Glycan Motif Profiling Reveals Plasma Sialyl-Lewis X Elevations in Pancreatic Cancers That Are Negative for Sialyl-Lewis A*§

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The sialyl-Lewis A (sLeA) glycan forms the basis of the CA19–9 assay and is the current best biomarker for pancreatic cancer, but because it is not elevated in ~25% of pancreatic cancers, it is not useful for early diagnosis. We hypothesized that sLeA-low tumors secrete glycans that are related to sLeA but not detectable by CA19–9 antibodies. We used a method called motif profiling to predict that a structural isomer of sLeA called sialyl-Lewis X (sLeX) is elevated in the plasma of some sLeA-low cancers. We corroborated this prediction in a set of 48 plasma samples and in a blinded set of 200 samples. An antibody sandwich assay formed by the capture and detection of sLeX was elevated in 13 of 69 cancers that were not elevated in sLeA, and a novel hybrid assay of sLeA capture and sLeX detected 24 of 69 sLeA-low cancers. A two-marker panel based on combined sLeA and sLeX detection differentiated 109 pancreatic cancers from 91 benign pancreatic diseases with 79% accuracy (74% sensitivity and 78% specificity), significantly better than sLeA alone, which yielded 68% accuracy (65% sensitivity and 71% specificity). Furthermore, sLeX staining was evident in tumors that do not elevate plasma sLeA, including those with poorly differentiated ductal adenocarcinoma. Thus, glycan-based biomarkers could characterize distinct subgroups of patients. In addition, the combined use of sLeA and sLeX, or related glycans, could lead to a biomarker panel that is useful in the clinical diagnosis of pancreatic cancer.

Précis: This paper shows that a structural isomer of the current biomarker for pancreatic cancer, CA19–9, is elevated in the plasma of patients who are low in CA19–9, potentially enabling more comprehensive detection and classification of pancreatic cancers. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.047837, 1323–1333, 2015.

A patient with an uncertain lesion of the pancreas typically is referred to a specialist for dedicated scans of the pancreas and, if available, additional procedures such as endoscopic imaging with fine-needle aspiration to obtain material for cytology. The diagnostic challenges include differentiating benign from neoplastic conditions and determining the type and potential aggressiveness of a neoplasm (1–4). Based on imaging and biopsy, each condition and type occasionally can mimic others, and obtaining definitive information from biopsy is not always possible (5). Molecular tests hold promise to improve this situation (6), as they could provide objective and detailed information about each patient’s condition. But molecular markers to diagnose incipient pancreatic cancer are not available despite decades of research; the current best marker for pancreatic cancer, the CA19–9 test, was discovered in 1979 (7, 8). CA19–9 is elevated in about 75% of pancreatic cancers (9), which is useful for certain purposes, such as monitoring response to treatment, but not for diagnosis.

The antigen detected by the CA19–9 test is a glycan, a tetrasaccharide known as the sialyl-Lewis A (sLeA)¹ antigen. The discovery that CA19–9 antibodies recognize a glycan (10, 11) further revealed the prevalent nature of glycosylation alterations in cancer. Researchers have uncovered other glycans that show up with high abundance in cancer (12, 13), some of which contribute to cancer cell function and carry information about cell differentiation. Glycans, therefore, have good potential to serve as biomarkers of cancer. But for the diagnosis of pancreatic cancer, glycan-based markers are not yet effective because we do not have markers to detect the

¹ The abbreviations used are: sLeA, sialyl-Lewis A; sLeX, sialyl-Lewis X; FUT 3, fucosyltransferase 3; CV, Coefficient of variation; EDTA, ethylenediaminetetraacetic acid.
cancers that are low in sLeA. A strategy for improving upon the CA19–9 test is to identify biomarkers that are elevated in the patients who are low in sLeA.

Previous research suggested that other glycans besides sLeA are overproduced in some cancers that are low in sLeA. All antibodies used in the CA19–9 assays primarily detect the sLeA glycan, which has the sequence Siaα2,3Galβ1,3(Fucα1,4)GlcNAc (where Sia is sialic acid, Gal is galactose, Fuc is fucose, and GlcNAc is N-acetylglucosamine), but some also detect other glycans (14, 15). The several available CA19–9 assays give divergent results for individual patients (15–17), indicating the occasional elevation of the off-target glycans. Additional evidence comes from the DUPAN2 antibody (18), which binds a non-fucosylated relative of sLeA called sialyl-Lewis C (19) (Siaα2,3Galβ1,3GlcNAc). DUPAN2 detection shows elevations in some pancreatic cancers that do not make sLeA (15, 20).

The results cited above raise the possibility that knowledge of the differences in specificities between antibodies could guide discovery of glycans that are produced in pancreatic cancers. In theory, one could compare the levels of binding to a patient sample between antibodies and make inferences about the glycans that are present, based on the specificities of the antibodies. For example, if two antibodies recognize overlapping but distinct sets of glycans, and if only one antibody binds glycans in certain samples, then the glycans uniquely recognized by the antibody showing binding could be elevated in the samples.

Several previous developments make such an approach possible. For one, we had detailed information about the specificities of glycan-specific antibodies available through glycan array technology. Glycan arrays enable measurements of the binding of antibodies or lectins to hundreds of different glycans in a single experiment (reviewed in references (21, 22)), from which one can derive the specificities of the glycan-binding proteins. We previously developed an algorithm and software to analyze glycan array data (23–25), along with a database of analyzed, publicly available glycan array data (26). With that information, we can select antibodies that target desired glycan motifs and precisely interpret measurements made using the antibodies. Secondly, antibody array technology gave the ability to efficiently test many antibody sandwich assays over multiple patient samples (27). Accordingly, we could acquire measurements from many glycan-binding antibodies over multiple samples and then use the information about the specificities of the antibodies to make predictions about the glycan motifs that are present in each sample. We previously developed an algorithm for that purpose, called motif prediction (28). We applied this approach, which we call motif profiling, to the problem of pancreatic cancer diagnostics, asking whether we could identify glycan motifs that are elevated in the cancer patients who are low in sLeA.

MATERIALS AND METHODS

Human Plasma and Tissue Samples—Plasma samples (using EDTA as the anticoagulant) were collected at the University of Pittsburgh School of Medicine from patients with pancreatic cancer, pancreatitis, or benign biliary obstruction and from healthy subjects. Early-stage cancer was defined as stages I and II, and late-stage cancer was defined as stages III and IV. The pancreatitis patients were a mixture of chronic and acute, and the healthy subjects had no evidence of pancreatic, biliary or liver disease. The samples were collected using a standard operating procedure based on the serum and plasma protocols from the Early Detection Research Network. All samples were stored at −80 °C and sent frozen on dry ice, and each aliquot had been thawed no more than three times before use.

In addition, the Van Andel Research Institute (VARI) Biospecimen facility provided formalin-fixed, paraffin-embedded tissue and matched EDTA-plasma specimens from patients who underwent pancreatic resections at a regional hospital affiliate in Grand Rapids, MI. All samples were collected under approved human-subjects protocols.

Biological Reagents—The buffers and biological solutions used in the microarray assays included: PBST0.5 or 0.1 (1X phosphate-buffed saline (PBS) + 0.5% or 0.1% Tween-20), 10X sample buffer (1X PBS + 1% Tween-20 + 1% Brij-35 (Thermo Scientific, Rockford, IL)); 4X IgG blocking mixture (400 µg/ml each of mouse, sheep, and goat IgG, 800 µg/ml rabbit IgG in 1X PBS; antibodies from Jackson ImmunoResearch, 872 West Baltimore Pike West Grove, PA); 10X protease inhibitor (Complete Tablet, Roche Applied Science, Indianapolis, IN); and 2X sample dilution buffer (2X sample buffer + 2X protease inhibitor + 2X IgG mixture in 1X PBS).

The antibodies and lectins were purchased from various sources (Table S1), except for the DUPAN-2 antibody, which was a kind gift from Dr. Tony Hollingsworth. The capture antibodies to be printed onto microarray slides were purified by dialysis (Slide-A-Lyzer, Pierce Biotechnology, Rockford, IL) to 1X PBS and ultracentrifuged. Biotinylation was performed using the EZ-Link-sulfo-NHS-LC-Biotin kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions.

Microarray Fabrication—We used a robotic microarray printer (2470, Aushon Biosystems, Billerica, MA) to spot the capture antibodies (Table S1) onto microscope slides coated with a thin layer of nitrocellulose (PATH slides, Grace Bio-Labs, Bend, OR). The antibodies were prepared at 250 µg/ml in 1X PBS with 0.005 Tween-20 and 15% glycerol. Each slide contained 48 identical arrays arranged in a 4 x 12 grid with 4.5 mm spacing between arrays, and each array had six replicate spots of each antibody. A wax-based hydrophobic border was imprinted to define boundaries between the arrays (Slide Imprinter, The Gel Company, San Francisco, CA). The printed slides were stored at 4 °C in a desiccated, vacuum-sealed slide box until use.

Microarray Assays and Analysis—We used protocols for the antibody microarray assays that are similar to those described previously (29, 30). The plasma samples were diluted twofold with 2X sample dilution buffer and incubated at 4 °C overnight with gentle agitation to induce IgG blocking. Unless otherwise stated, all the following steps were conducted at room temperature. The next day, the PATH slides were blocked with 1% bovine serum albumin (BSA, Fisher Scientific, Fair Lawn, NJ) in PBST0.5 for 1 h, washed in three changes of PBST0.5 for 3 min each, and dried by brief centrifugation at 160 × g. We did not chemically derivatize the glycans on the capture antibodies as previously described (29) because in control experiments we observed very little binding of the detection reagents to the glycans on the capture antibodies.

We applied 6 µl of each plasma sample to each array and let the sample incubate for 1 h. Each unique sample was applied to three
separate arrays. The slides were washed three times for 3 min each in PBST0.1 and spin-dried. We applied 6 μl of biotinylated detection antibody (3 μg/ml in PBST0.1 with 0.1% BSA) to each array for 1 h and then washed and dried the slides as above. Finally we applied Cy5-labeled streptavidin (cat. # 43-4316, Invitrogen, Camarillo, CA) at 2 μg/ml in PBST0.1 with 0.1% BSA for 1 h and washed and dried the slides. We scanned the slides for fluorescence using 633 nm excitation (LS Reloaded, TECAN, 9401 Globe Center Drive Suite 140 Morrisville NC).

We quantified the resulting images with the GenePix Pro 5.0 software (Molecular Devices, Sunnyvale, CA), using both automatic and manual spot finding features. The local background was subtracted from the median intensity of each spot. We used a custom script to remove any outliers from the six replicate spots according to the Grubbs' test. The script calculates the Grubbs' statistic for the spot farthest from the mean of the replicates and rejects the spot if the Grubbs' statistic exceeds a preset threshold, here using $p < .1$. The script repeatedly removes spots until no outliers remain or to a minimum of four spots. It then calculates the geometric mean of the remaining replicate spots as the final output for each array.

The program also averages values between replicate arrays and reports the associated coefficient of variation, which is used for quality control. We repeated assays for measurements that had a CV > 0.4 and signals in the quantifiable response range of the assay (determined by dilution series of pooled samples) (31). For the large-scale screening studies, we could not calculate CVs because we ran the experiments in duplicate instead of triplicate owing to practical limitations arising from the large number of conditions. Instead, we evaluated reproducibility using correlations between the duplicates. The duplicate measurements showing signals above background had correlations greater than 0.94, confirming good consistency within the assay.

**RESULTS**

Reagents to Profile Motifs Related to Sialyl-Lewis A—The first step was to identify an appropriate suite of antibodies against sLeA and related glycans. We previously developed a database, called GlycanBinder (25, 26), consisting of analyzed glycan array data and metadata from over 3,000 glycan array experiments conducted by the Consortium for Functional Glycomics (Fig. 1A). We searched the database for binders against sLeA (Fig. 1B), reasoning that among binders with specificity for sLeA we would observe variation in cross-reactivity for related glycans, which we could use to get information about the expression of the related glycans. A query for reagents with a high motif score for sLeA (an estimate of specificity for sLeA (23)) or with the terms “sialyl Lewis A” or “CA19-9” in the metadata produced an assortment of antibodies and proteins with both predicted and unpredicted binding to sLeA (Table S2).

All antibodies and proteins had significant binding to the sLeA motif, but we saw diversity in their binding to other motifs (Fig. S1). For example, the 9L426 clone has the narrowest specificity, binding only Neu5Acα2,3-terminated sLeA (not Neu5Gc); the M081221 clone does not bind sLeA terminated with Neu5Gc nor to sialyl-Lewis C, but it does bind to sialyl-Lewis X; and the SLE121 antibody binds sLeA as well as sialyl-Lewis C in certain glycans (Fig. 1C). Based on this analysis, we selected three antibodies to give primary specificity for sLeA but diversity in the extent of binding to other motifs. In addition to the sialyl-Lewis A antibodies, we selected antibodies against Lewis A and sialyl-Lewis C (Fig. 1B, complete antibody information in Table S1).

Motif Profiling in Human Plasma—We selected 21 plasma samples, based on a previous analysis of sLeA levels (32), to include samples from cancer patients with either high or low sLeA and from control subjects. The low samples were among the bottom 15% of all cancer samples in sLeA, which are well below the clinical cutoff of 37 U/ml, and the high samples were among the top 15% in sLeA. We used antibody arrays to obtain measurements for each sample from every combination of capture and detection antibody (Fig. 2A). Each assay is defined by a capture:detection pair of antibodies, so with five capture and five detection antibodies, we obtained a set of 25 unique, pairwise measurements.

We found that a few antibody pairs showed significant differences between the groups (Fig. 2B and Fig. S2). The samples previously identified as having high sLeA were strongly elevated, relative to the other samples, only in combinations using the 9L426 clone as a capture antibody (Fig. S2). The fact that the 9L426 antibody is most specific for sLeA suggests sLeA is the dominant, cancer-specific epitope in these samples. Combinations including M081221, 9L426, and SLE121 were significantly increased for the cancer patients with low sLeA (Fig. 2B). The pairwise antibody combination that is most specific for sLeA, 9L426 capture and detection, did not show elevation in that group. Therefore, the slight elevation observed in some other pairs could be due to the secondary specificities that are unique to those antibodies.

Motif Prediction—Given the detailed knowledge available from the glycan array data about the specificities of the antibodies, it could be possible to quantitatively integrate information from all antibodies to predict the motifs that are present in each sample. We previously introduced an algorithm for that purpose called motif prediction (28). The steps in the algorithm are to multiply the normalized fluorescence measurement for a detection antibody by the antibody’s motif score for a given motif, repeat for each detection antibody, and then sum the products over all detection antibodies (Fig. S3). The motif scores, indicating the estimated preference of each antibody for each motif, were derived from glycan array data (see Supplementary Information). Performing this calculation led to a “motif prediction score” for each motif and each sample.

We asked whether any particular motifs were predicted to be significantly different between the cancer patients with low sLeA and the control groups (Fig. S4). Among the 215 motifs defined in our glycan array analysis (25, 26), the algorithm predicted higher ($p < .05$) sialyl-Lewis A and sialyl-Lewis X for the 9L426 and M081221 capture antibodies, as well as higher sialyl-Lewis C, higher internal neo-poly-LacNAc, and lower...
FIG. 1. Identifying binders to sialyl-Lewis A. (A) We used a database of glycan array data to search for binders to sialyl-Lewis A. To develop the database, we processed all the available datasets from the Consortium for Functional Glycomics using the motif segregation algorithm, and stored the metadata, raw data, analyzed data, and motifs. (B) Two motifs up-regulated in pancreatic cancer are sialyl-Lewis A and sialyl-Lewis C, each detectable by a monoclonal antibody. (C) We selected anti-sLeA clones SLE121, M081221, and 9L426 for use in profiling experiments based on their diversity in specificity. The numbers are derived from glycan array experiments and represent binding to the indicated glycan in fluorescence units. The number above each column is the concentration of the antibody incubated on the array, in μg/ml. Green cells indicate high binding, yellow is medium binding, and orange is low binding.
terminal neo-poly-LAcNAc for the M081221 capture antibody. The motif predicted to be elevated for both capture antibodies, besides sLeA, was sialyl-Lewis X (sLeX).

Testing the Prediction of Elevated Plasma sLeX in sLeA-Low Cancers—To test the above prediction, we probed the samples with antibodies that have primary specificity for sLeX. A search of the GlycanBinder database found clones KM93 and CSLEX1, which are anti-sialyl-Lewis X antibodies that have been analyzed by glycan array and have good specificity for sLeX (analysis not shown). We produced antibody arrays containing all the antibodies used initially plus the sLeX antibodies, and we repeated the profiling of the serum samples.

Using the CSLEX1 antibody as capture (the use of CSLEX1 as a detection antibody had not been validated for this initial screen), we observed higher signals in the sLeA-low cancer group when we detected either with the SLE121 or M081221 clones (Fig. 3A). These antibody combinations produced the highest differences in signals of any capture:detection pair tested, supporting the idea that at least some sLeA-low cancer patients within the sLeA-low group produced sLeX instead of sLeA.

We further tested this relationship with a set of 48 samples that included 15 low sLeA and seven high sLeA cancer cases (among the bottom and top 15% in sLeA, respectively) and 26 control subjects comprising healthy individuals and patients with benign pancreatic diseases. With optimization of the assay conditions using cell lines expressing sLeX as controls (not shown), we achieved functioning assays using CSLEX1 as the detection antibody. An assay consisting of capture and detection using anti-sLeX showed elevations in seven of the 15 sLeA-low cancer patients at a threshold that was above all the sLeA-high cancer patients and all but two of the control patients (Fig. 3B). The difference was significant ($p = .01$, Mann-Whitney test) for the comparison of sLeA-low to sLeA-high cancers and borderline significant ($p = .1$) for the comparison of sLeA-low cancers to controls, owing to the extremely high sLeX levels in two control samples from chronic pancreatitis patients (see Table S3 for details on the samples and individual values). This result supports the concept that at least some of the sLeA-low pancreatic cancers show elevations in sLeX. Furthermore, sLeX elevations can occur in early-stage cancer: The 15 sLeA-low cancers included nine that were early stage (stage I or II), and four were elevated in sLeX (Table S3).

Validation of Plasma sLeX Elevations in sLeA-Low Cancers—We further tested the hypothesis in a blinded set of 200
samples, consisting of samples from pancreatic cancer patients and patients with chronic or acute pancreatitis or benign biliary stricture. We probed the antibody arrays with the anti-sLeA (9L426) and anti-sLeX (CSLEX1) antibodies, and we calibrated the sLeA measurements using a CA19–9 standard. Upon unblinding (after data were collected and sent to a third party for initial analysis), we observed trends consistent with the foregoing experiments (Fig. 4A).

Plasma sLeA levels were elevated in 58/109 cancer patients (53% sensitivity (95% CI: 44.0–62.4%)) and 18/91 control subjects (80% specificity (95% CI: 71.4–87.9%)) based on a cutoff of 37 U/ml, as used clinically to define CA19–9 elevation. Of the cancer patients below 37 U/ml in sLeA, 7/51 (14%) were elevated in sLeX, and of the cancer patients below an alternative cutoff in sLeA of 100 U/ml (used clinically to give higher specificity for cancer), 13/69 (19%) were elevated in sLeX, based on thresholds in sLeX that produced no additional elevations of control subjects (Fig. 4A and Table I). In addition, sLeX levels were significantly different (p < .001, Mann-Whitney test) between sLeA-low (<37 U/ml) cancers and all controls (Table I). Therefore, divergent groups of cancer patients showed sLeA and sLeX elevations, while most of the control subjects showed no elevations in either.

The antibody arrays contained additional capture antibodies (Table I), so we asked whether any other combinations of
capture:detection assays could improve discrimination of cancers from controls. The assay showing the greatest difference between sLeA-low cancers and controls was anti-sLeA capture and anti-sLeX detection (Table I and Fig. 4B), which represents the co-expression of sLeA and sLeX on the same molecule. Of the cancer cases that were below the cutoff of 100 U/ml in sLeA, 24/69 (35%) were elevated in sLeA:sLeX using a cutoff in that marker that added only one new false positive detection. If we classify as a case any sample that was elevated in either the sLeX sandwich or the hybrid sLeA:sLeX sandwich, using thresholds chosen to optimize accuracy (Fig. 4C), the two-marker panel had a sensitivity of 76% (83/109 cases, 95% CI: 59.6–89.0%), specificity of 78% (71/91 controls, 95% CI: 65.9–97.8%), and accuracy of 77% (154/200 correct, 95% CI: 74.0–84.0%), significantly better (p = .009) than the best accuracy of 68% (136/200 correct) for sLeA.

**Table I**

| Marker | p value* | p value* |
|--------|----------|----------|
| Sialyl Lewis A(9L426):sialyl Lewis X(CSLEX1) | 9.2E-06 | 3.09E-13 |
| Sialyl Lewis X(CSLEX1):sialyl Lewis X(CSLEX1) | 2.24E-04 | 4.06E-08 |
| Lewis A(7LE):sialyl Lewis X(CSLEX1) | NS | 1.08E-03 |
| Sialyl Lewis A(121SLE):sialyl Lewis X(CSLEX1) | NS | 1.01E-02 |
| Sialyl Lewis A(M081221):sialyl Lewis X(CSLEX1) | NS | NS |
| Sialyl Lewis X(9L648):sialyl Lewis X(CSLEX1) | NS | NS |
| DUPAN2:sialyl Lewis X(CSLEX1) | NS | NS |
| Lewis X(P12):sialyl Lewis X(CSLEX1) | NS | NS |

**Fig. 4.** sLeX and dual expression of sLeA and sLeX are elevated in some sLeA-low cancers. (A) The graph shows the correlation between sLeA (using anti-sLeA clone 9L426 as capture and detection, y axis) and sLeX (using anti-sLeX clone CSLEX1 as capture and detection, x axis). Each point represents a patient sample, and each value is the average of three replicates. The dashed lines represent thresholds defining elevations for each assay. (B) The y axis indicates the sLeA measurements, and the y axis indicates measurements of dual expression of sLeA and sLeX, using anti-sLeA capture and anti-sLeX detection. At the thresholds defined by the dashed lines, many cancers are elevated only in the sLeA sandwich assay or the sLeA:sLeX sandwich assay. (C) The graph depicts measurements from the capture:detection combinations indicated on the axis labels, and the right graph is a zoomed portion of the left. At the thresholds defined by the dashed lines, many cancers are elevated only in the sLeX sandwich assay or the sLeA:sLeX sandwich assay.

**Statistical comparisons between the patient groups.** Each marker is indicated by the capture:detection antibodies. *The first p value is based on the Mann-Whitney test for comparing sLeA-low (<37 U/ml) cancers (n = 51) to all controls (n = 91). The second p value is for comparison all cancers (n = 109) to all controls (n = 91). NS, not significant.
It should be noted that the controls in this experiment do not include healthy subjects, so the performance of sLeX is slightly worse than in studies comparing cancer to healthy people. The controls included 30 patients with benign biliary obstruction; the standard CA19–9 assay was elevated in three of those patients at the 37 U/ml cutoff, and sLeX also was elevated in three (Table S4). Of the 18 acute pancreatitis patients, one was elevated in sLeX and zero in CA19–9, and of the 41 chronic pancreatitis patients, four were elevated in CA19–9 and three were elevated in sLeX, one of which was not elevated in CA19–9 (Table S4). Therefore, the rate of additional elevations from benign inflammatory conditions is not great using sLeX.

About 5% of people harbor homozygous inactivating mutations in the FUT3 gene, resulting in inability to produce the sLeA glycan (33). Six of the 109 (5.5%) of the cancer patients in our cohort had such mutations, all of whom were low in sLeA, as expected. Four of the 6 were elevated in plasma sLeX (Table S4). This finding further supports the ability of sLeX to provide added value to sLeA.

**Diversity in Glycan Expression and Histomorphologies of the Tumors**—To gain more information on the origin of plasma sLeX elevations in pancreatic cancers, particularly those without plasma sLeA elevations, we analyzed matched tissue and plasma samples from 14 subjects with pancreatic tumors, randomly selected among pancreatic resections at a regional hospital affiliate in Grand Rapids, MI. The respective rates of sLeA and sLeX elevations in the serum generally matched those in the preceding experiments, with patients variously elevated in one, both, or neither of the markers (Fig. 5). The localization of sLeX was very similar to that of sLeA, with staining in the luminal and cytoplasmic areas of the ducts and very little staining in uninvolved regions of the pancreas except for in centroacinar cells and small ducts (not shown).

We observed a general agreement between the plasma levels and tissue staining in the ductal epithelia, at least...
among the moderately and well-differentiated cancers. The patients with high plasma sLeA showed moderately or well-differentiated ducts with surrounding fibrotic stroma and heavy staining for sLeA, but the cancers not showing plasma sLeA elevation showed little gland formation and were marked by undifferentiated cells, tightly knit clusters of invasive cells, squamous formations, or high necrosis (Fig. 5). Those that had plasma sLeX elevation showed sLeX staining in the neoplastic cells or in the stroma. The set included three cases of neuroendocrine tumor, a condition that usually does not elevate plasma sLeA. Two of the three had elevated plasma sLeX, and all three showed sLeX staining in the neoplastic tissue.

The above analysis suggests that pancreatic cancers that do not elevate plasma sLeA tend toward poorly differentiated ductal adenocarcinoma. Among 10 of the University of Pittsburgh cancer cases with surgical pathology available (Fig. S5), the five that had elevated plasma sLeA showed well-differentiated ductal adenocarcinoma, characterized by invasive glands surrounded by desmoplastic stroma. The other five, two of which had elevated sLeX, showed moderately differentiated or poorly differentiated adenocarcinoma or adenosquamous carcinoma (one case). This relationship suggests that histomorphologies other than well-differentiated ductal adenocarcinoma tend not to elevate plasma sLeA but maintain the potential to elevate plasma sLeX.

**DISCUSSION**

The goal of this research was to test the hypothesis that some patients with low plasma sLeA have elevations in other, related glycan markers. We found that sLeX, a structural isomer of sLeA, was elevated in the plasma of 14–19% of patients with low sLeA and that a novel marker formed by the capture of sLeA and the detection of sLeX was elevated in ~35% of the sLeA-low cancers with low false-positive detection. If validated in larger studies, these findings could provide the foundation for a biomarker panel that improves upon CA19–9 and that could be used in the clinical diagnosis of pancreatic cancer. Furthermore, this research establishes a novel approach to screen for glycan-based biomarkers, called motif profiling. The method uses detailed analyses of antibody specificity, available from glycan array data, to predict differences between patients in particular glycan motifs—differences that would not be discernable based on a less-detailed knowledge of the antibody specificities. We envision that the approach will be particularly useful using antibodies or lectins with complex fine specificities or for integrating information over many reagents because such analyses would be unmanageable without a software tool.

The present work expands on previous studies investigating the idea that CA19–9-negative cancers make glycans that are related to sLeA. The studies mainly used monoclonal antibodies such as CA 50 (34), Span-1 (34), CA 242 (35), and others, generally produced minimal added value over CA19–9 alone. Perhaps the most promising antibody for this purpose was DUPAN2. Separate studies demonstrated that some of the CA19–9-low patients show elevations in DUPAN-2 (20, 36), but a biomarker panel based on these assays never became established. Miyamoto and coworkers found increased levels of a novel glycan, 6-sialylated, type-1 H antigen, in three out of six pancreatic tumors that were negative for CA19–9 (37), but the researchers did not demonstrate this structure as a blood biomarker.

An important consideration for the clinical use of a biomarker is the rate of false positive detection. The new markers showed elevations in some of the control subjects with benign disease but at levels lower than the cancer patients. Higher cutoff would give higher specificity, as with the current use of the standard CA19–9 assay. CA19–9 levels are above 37 U/ml for 18–21% of chronic pancreatitis patients (34, 38), but a higher threshold of 100 U/ml gives nearly perfect discrimination of pancreatic cancer from chronic pancreatitis (9). The present results provide a foundation for achieving both high sensitivity and high specificity through complementary biomarkers, each of which enable specific detection of a subgroup of pancreatic cancer. In order to further improve sensitivity, a viable strategy would be to search for markers elevated in the pancreatic cancers did not elevate either sLeA or sLeX.

An extensive study of the tumor tissue was not in the scope of the present work, but we achieved some preliminary insights into the nature of the tumors producing each type of marker elevation. All patients with high plasma sLeA for which tissue was available had well or moderately differentiated ductal adenocarcinoma, with the ducts staining heavily for sLeA. In contrast, the patients with low plasma sLeA, some of which also had high plasma sLeX, had moderately or poorly differentiated adenocarcinoma or adenosquamous carcinoma. The correspondence between high ductal staining and elevated plasma levels supports a direct connection between the two and also suggests that the ductal secretions disseminate into the stroma, lymph, and peripheral circulation—a model that is probable because the proliferating ducts do not typically have outlets to the main pancreatic duct. Results from a previous study also support this link: The molecular form of the sLeA-containing material from the blood matched that from the tumors (39). Some of the tumors with low plasma sLeA nevertheless showed high sLeA staining in the cancer cells (Fig. 5B), suggesting that these tumors did not secrete the marker enough to be detected in the circulation. The lack of differentiated ductal structures potentially reduces such secretions. Taken together, the current results give rise to the intriguing hypothesis that specific glycans characterize distinct subgroups of cancers. At this point, we will need a larger study to establish the connections between cancer-cell se-
creations, histomorphologies, and plasma elevations of sLeA and sLeX.

Previous studies also found overexpression of sLeX in some pancreatic tumors but not in the plasma nor in relation to cancers that are low in sLeA. In an Immunohistochemistry (IHC) study of 30 patients with pancreatic cancer, sLeX staining was present in 30% of patients, relative to sLeA staining in 87% of patients (40). Co-expression of LeA and LeX was observed in 23% of patients, indicating that 7% of patients had staining in sLeX only. This relative rate of sLeA and sLeX staining generally agrees with our findings, but the study did not examine details of histomorphology. Other studies demonstrated general agreement between blood levels and tissue levels of sialyl-Lewis A (20, 41), but the studies did not examine the relationships to various histomorphologies or the relationships with sLeX.

The glycans that the cancer cells produce could tell us something about the pathways at work in the cell and about their interactions with their environment, particularly in regard to affecting metastasis and recognition by immune cells. For example, higher sLeX expression on cancer cells in mice leads to higher metastasis but also greater rejection by Natural killer cell (NK) cells (42). The role of sLeX in metastasis likely is mediated through interactions with E-selectin receptors (43). Sialyl-Lewis A is also a ligand for E-selectin (44), so their interactions with sLeX likely is mediated through interactions with E-selectin receptors.

In summary, we show that a significant group of patients with low plasma sLeA have elevations in plasma sLeX. A novel assay formed by the capture of sLeA and detection of sLeX was particularly useful for detecting many sLeA-low cancers with a low rate of false-positive elevation. The sLeA and sLeX glycans showed similar expression patterns in well and moderately differentiated ductal adenocarcinomas but divergent patterns in other histomorphologies that do not yield elevated plasma sLeA, thus supporting the concept of glycans as indicators of biological subtypes of disease. The present findings suggest that further development of panels of complementary glycans could enable the detection of a broader range of pancreatic cancers than previously possible using CA19–9. This capability could have value beyond early diagnosis, such as determining who should undergo surgery for neoadjuvant chemotherapy (chemotherapy applied prior to surgery) and detecting disease resurgence. Each use of CA19–9 could benefit from additional biomarkers that are applicable to tumors that do not secrete high levels of sLeA.

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[5] This article contains supplemental material Tables S1–S4 and Figs. S1–S5.

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