−	++	+++	−	−
ASPC-1	ND	+++	−	−	++	−	+++	−	−
HCG25	PD	+++	−	−	−	−	−	−	−
T3M4	MD	++	+++	−	+++	−	+	−	−
Colo357	WD	++++	−	−	+++	++	+++	−	−
BxPC3	MD	+++	++	−	+++	−	+	+++	−
MiaPaCa	ND	+	−	−	−	−	−	−	−
HPAC	ND	++++	−	−	+++	++	−	−	−
QGP1	ND	+	−	−	+++	−	−	−	−
HS766T	ND	+	+	−	−	+++	++	++	−
Panc89	MD	ND	++	−	+++	++	++	−	−
Pancreas[c]		+++	−	−	−	+++	+++	++++	−
Trachea[c]		++	++	−	+++	++++	++++	−	−
S.I.[c]		+	++++	++++	+	−	−	++++	−
S.G.[c]		ND	−	−	+++	−	++	−	++++

[a] D.S., differentiation stage; PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; ND, not determined.
[b] −, no expression; +, low level; ++, moderate level; +++, high level; ++++, very high level; ND, not determined.
[c] Normal tissue samples were included as controls. S.I., small intestine; S.G., salivary gland.

Table 2 Expression of MUC genes in pancreatic adenocarcinoma by RT-PCR analysis

Total RNA from 16 pancreatic tumor samples was isolated and subjected to semiquantitative RT-PCR as described in "Materials and Methods." Amplification products were resolved on 1% agarose gel containing ethidium bromide. After exposure to UV light, the density of DNA bands was determined using the GelExpert software system (Nucleotech). Density values for MUC-specific amplification products were normalized against those for the internal control, *GAPDH*.

	Clinical data				Expression level[c]							
Sample	Age (yr)	Sex	D.S.[a]	TNM[b]	*MUC1*	*MUC2*	*MUC3A*	*MUC4*	*MUC5AC*	*MUC5B*	*MUC6*	*MUC7*
1	84	F	3	200	+++[c]	−	−	−	−	+	++++	−
2	67	M	2	100	+++	−	−	+	−	+	+	−
3	73	F	2	210	++++	−	−	−	−	+	+	−
4	57	M	2	210	++	−	−	−	−	+	+	−
5	74	F	3	310	++++	−	−	+++	−	++	+	−
6	74	F	2	310	+++	−	−	+	−	+	++	−
7	63	M	3	310	++	−	−	+	−	+	+	−
8	60	F	2	210	++++	−	−	+	++	++++	++	−
9	72	F	3	300	+++	−	−	+	−	+	+	−
10	50	F	2	210	+++	−	−	++	−	+	++	−
11	43	F	2	300	+++	−	−	++++	−	+	++	−
12	75	F	2	310	++++	−	−	++++	−	+	+	−
13	73	F	1	300	++	−	−	−	−	+	+	−
14	64	M	1	100	+++	−	−	++	−	+++	+++	−
15	77	F	4	410	++++	−	−	++	−	+	+	−
16	62	M	3	310	+	−	−	+	−	+	++++	−

[a] D.S., differentiation stage.
[b] Tumor-Node-Metastasis classification.
[c] −, no expression; +, low level; ++, moderate level; +++, high level; ++++, very high level.

the other hand, on the basis of semiquantitative RT-PCR analyses of tumor cell lines, a correlation appeared to exist between the level of *MUC4* expression and the degree of differentiation of the cell lines under study. This may further facilitate the detection of *MUC4* in biopsies, because a more elevated expression of *MUC4* was observed in moderately and well-differentiated cell lines as compared with their poorly differentiated counterpart. Studies using "real-time" PCR are under way in our laboratory to confirm these observations and to evaluate the situation in tumor tissues.

Table 3 Expression of MUC genes in chronic pancreatitis by RT-PCR analysis

Total RNA from 10 chronic pancreatitis samples was isolated and subjected to semiquantitative RT-PCR as described in "Materials and Methods." Amplification products were resolved on 1% agarose gel containing ethidium bromide. After exposure to UV light, the density of DNA bands was determined using the GelExpert software system (Nucleotech). Density values for MUC-specific amplification products were normalized against those for the internal control, *GAPDH*.

	Expression level[a]							
Sample	*MUC1*	*MUC2*	*MUC3A*	*MUC4*	*MUC5AC*	*MUC5B*	*MUC6*	*MUC7*
1	+[a]	−	−	−	−	++++	++	−
2	+	−	−	−	−	+++	++	−
3	+	−	−	−	−	−	++	−
4	+	−	−	−	++	++	++++	−
5	+	−	−	−	−	++	++	−
6	+	−	−	−	−	+	++	−
7	++++	−	−	−	−	++	++	−
8	+	−	−	−	−	+	++++	−
9	+	−	−	−	−	++	+++	−
10	+	−	−	−	−	++	++	−

[a] −, no expression; +, low level; ++, moderate level; +++, high level; ++++, very high level.

Previous studies have documented the expression profiles of MUCs in the human pancreas under normal and pathological conditions (32, 45–50). The analytical methods used in these studies primarily included immunohistochemistry, *in situ* hybridization, and Northern blotting. On one hand, despite the valuable information that can be obtained from immunohistochemical and *in situ* hybridization analyses, these techniques are inherently characterized by a restricted versatility, because they do not generally allow for a concomitant examination of multiple MUCs in individual samples. On the other hand, the use of Northern blotting may help overcome such a limitation; however, the repetitive nature of the probe sequences used in earlier studies is likely to compromise the specificity of this technique to some extent. Here, our experiments were primarily based on the RT-PCR protocol, which has provided us with the advantage of using primers contained within unique, nonrepetitive MUC sequences, thereby conferring enhanced sensitivity and specificity to our assays. Indeed, under our experimental conditions (RT-PCR), we were unable to detect *MUC3A* in any of the pancreatic samples (normal and diseased) tested. This was in conflict with the apparently nonspecific positive signal from assays on the basis of repetitive-sequence probes (Fig. 2; Refs. 32 and 50). In consideration of the sequence similarities among *MUC3A*, *MUC11*, and *MUC12* (19), we suspect that the detection of *MUC3A* in pancreatic samples reported in previous studies may have resulted from a cross-hybridization with *MUC11* and/or *MUC12*. In support of these statements, we have screened a large number of pancreatic tissue samples (normal and tumor) for *MUC11* and *MUC12* expression and have consistently detected transcripts for these MUCs in all specimens analyzed.[5]

In conclusion, we propose that MUC4 is a tumor-associated MUC with potential use as a diagnostic molecular marker. In addition, our work introduces a novel avenue to discriminate between pancreatic adenocarcinoma and chronic pancreatitis. These findings may impact the development of highly specific techniques that allow the detection of the MUC4 antigen in the circulation, which, in turn, may represent a significant step toward the diagnosis of pancreatic adenocarcinoma at its earliest stage of development. In that regard, the results from another study conducted in our laboratory strongly corroborate these statements. Indeed, we have demonstrated through a RT-PCR-based screening procedure that *MUC4* mRNA was expressed in the peripheral blood mononuclear cells of pancreatic cancer patients but remained undetectable in those of healthy individuals and of patients affected by other types of cancer (51). These observations constitute a clear indication of the potential use of MUC4 as a powerful tumor cell-independent marker of pancreatic cancer. It should be noted, however, that although the aberrant expression of *MUC4* in pancreatic tumors and tumor cell lines was shown to occur at a relatively high frequency, a comparatively smaller, yet not negligible, fraction of these pathological samples failed to express transcripts of the target molecule, thereby evading the detection system (Tables 1 and 2). To minimize the incidence of potential false-negative results, a combination of the MUC4-based detection assay with some of the protocols established previously (12, 52–54) may be necessary.

Overall, further studies on the biological characterization of this molecule should yield valuable information that may help elucidate the mechanisms underlying human pancreatic carcinogenesis and that may facilitate the design of therapeutic strategies.

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REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P.A. Cancer statistics. CA Cancer J. Clin., *49:* 8–31, 1999.

2. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1997. CA Cancer J. Clin., *47:* 5–27, 1997.

3. Warshaw, A. L., and Fernandez-del, C. C. Pancreatic carcinoma. N. Engl. J. Med., *326:* 455–465, 1992.

4. Reber, H. A. Pancreatic Cancer: Pathogenesis, Diagnosis, and Treatment. Totowa, NJ: Humana Press, 1998.

5. Lowenfels, A. B., Maisonneuve, P., and Lankisch, P. G. Chronic pancreatitis and other risk factors for pancreatic cancer. Gastroenterol. Clin. North Am., *28:* 673–685, 1999.

6. DelMaschio, A., Vanzulli, A., Sironi, S., Castrucci, M., Mellone, R., Staudacher, C., Carlucci, M., Zerbi, A., Parolini, D., and Faravelli, A. Pancreatic cancer *versus* chronic pancreatitis: diagnosis with CA 19-9 assessment, US, CT, and CT-guided fine-needle biopsy. Radiology, *178:* 95–99, 1991.

7. Mahvi, D. M., Seigler, H. F., Meyers, W. C., Kalthoff, H., Schmiegel, W. H., and Metzgar, R. S. DU-PAN-2 levels in serum and pancreatic ductal fluids of patients with benign and malignant pancreatic disease. Pancreas, *3:* 488–493, 1988.

8. Fernandez-del, C. C., and Warshaw, A. L. Diagnosis and preoperative evaluation of pancreatic cancer, with implications for management. Gastroenterol. Clin. North Am., *19:* 915–933, 1990.

9. Schmiegel, W. Tumor markers in pancreatic cancer—current concepts. Hepatogastroenterology, *36:* 446–449, 1989.

10. Luo, J. C., Neugut, A. I., Garbowski, G., Forde, K. A., Treat, M., Smith, S., Carney, W. P., and Brandt-Rauf, P. W. Levels of p53 antigen in the plasma of patients with adenomas and carcinomas of the colon. Cancer Lett., *91:* 235–240, 1995.

11. Marxsen, J., Schmiegel, W., Roder, C., Harder, R., Juhl, H., Henne-Bruns, D., Kremer, B., and Kalthoff, H. Detection of the anti-p53 antibody response in malignant and benign pancreatic disease. Br. J. Cancer, *70:* 1031–1034, 1994.

12. Lowenfels, A. B., and Maisonneuve, P. Pancreatic cancer: development of a unifying etiologic concept. Ann. NY Acad. Sci., *880:* 191–200, 1999.

13. Gendler, S. J., and Spicer, A. P. Epithelial mucin genes. Annu. Rev. Physiol., *57:* 607–634, 1995.

14. Lapensee, L., Paquette, Y., and Bleau, G. Allelic polymorphism and chromosomal localization of the human oviductin gene (*MUC9*). Fertil. Steril., *68:* 702–708, 1997.

15. Porchet, N., Buisine, M. P., Desseyn, J. L., Moniaux, N., Nollet, S., Degand, P., Pigny, P., Van, S., I, Laine, A., and Aubert, J. P. MUC genes: a superfamily of genes? Towards a functional classification of human apomucins. J. Soc. Biol., *193:* 85–99, 1999.

16. Pratt, W. S., Crawley, S., Hicks, J., Ho, J., Nash, M., Kim, Y. S., Gum, J. R., and Swallow, D. M. Multiple transcripts of MUC3: evidence for two genes, *MUC3A* and *MUC3B*. Biochem. Biophys. Res. Commun., *275:* 916–923, 2000.

17. Seregni, E., Botti, C., Massaron, S., Lombardo, C., Capobianco, A., Bogni, A., and Bombardieri, E. Structure, function and gene expression of epithelial mucins. Tumori, *83:* 625–632, 1997.

18. Shankar, V., Pichan, P., Eddy, R. L. J., Tonk, V., Nowak, N., Sait, S. N., Shows, T. B., Schultz, R. E., Gotway, G., Elkins, R. C., Gilmore, M. S., and Sachdev, G. P. Chromosomal localization of a human mucin gene (*MUC8*) and cloning of the cDNA corresponding to the carboxyl terminus. Am. J. Respir. Cell Mol. Biol., *16:* 232–241, 1997.

19. Williams, S. J., McGuckin, M. A., Gotley, D. C., Eyre, H. J., Sutherland, G. R., and Antalis, T. M. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. Cancer Res., *59:* 4083–4089, 1999.

20. Williams, S. J., Wreschner, D. H., Tran, M., Eyre, H. J., Sutherland, G. R., and McGuckin, M. A. MUC13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J. Biol. Chem., *276:* 18327–18336, 2001.

21. Aplin, J. D. MUC-1 glycosylation in endometrium: possible roles of the apical glycocalyx at implantation. Hum. Reprod., *14* (Suppl. 2)*:* 17–25, 1999.

22. Komatsu, M., Jepson, S., Arango, M. E., Carothers, C. C., and Carraway, K. L. Muc4/sialomucin complex, an intramembrane modulator of ErbB2/HER2/Neu, potentiates primary tumor growth and suppresses apoptosis in a xenotransplanted tumor. Oncogene, *20:* 461–470, 2001.

23. von Mensdorff-Pouilly, S., Snijdewint, F. G., Verstraeten, A. A., Verheijen, R. H., and Kenemans, P. Human MUC1 mucin: a multifaceted glycoprotein. Int. J. Biol. Mark., *15:* 343–356, 2000.

24. Wesseling, J., van der Valk, S. W., Vos, H. L., Sonnenberg, A., and Hilkens, J. Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J. Cell Biol., *129:* 255–265, 1995.

25. Wesseling, J., van der Valk, S. W., and Hilkens, J. A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1. Mol. Biol. Cell, *7:* 565–577, 1996.

26. Filipe, M. I. Mucins in the human gastrointestinal epithelium: a review. Investig. Cell Pathol., *2:* 195–216, 1979.

27. Filipe, M. I., Potet, F., Bogomoletz, W. V., Dawson, P. A., Fabiani, B., Chauveinc, P., Fenzy, A., Gazzard, B., Goldfain, D., and Zeegen, R. Incomplete sulphomucin-secreting intestinal metaplasia for gastric cancer. Preliminary data from a prospective study from three centres. Gut, *26:* 1319–1326, 1985.

28. Hakomori, S. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. Adv. Cancer Res., *52:* 257–331, 1989.

29. Ho, J. J., and Kim, Y. S. Serological pancreatic tumor markers and the MUC1 apomucin. Pancreas, *9:* 674–691, 1994.

30. Satoh, S., Hinoda, Y., Hayashi, T., Burdick, M. D., Imai, K., and Hollingsworth, M. A. Enhancement of metastatic properties of pancreatic cancer cells by *MUC1* gene encoding an anti-adhesion molecule. Int. J. Cancer, *88:* 507–518, 2000.

31. Yonezawa, S., and Sato, E. Expression of mucin antigens in human cancers and its relationship with malignancy potential. Pathol. Int., *47:* 813–830, 1997.

32. Balague, C., Audie, J. P., Porchet, N., and Real, F. X. *In situ* hybridization shows distinct patterns of mucin gene expression in normal, benign, and malignant pancreas tissues. Gastroenterology, *109:* 953–964, 1995.

33. Choudhury, A., Singh, R. K., Moniaux, N., El-Metwally, T. H., Aubert, J. P., and Batra, S. K. Retinoic acid-dependent transforming growth factor-β2-mediated induction of MUC4 mucin expression in human pancreatic tumor cells follows retinoic acid receptor-aα signaling pathway. J. Biol. Chem., *275:* 33929–33936, 2000.

34. Hollingsworth, M. A., Strawhecker, J. M., Caffrey, T. C., and Mack, D. R. Expression of MUC1, MUC2, MUC3 and MUC4 mucin mRNAs in human pancreatic and intestinal tumor cell lines. Int. J. Cancer, *57:* 198–203, 1994.

35. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, *18:* 5294–5299, 1979.

36. Debailleul, V., Laine, A., Huet, G., Mathon, P., d'Hooghe, M. C., Aubert, J. P., and Porchet, N. Human mucin genes *MUC2*, *MUC3*, *MUC4*, *MUC5AC*, *MUC5B*, and *MUC6* express stable and extremely large mRNAs and exhibit a variable length polymorphism. An improved method to analyze large mRNAs. J. Biol. Chem., *273:* 881–890, 1998.

37. Choudhury, A., Moniaux, N., Winpenny, J. P., Hollingsworth, M. A., Aubert, J. P., and Batra, S. K. Human MUC4 mucin cDNA and its variants in pancreatic carcinoma. J. Biochem. (Tokyo), *128:* 233–243, 2000.

38. Lan, M. S., Batra, S. K., Qi, W. N., Metzgar, R. S., and Hollingsworth, M. A. Cloning and sequencing of a human pancreatic tumor mucin cDNA. J. Biol. Chem., *265:* 15294–15299, 1990.

39. Nguyen, V. C., Aubert, J. P., Gross, M. S., Porchet, N., Degand, P., and Frezal, J. Assignment of human tracheobronchial mucin gene(s) to

11p15 and a tracheobronchial mucin-related sequence to chromosome 13. Hum. Genet., *86:* 167–172, 1990.

40. Farrell, T. J., Barbot, D. J., and Rosato, F. E. Pancreatic resection combined with intraoperative radiation therapy for pancreatic cancer. Ann. Surg., *226:* 66–69, 1997.

41. Link, K. H., Gansauge, F., Gorich, J., Leder, G. H., Rilinger, N., and Beger, H. G. Palliative and adjuvant regional chemotherapy in pancreatic cancer. Eur. J. Surg. Oncol., *23:* 409–414, 1997.

42. Colcher, D., Pavlinkova, G., Beresford, G., Booth, B. J., and Batra, S. K. Single-chain antibodies in pancreatic cancer. Ann. NY Acad. Sci., *880:* 263–280, 1999.

43. Miknyoczki, S. J., Dionne, C. A., Klein-Szanto, A. J., and Ruggeri, B. A. The novel Trk receptor tyrosine kinase inhibitor CEP-701 (KT-5555) exhibits antitumor efficacy against human pancreatic carcinoma (Panc1) xenograft growth and *in vivo* invasiveness. Ann. NY Acad. Sci., *880:* 252–262, 1999.

44. Jones, L., Ghaneh, P., Humphreys, M., and Neoptolemos, J. P. The matrix metalloproteinases and their inhibitors in the treatment of pancreatic cancer. Ann. NY Acad. Sci., *880:* 288–307, 1999.

45. Balague, C., Gambus, G., Carrato, C., Porchet, N., Aubert, J. P., Kim, Y. S., and Real, F. X. Altered expression of *MUC2*, *MUC4*, and *MUC5* mucin genes in pancreas tissues and cancer cell lines. Gastroenterology, *106:* 1054–1061, 1994.

46. Buisine, M. P., Devisme, L., Degand, P., Dieu, M. C., Gosselin, B., Copin, M. C., Aubert, J. P., and Porchet, N. Developmental mucin gene expression in the gastroduodenal tract and accessory digestive glands. II. Duodenum and liver, gallbladder, and pancreas. J. Histochem. Cytochem., *48:* 1667–1676, 2000.

47. Masaki, Y., Oka, M., Ogura, Y., Ueno, T., Nishihara, K., Tangoku, A., Takahashi, M., Yamamoto, M., and Irimura, T. Sialylated MUC1 mucin expression in normal pancreas, benign pancreatic lesions, and pancreatic ductal adenocarcinoma. Hepatogastroenterology, *46:* 2240–2245, 1999.

48. Monges, G. M., Mathoulin-Portier, M. P., Acres, R. B., Houvenaeghel, G. F., Giovannini, M. F., Seitz, J. F., Bardou, V. J., Payan, M. J., and Olive, D. Differential MUC 1 expression in normal and neoplastic human pancreatic tissue. An immunohistochemical study of 60 samples. Am. J. Clin. Pathol., *112:* 635–640, 1999.

49. Reid, C. J., and Harris, A. Developmental expression of mucin genes in the human gastrointestinal system. Gut, *42:* 220–226, 1998.

50. Terada, T., Ohta, T., Sasaki, M., Nakanuma, Y., and Kim, Y. S. Expression of MUC apomucins in normal pancreas and pancreatic tumours. J. Pathol., *180:* 160–165, 1996.

51. Ringel, J., Faulmann, G., Brandt, R., Moniaux, N., Tarantolo, St., Joshi, S., Loehr, M., and Batra, S. K. MUC4 mRNA in peripheral blood mononuclear cells (PBMC) as a potential tumor marker for pancreatic cancer. Proc. Am. Assoc. Cancer Res., *42:* 616, 2001.

52. Eskelinen, M. J., and Haglund, U. H. Prognosis of human pancreatic adenocarcinoma: review of clinical and histopathological variables and possible uses of new molecular methods. Eur. J. Surg., *165:* 292–306, 1999.

53. Adamek, H. E., Albert, J., Breer, H., Weitz, M., Schilling, D., and Riemann, J. F. Pancreatic cancer detection with magnetic resonance cholangiopancreatography and endoscopic retrograde cholangiopancreatography: a prospective controlled study. Lancet, *356:* 190–193, 2000.

54. Hosten, N., Lemke, A. J., Wiedenmann, B., Bohmig, M., and Rosewicz, S. Combined imaging techniques for pancreatic cancer. Lancet, *356:* 909–910, 2000.

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Mahefatiana Andrianifahanana, Nicolas Moniaux, Bruno M. Schmied, et al.

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