PAM4- Reactive MUC1 Is a Biomarker for Early Pancreatic Adenocarcinoma

David V. Gold,1 Zarir Karanjawala,2 David E. Modrak,1 David M. Goldenberg,1 and Ralph H. Hruban2

Abstract

Purpose: The anti-MUC1 monoclonal antibody (MAb), PAM4, has a high specificity for pancreatic adenocarcinoma compared with other cancers, normal tissues, or pancreatitis. In order to assess its role in early pancreatic cancer development, we examined the expression of the PAM4-reactive MUC1 in the noninvasive precursor lesions, pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasia (IPMN).

Experimental Design: Tissue microarrays prepared from formalin-fixed, paraffin-embedded specimens were assessed by immunohistology for expression of the PAM4-reactive, non–variable number of tandem repeats (VNTR), MUC1 epitope, and the VNTR epitope bound by the MA5 MAb.

Results: The PAM4-reactive MUC1 epitope was not detected in normal pancreas but was expressed in 87% (48 of 55) of invasive pancreatic adenocarcinomas, including early stage 1 disease: PAM4 labeled 94% (44 of 47) of the earliest PanIN lesions, PanIN-1A and 1B, along with 91% (10 of 11) of PanIN-2, 40% (2 of 5) of PanIN-3, and 86% (31 of 36) of intraductal papillary mucinous neoplasia lesions. A mostly diffuse pattern of labeling was observed. A second, unrelated, anti-MUC1 MAb, MA5, showed considerably less sensitivity with early PanIN-1 lesions; only 61% (25 of 41) were positive and the labeling did not differentiate normal pancreas from PanINs.

Conclusions: The results suggest that expression of the PAM4-reactive antigen may represent an early event in the development of invasive pancreatic adenocarcinoma, and is unrelated to the VNTR peptide core epitopes of MUC1. Detection of this biomarker using immunohistology, in vitro immunoassays, and in vivo antibody–based imaging may provide new opportunities for the early detection and improved diagnosis of pancreatic cancer.

Most patients with pancreatic cancer do not develop symptoms until after the disease has metastasized. The early detection and diagnosis of pancreatic cancer, as well as appropriate staging of the disease, would almost certainly provide a survival advantage. With few options currently available, there remains a critical need for the development of procedures for the accurate detection of early pancreatic cancer.

A growing body of evidence supports the view that pancreatic adenocarcinoma can arise from histologically well-defined noninvasive lesions within the pancreatic ducts (1–3). These precursor lesions include pancreatic intraepithelial neoplasias (PanIN), and the larger intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (3). These precursors are true neoplasms that share many of the aberrant genetic alterations and expression of biomarkers characteristic of invasive adenocarcinoma (4–8). It has been proposed that PanIN lesions progress from the early PanIN-1A (flat epithelium with minimal atypia) and PanIN-1B (papillary epithelium with minimal atypia) to PanIN-2 (papillary epithelium with moderate atypia) to PanIN-3 (papillary epithelium with marked atypia), and eventually invasive adenocarcinomas.

In an effort to improve the early detection of pancreatic neoplasia, several investigators have examined PanIN lesions for specific genetic and protein biomarkers. These include survivin (9), Her2neu (10, 11), mesothelin (2), p53 (2), DPC4 (2, 12), and several mucin species (MUC1, MUC4, MUC5AC, etc. refs. 2, 6, 13–15) among others. For the most part, detection of these biomarkers has been shown by the use of immunohistochemical labeling of tissues. Certain biomarkers (e.g., mucins) are highly complex molecules that may, or may not, display specific epitope structures at varying stages in the progression from PanIN-1 to invasive adenocarcinoma. Indeed, discrepancies are apparent when MUC1 is identified by the use of monoclonal antibodies (MAb) that react with the variable number of tandem repeats (VNTR) core peptide versus MAbs reactive with glycosylated structures. MUC1 may be reported as present or absent from normal tissues, precursor lesions, and/or adenocarcinoma based on the specific MAb being used. Thus, caution is needed in determining which events (i.e., expression
or suppression of specific genes and/or proteins) occur at early or late stages of neoplastic progression.

We have developed and characterized the PAM4 MAb, which shows high specificity for a MUC1 expressed by pancreatic adenocarcinoma as compared with other forms of cancer, normal pancreas or other organs, or pancreatitis (16). We have also reported its sensitivity and specificity for pancreatic cancer in a serum immunoassay that showed differentiation from pancreatitis (17), its use for nuclear imaging of invasive pancreatic adenocarcinoma (18–20), and its potential for a targeted therapy of this disease (21, 22). In the present studies, we evaluated the expression of PAM4-reactive MUC1 in early pancreatic neoplasia, PanIN and IPMN lesions, two well-defined noninvasive precursors of pancreatic adenocarcinoma, in order to reveal the pattern of epitope expression in association with the development of invasive carcinoma. We found that, although absent from normal ductal epithelium, PAM4-reactive MUC1 is abundantly present in the earliest stages of disease (i.e., PanIN-1A), and that its expression remains high in all PanIN stages. Furthermore, we have confirmed and significantly extended our previous observations regarding the tissue-specific nature of the PAM4-reactive epitope as a biomarker for invasive pancreatic adenocarcinoma.

### Materials and Methods

**Tissue specimens.** Tissue microarrays were created from a total of 63 PanIN and 36 IPMN lesions identified within formalin-fixed, paraffin-embedded tumor specimens from patients who underwent pancreatectomy for pancreatic cancer at the Johns Hopkins Hospital (2, 3). These
samples were collected with the approval of the Johns Hopkins Institutional Review Board. All other tissue microarrays were purchased, either from ISUABXIS through Accurate Chemical & Scientific Corp. (pancreatic cancer A207-II and -IV, hepatocellular carcinoma A204, stomach cancer A209-II, colon cancer A203, ovarian cancer A213, breast cancer A202, and lung cancer A206-III) or USBiomax, Inc. (FDA-801 and FDA-802, normal adult tissues).

**Immunohistochemistry.** Immunohistochemistry was done essentially as described previously (2). Unstained sections were deparaffinized by routine methods. The microarrays were then heated to 95°C for 20 min in a pH 6.0 citrate buffer, Target Retrieval Solution (Dako), allowed to cool to room temperature, and then quenched with 3% H2O2 for 15 min at room temperature. Primary antibodies PAM4 anti-MUC1 (16), MA5 anti-MUC1 (23), and nonbinding control Ag8 were then used at 1 μg/mL with an ABC Vectastain kit (Vector Laboratories) for labeling the tissues. The scoring criteria used were consistent with that reported for earlier studies on biomarkers in pancreatic adenocarcinoma (2): 0—negative, <1% of the tissue core is labeled; 1—a weak, focal labeling of between 1% and 25% of the tissue; 2—a strong, focal labeling of between 1% and 25% of the tissue; 3—a weak, diffuse labeling of >25% of the tissue; 4—a strong, diffuse labeling of >25% of the tissue. Only the appropriate tissue components (e.g., adenocarcinoma cells, normal ducts, etc.) were considered for assessment.

**Statistical analyses.** Statistical differences were determined by comparison of antigen content as defined by the above scoring paradigm. Comparison of any two groups was done by use of a Student’s t test. Receiver operating characteristic curves were generated by use of the Med-Calc statistical software package (version 7.5; Med-Calc).

---

### Table 1. Expression of PAM4- and MA5-reactive MUC1 in normal and neoplastic pancreatic tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>PAM4</th>
<th>MA5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Focal</td>
<td>Diffuse</td>
</tr>
<tr>
<td>Normal</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PanIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-1A</td>
<td>27/25</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>-1B</td>
<td>20/16</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>-2</td>
<td>11/8</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>-3</td>
<td>5/7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>63/56</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>IPMN</td>
<td>36/35</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>13</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Moderately different</td>
<td>24/25</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>18</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>55/56</td>
<td>13</td>
<td>35</td>
</tr>
</tbody>
</table>

**NOTE:** Values for n/n (number of specimens examined) reflect different numbers of specimens examined with the antibodies PAM4 and MA5. Focal, 1% to 25% of the appropriate tissue components labeled with the indicated MAb; Diffuse, >25% of the appropriate tissue components labeled with the indicated MAb; Total, focal + diffuse.
Results

We evaluated tissue microarrays containing cores from normal pancreas, predominantly, surgically removed, non-neoplastic tissue adjacent to tumor, PanIN, IPMN, and invasive adenocarcinoma for the expression of PAM4-reactive MUC1. In no instance was MAb-PAM4 reactive with normal pancreatic tissues, including acinar, ductal, and islet cells. However, MA5, an anti-MUC1 antibody against the VNTR peptide core, reacted with all but one of the normal pancreas specimens, detecting a pattern of MUC1 expression in the cytoplasm and cell surface of both acinar and ductal cells, but not islet cells (Fig. 1A and B).

In contrast to the absence of labeling observed in normal pancreatic tissues, PAM4-reactive MUC1 was identified in 87% of invasive adenocarcinomas examined (n = 55; Fig. 1C and D). Statistically significant differences in labeling were observed for specimens grouped by grade of differentiation, with a trend towards higher expression of the specific PAM4 epitope within glandular structures within other MUC1-producing organs.

Only a few specimens from nonpancreatic, MUC1-producing cancers were considered positive with PAM4 (Table 3). None of the hepatocellular or breast cancers were positive. However, PAM4 was reactive with small numbers of adenocarcinomas (4 of 19) and signet ring (2 of 14) gastric cancers, colorectal adenocarcinomas (7 of 76), as well as mucinous ovarian (4 of 5) and lung adenocarcinomas (4 of 24). In contrast to the mostly intense, diffuse staining pattern observed with pancreatic neoplasms, labeling patterns within these nonpancreatic specimens were predominantly focal and of low intensity. It should be noted that PAM4 was previously reported as reactive with 10 of 26 (38%) colorectal cancers, whereas for the present study, the percentage of positive specimens was <10%. Differences in the labeling procedure and scoring rubric used might account for this discrepancy; however, the significantly larger number of specimens examined for the current studies, coupled with use of the control MA5, anti-MUC1 MAb, supports the conclusion that PAM4 is relatively nonreactive with colorectal cancers.

A total of 320 invasive cancer specimens were examined in this study. Receiver operating characteristic curves were generated to compare PAM4 reactivity in pancreatic adenocarcinoma versus all nonpancreatic cancers (Fig. 3). A positive

| Table 2. Expression of PAM4- and MA5-reactive MUC1 in normal tissues |
|-------------------|---|---|---|---|---|---|
| Tissue          | n | Focal | Diffuse | Total | Focal | Diffuse | Total |
| Pancreas        | 11 | 0   | 0   | 0   | 2    | 8     | 10    |
| Liver           | 7  | 0   | 0   | 0   | 0    | 0     | 0     |
| Salivary gland  | 3  | 0   | 0   | 0   | 1    | 0     | 1     |
| Esophagus       | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Stomach         | 11 | 0   | 0   | 10  | 0    | 5     | 5     |
| Intestine       | 3  | 0   | 0   | 0   | 1    | 0     | 1     |
| Colon           | 16 | 2   | 2   | 4   | 3    | 6     | 9     |
| Breast          | 3  | 0   | 0   | 0   | 3    | 0     | 3     |
| Ovary           | 8  | 0   | 0   | 0   | 0    | 0     | 0     |
| Uterus          | 3  | 0   | 0   | 0   | 1    | 2     | 3     |
| Cervix          | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Prostate        | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Testis          | 3  | 0   | 0   | 0   | 0    | 2     | 2     |
| Heart           | 2  | 0   | 0   | 0   | 0    | 0     | 0     |
| Kidney          | 3  | 0   | 0   | 0   | 0    | 3     | 3     |
| Lung            | 11 | 0   | 0   | 0   | 1    | 9     | 10    |
| Bone            | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Marrow          | 30 | 0   | 0   | 0   | 0    | 0     | 0     |
| Spleen          | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Thymus          | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Striated Muscle | 3  | 0   | 0   | 0   | 0    | 0     | 0     |
| Skin            | 3  | 0   | 0   | 0   | 0    | 0     | 0     |

NOTE: Focal, 1% to 25% of the appropriate tissue components labeled with the indicated MAb; Diffuse, >25% of the appropriate tissue components labeled with the indicated MAb. Total, Focal + Diffuse.
PAM4 reaction (i.e., score >0) gave sensitivity and specificity values of 87.3% (95% confidence interval, 75.5–94.7%) and 92.1% (95% confidence interval, 88.1–95.0%), respectively, with a positive diagnostic likelihood ratio of 11.0, for the diagnosis of invasive pancreatic carcinoma.

Discussion

An estimated 37,000 new cases of pancreatic cancer will be diagnosed in the United States in 2007. Unfortunately, the overwhelming majority of these patients present with advanced disease and, consequently, will not survive. Development of accurate procedures for the early detection and diagnosis, as well as accurate staging, of pancreatic cancer would likely have a major effect on treatment outcomes. Although early detection and diagnosis are not necessarily the same, they both require the presence of a marker, or set of markers, that can be identified at an early stage of the disease process. In the current report, we provide evidence that the PAM4-reactive, MUC1 epitope is such a marker. The PAM4 epitope is not present in normal adult pancreatic tissue, but is expressed at the earliest stages of neoplastic progression, PanIN-1, and remains present throughout the sequence of events that leads to invasive adenocarcinoma. In addition, the PAM4-reactive, target epitope exhibits a high level of specificity, being expressed almost exclusively within pancreatic neoplasms, although there remains the caveat of our limited examination of other tissues and the potential sampling biases that may exist within tissue microarrays.

Although there is considerable ongoing research to identify biomarkers for use in the early detection of pancreatic cancer, with much of it currently based on proteomic and/or genomic approaches to identify circulating markers (24–26), the most widely studied biomarkers are the mucins, and in particular for pancreatic cancer, mucins 1, 4, and 5ac. Mucins are high-molecular weight glycoproteins expressed by a variety of solid and hematologic tissues. In general, they consist of a protein core containing a VNTR domain with high levels of glycosylation. Mucins may exist as either a secreted or membrane-bound form. A major confounding property of these glycoproteins is their extreme microheterogeneity. As such, it is likely that no chemical, physical or immunochemical means for detection will identify all molecules of a particular mucin species or, for that matter, mucin species synthesized within a single tissue. Thus, the means used to identify and/or quantify a mucin will be reflected in the sensitivity and specificity of detection.

Several investigators have reported the use of mucin-reactive antibodies for the identification of invasive pancreatic adenocarcinoma, as well as its precursor lesions, including the expression of MUC1 by use of antibodies reactive with the VNTR-domain [e.g., MAb DF3 (27), C595 (28), and Ma645 (13)]. In general, a high reactivity has been observed with invasive pancreatic adenocarcinoma. However, none of these MAbs is specific for pancreatic cancer, but instead react with diverse cancer types. In addition, the anti–VNTR domain MAbs DF3, C595 and, as reported here, MA5, each react with normal pancreatic tissues. Expression of the VNTR epitopes is
significantly decreased in early grade PanINs with enhanced reexpression (or more likely, uncovering) of the peptide core epitopes in association with increased cellular atypia. Similar results have been reported with antibodies to MUC4, demonstrating increased MUC4 expression associated with PanIN progression to invasive adenocarcinoma (6). However, in contrast to MUC1, MUC4 usually is not expressed within normal pancreatic tissues, as identified both by the use of MAbs and reverse transcription-PCR (6, 29). On the other hand, immunohistochemical data suggests that MUC5ac, as identified by MAb-CLH-2 (2), is expressed early in the development of pancreatic neoplasia, similar to the MUC1 epitope recognized by PAM4.

The CA19-9 epitope, sialyl-Lea, could be associated with MUC1 (as well as several other mucins and glycolipids), and is considered the current biomarker of choice for the management of pancreatic cancer (30, 31). Other sialylated carbohydrate structures (e.g., sialyl-Lex and sialyl-Tn) that can reside within the MUC1 glycome have been reported to be expressed at higher levels in pancreatic neoplasms as compared with normal tissues (32, 33). To the best of our knowledge, detailed studies of these epitopes within precursor lesions have not been reported.

During fetal development of the pancreas, epithelial cells of the foregut expand into multiple buds that proceed to form the tubules around which the pancreatic tissue develops (34). Whereas the fetal stomach produces MUC1, MUC4, and MUC5ac as early as 8 weeks, and continues to do so throughout adulthood, evidence indicates that these mucins are absent from the fetal pancreas, with MUC1 and MUC5ac being expressed only at term (35–37). Our observation that the normal adult gastric mucosa consistently expresses the PAM4 epitope is of interest because it is possible that during the process of carcinogenesis, pancreatic cells reexpress certain characteristics of primordial gut epithelial cells (5).

MUC1, MUC4, and other transmembrane mucins function, at least in part, as mediators of cell signaling events; for example, the MUC1 cytoplasmic tail is reported to interact with Ras, p53, and β-catenin pathways, among others (38). In addition, both MUC1 and MUC4 may have a more direct role in specific cellular behaviors, including inhibition of cell-binding to matrix proteins and enhancement of invasion (39–41). The use of specific MAbs to identify structural changes within MUC1, in association with early or late stages of neoplastic progression may provide added information regarding structure/function relationships. Although the detailed structure of the PAM4 epitope is not known, it is thought to be dependent on MUC1 glycosylation status (data not presented). Therefore, altered glycosylation, perhaps via reprogramming of glycosyltransferase expression, may represent an early event in the progression to invasive adenocarcinoma.

We could speculate on several potentially important PAM4-based applications for the management of patients with pancreatic cancer. For example, several institutions using sensitive imaging technologies are reporting the ability to discover small (<2 cm) localized pancreatic cancers as well as their precursor lesions, both in the general public and in high-risk groups undergoing targeted screening protocols (42–45). Detection, diagnosis, and management at this stage should provide significantly improved patient outcomes. However, the asymptomatic, noninvasive precursors represent a significant challenge for the accurate diagnosis of benign versus malignant lesions, with implications for subsequent treatment. Although we have pursued, and continue to develop, both an in vitro blood immunoassay (17) and in vivo nuclear imaging technologies (18–20) for early detection and diagnosis of pancreatic adenocarcinoma, these technologies may not prove useful for the investigation of noninvasive precursor lesions.

The presence of an intact basement membrane may not permit the leakage of MUC1 into the circulation for detection by immunoassay and, conversely, the intact membrane may not permit radiolabeled PAM4, whole IgG, access to the antigen-expressing lesion for external imaging. This latter conjecture may be evident by our inability to observe radiolabeled PAM4 targeting to gastric mucosa in any of the clinical studies reported to date (18, 19), even though the tissue expresses PAM4-reactive MUC1. Thus, the ability to diagnose pancreatic lesions as neoplastic by use of immunohistochemical labeling of tissue biopsy and/or fine-needle aspirates may have important clinical value.

In another setting, immunohistochemical labeling of tissue may assist in the detection of minimal residual disease and micrometastases within resected tissue, and in particular, regional lymph nodes, to support decisions on prognosis and further management of the cancer patient. This has been the case for several solid tumors, including, for example, breast and lung cancers (for review, see ref. 46). Indeed, antibodies to MUC1 have been studied for the detection of breast cancer cells in bone marrow (47). Unfortunately, the use of these particular anti-MUC1 antibodies did not prove beneficial, due to the fact that the antibodies were also reactive with normal bone marrow cells. Similar studies being pursued with pancreatic adenocarcinoma, using antibodies to K-Ras, MUC1, cytokeratins, and other potential markers have reported that accurate
staging via immunohistochemical detection of minimal residual disease in regional nodes can provide better prognostic information for patient management (48, 49). Based on the specificity and sensitivity of PAM4 for pancreatic cancer, as reported herein, we suggest that immunolabeling with PAM4 could provide clinically relevant data for the prognosis and management of pancreatic cancer.

As mentioned, we have reported the development of an enzyme immunoassay to quantitate the PAM4-bound antigen in the circulation (17). The sensitivity of the assay for pancreatic cancer was 77%, with a specificity of 95% and ongoing studies are investigating the potential of this immunoassay for the detection of early stage disease. We are also exploring another means for detection of this biomarker by in vitro imaging with radiolabeled PAM4-MAB, and have reported successful clinical imaging of invasive pancreatic adenocarcinoma with 131I- and 99mTc-labeled murine-PAM4 IgG and, subsequently, 111In-labeled humanized-PAM4 IgG (18, 19, 50). We are currently exploring the potential of a next-generation recombinant bispecific MAB for pretargeted imaging of pancreatic cancer, and hope to translate this imaging system to clinical studies to determine if the specific PAM4-reactive, MUC1 biomarker can indeed prove useful for early detection of disease by external scintigraphy. Finally, the humanized PAM4 MAB may also prove useful for targeting therapeutic agents to pancreatic cancer, as suggested by a current phase I clinical study with a 90Y immunonjugate (50). Current and future studies are aimed at how best to detect and quantitate this biomarker for specific clinical applications, be it immunohistochemical labeling of tissue specimens (as described herein), in vitro quantitation of circulating antigen, or in vivo antibody-targeted imaging strategies.

References


PAM4-Reactive MUC1 Is a Biomarker for Early Pancreatic Adenocarcinoma

David V. Gold, Zarir Karanjawala, David E. Modrak, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/24/7380

Cited articles
This article cites 48 articles, 12 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/24/7380.full.html#ref-list-1

Citing articles
This article has been cited by 16 HighWire-hosted articles. Access the articles at:
/content/13/24/7380.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.