

Research Article

Development of a Serum Biomarker Assay That Differentiates Tumor-Associated MUC5AC (NPC-1C ANTIGEN) from Normal MUC5AC

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A serum ELISA using a monoclonal antibody that detects a MUC5AC-related antigen (NPC-1C antigen) expressed by pancreatic and colorectal cancer was developed. The NPC-1C antibody reacts with specific epitopes expressed by tumor-associated MUC5AC that does not appear on MUC5AC from normal tissues. Based on observations of a highly specific antibody, we tested the ELISA to differentiate serum from healthy blood donors compared to serum from patients with colorectal or pancreatic cancer. Additionally, patient tumor tissue was stained to examine the expression pattern of MUC5AC-related antigen in pancreatic and colorectal cancers. The results indicate the NPC-1C antibody ELISA distinguished serum of cancer patients from normal donors with very good sensitivity and specificity. Most patient's tumor biopsy exhibited NPC-1C antibody reactivity, indicating that tumor-associated MUC5AC antigen from tumor is shed into blood, where it can be detected by the NPC-1C antibody ELISA. This serum test provides a new tool to aid in the diagnosis of these cancers and immune monitoring of cancer treatment regimens.

1. Introduction

The early diagnosis of colorectal and pancreatic cancers remains an area of high unmet medical need, as underscored by the U S estimated combined, annual death rate of >89,000 [1]. Although the serum marker CA19-9 is elevated in the majority of pancreatic cancer patients, the specificity of CA19-9 is limited. CA19-9 is frequently elevated in patients with various benign pancreaticobiliary disorders [2–4]. As a result of all of these issues, CA19-9 is not recommended as a screening test for pancreatic cancer [5]. The American College of Gastroenterology (ACG) recommends colonoscopy as the preferred screening/prevention test for colorectal cancer. Noninvasive fecal immunochemical tests are only recommended for patients who decline cancer prevention tests [6]. Currently, there is no consensus for screening for the early detection of pancreatic cancer. Unlike colorectal cancer, the majority cases of pancreatic cancer are detected when a patient is symptomatic which often times represents late stage cancer, resulting in an overall 5 year survival of less than 5% [1]. The majority of colorectal and pancreatic

cancer patients are diagnosed utilizing invasive procedures that are expensive, and usually reveal the diagnosis later in the disease process. Newer approaches are being investigated that could allow for earlier detection of disease, in a cost-effective manner, that furthermore could result in better outcomes for patients with these diseases.

As an alternative diagnostic approach, we developed an ELISA using a promising novel tumor-specific monoclonal antibody generated against a clinically tested human colon cancer vaccine. NPC-1 is a monoclonal antibody that was derived from a Tumor Associated Antigen- (TAA-) based vaccine that was previously tested in Phase I-II clinical trials performed in the United States [7–9]. The TAA utilized in these studies was derived from pooled allogeneic colon cancer specimens from multiple patients, which was obtained postoperatively. Cell membranes were isolated from the tumor, and proteins from solubilized membranes were prepared by sonication and Sephadex G-200 chromatography. Semipurified TAAs were identified by *in vitro* and *in vivo* testing in colon cancer patients and healthy volunteers for cell-mediated immunoreactivities. The colon TAA was

detected in fetal intestine and cell membranes, and was localized on tumor cell membranes. Using discontinuous, gradient gel electrophoresis, both colon TAA and CEA were separated and cross-compared. The TAA was shown to be distinct from CEA [8]. The cDNA encoding the NPC-1 antibody was cloned from hybridoma cells, chimerized by genetic engineering, and expressed in a heterologous expression system (Chinese hamster ovary cells). The purified recombinant chimeric antibody is denoted NPC-1C.

The NPC-1C antibody binds to a protein antigen biomarker expressed by human colorectal and pancreatic tumors. In immunohistochemical testing, NPC-1C did not react significantly with tissues from healthy donors or other types of cancer. Furthermore, as discussed below, the NPC-1C antibody ELISA developed can distinguish serum of patients with colorectal or pancreatic cancer from healthy volunteers, thereby providing the rationale for accelerated development and testing of the variant MUC5AC (NPC-1C antigen) detection assay. The test may have application in diagnosis and treatment monitoring of patients with pancreatic or colorectal cancers.

2. Materials and Methods

2.1. ELISA Test. A sandwich ELISA was developed using NPC-1C antibody as the capture reagent. Biotin-labeled NPC-1C was used as the detection antibody. This homologous antibody format was possible due to the discovery of multiple NPC-1C antigen-binding sites expressed by the cancer-associated MUC5AC-related (NPC-1C) antigen. Serum samples were procured from various commercial and private sources under appropriate IRB-reviewed protocols. The assay developed here used serum from colorectal and pancreatic cancer patients, and serum from healthy blood donors.

Microtiter plates (96-well Nunc Maxisorp) were coated with purified unlabeled NPC-1C antibody at 10 µg/mL in 0.5 M sodium carbonate pH 9.5 overnight at 25°C. Plates were then blocked with 1% skim milk made in Tris-Buffered Saline (TBS) containing 5 mM EDTA and 1% sucrose for 4 hours at 25°C. Plates prepared in this manner could be stored dried and sealed for at least 12 months. All dilutions were made in ImmunoBooster buffers (Bioworld Consulting Laboratories, LLC) supplemented with 20 mM EDTA. Wash buffer was TBS containing 0.05% Tween-20 nonionic detergent. A detergent extract of cultured human LS174T colorectal tumor cells was used as a source of NPC-1C antigen to derive a standard curve. Units were in cells/well. Extracts derived from human pancreatic CFPAC-1 tumor cells or human lung A549 tumor cells were generated similarly. All tumor cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI medium containing 10% FBS (heat-inactivated) with 8 mM glutamine. To measure direct binding of NPC-1C antibody to the variant MUC5AC (NPC-1C) antigen, CFPAC-1 cells were grown in serum-free medium for 5 days and the conditioned medium was filtered and stored in one large lot at 4°C.

The sandwich ELISAs were performed by diluting the cell extract standard on each plate, next to patient or normal serum samples diluted 1:24 in the diluent. All incubations were performed at 25°C and all volumes were 100 µL per well. The plates were incubated for 15 minutes and washed three times with wash buffer. The biotin-labeled NPC-1C antibody was then added to the wells at 1 µg/mL, incubated for 15 minutes, and plates were washed three times. Peroxidase-conjugated streptavidin (1:5,000 dilution) was added to the plates for 15 minutes, and plates were washed three times with wash buffer and two times with TBS. The assay was developed by the addition of TMB substrate (BioFX Laboratories Inc.) to the plates, incubation for 15 minutes, then the color reaction was stopped with the addition of 0.5 M sulfuric acid. The data was acquired by measuring absorbance at 450 nm. The data was analyzed using GraphPad Prism or Microsoft Excel software programs.

NPC-1C antibody-binding competition assays were performed by coating microtiter plates with serum-free conditioned medium from CFPAC-1 tumor cells shown to secrete the variant MUC5AC antigen into the culture medium. Following a blocking step as described above, a solution of 1 µg/mL NPC-1C antibody was mixed with serial dilutions of conditioned medium from CFPAC-1, LS174T, and A549 tumor cells. The ELISA was developed using anti-human IgG peroxidase-conjugated antibody followed by TMB substrate incubation as described above. Inhibition curves were plotted using Microsoft Excel.

2.2. Immunohistochemistry. Tumor biopsy specimens from colorectal, pancreatic, or lung cancer patients were deparaffinized at 60°C for 30 minutes prior to staining with NPC-1C antibody. Subsequently, all staining steps were carried out at 25°C. Slides (4 microns thick) were blocked with Peroxo-Bloc inhibitor (Zymed Laboratories) for 2 minutes, rinsed with phosphate-buffered saline (PBS), and blocked with CAS (Zymed Laboratories) for an additional 10 minutes. Slides were stained with 10 µg/mL of biotin-labeled NPC-1C antibody for 1 hour, and washed three times with PBS containing 0.05% Tween-20 nonionic detergent. Previous titration of biotinylated-NPC-1C antibody demonstrated 10 µg/mL to be an optimal concentration for immunohistochemical detection of the variant MUC5AC antigen. A 1:400 dilution of peroxidase-conjugated streptavidin (Dako North America, Inc.) was then applied to the slides for 30 minutes and slides were washed 3 times. A solution of DAB (Zymed Laboratories) was applied for 3 minutes then rinsed with PBS. A solution of hematoxylin was then applied for 3 minutes and rinsed with tap water until clear. The slides were dehydrated with xylene and a coverslip was added using Permount mounting medium. Additional consecutive slides were stained with human cytokeratin AE1/AE3 (Abcam plc) as a positive control, and human IgG1 isotype as a negative control (AXXORA, LLC). The anti-MUC5AC antibody (clone 45M1) used to stain lung tumor tissue (10 µg/mL) was purchased from Abcam plc.

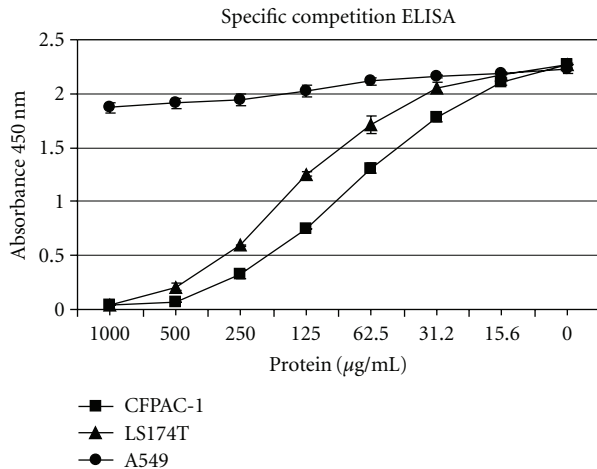


FIGURE 1: NPC-1C antibody reacts with a colorectal and pancreatic cancer-specific antigen. NPC-1C antigen secreted by serum-starved CFPAC-1 cells was used to coat a 96-well microtiter plate. Following a blocking step, 1 μ g/mL of NPC-1C antibody was added to the wells in the absence or presence of increasing amounts of conditioned medium from CFPAC-1 (pancreatic), LS174T (colorectal), or A549 (lung) cells to compete for NPC-1C binding to the coated antigen. The ELISA was developed by adding anti-human IgG-conjugated peroxidase reagent followed by TMB substrate.

3. Results

The NPC-1 antibody was generated in mice immunized with a preparation of pooled human colon tumor tissue extract. Hundreds of hybridomas were screened for cancer-specific characteristics such as binding to tumor tissues and cell lines, with no cross-reactivity to normal human tissues. The original murine IgG, NPC-1, was cloned and chimerized by genetically engineering the mouse variable regions of heavy and light chains with human IgG1 constant regions of the heavy and light chains. The resulting chimeric antibody, NPC-1C, was then expressed in a recombinant Chinese hamster ovary cell clone for further preclinical and clinical development.

The NPC-1C antibody was used as an affinity chromatography tool to isolate and identify the target antigen expressed by colorectal and pancreatic tumor cells. It was shown early that although the murine NPC-1 antibody was generated against a preparation of colorectal tumor-associated antigens, the NPC-1 antibody cross-reacted with pancreatic cancer tissues and cell lines. The target antigen recognized by NPC-1C was determined to be related to the mucin 5ac (MUC5AC) protein, a member of the secreted mucin family of glycoproteins (reviewed in [10]). We rapidly determined that the variant MUC5AC antigen was shed into the supernatant of many cultured human colorectal and pancreatic tumor cells, but not by other tumor cell lines such as prostate or lung tumor cells. We therefore examined whether the NPC-1C antibody could be useful to detect the target MUC5AC-related antigen in humans afflicted with colorectal or pancreatic cancer.

To demonstrate by example that the secreted variant MUC5AC antigen recognized by NPC-1C antibody was expressed specifically by colorectal and pancreatic tumor cells, supernates from LS174T, and CFPAC-1 were admixed with NPC-1C in a competitive ELISA format. Figure 1 shows that the soluble variant MUC5AC antigen secreted by LS174T and CFPAC-1 tumor cells could compete effectively with binding to NPC-1C antibody when the variant MUC5AC antigen was coated on microtiter plates. In contrast, supernates from human lung A549 tumor cells that are known to secrete normal MUC5AC did not compete for binding to NPC-1C antibody. Similar competition curves were shown with other colorectal and pancreatic tumor cell lines, but not with another squamous tumor cell line (data not shown). Together the results demonstrate that NPC-1C binds specifically to a variant MUC5AC antigen expressed by colorectal and pancreatic tumor cells, but not MUC5AC secreted by other tumor cell types.

Chemical and enzymatic digestion of the NPC-1C antibody purified variant MUC5AC antigen revealed that each molecule of the NPC-1C target contained multiple epitopes for NPC-1C binding (data not shown). Therefore, we reasoned that it would be possible to use NPC-1C as a specific reagent for both the capture and detection antibody in a homologous format ELISA. Biotin-labeled NPC-1C antibody was prepared as the detection reagent and tested with variant MUC5AC antigen expressed by LS174T cells. Figure 2 shows that NPC-1C antibody was capable of measuring the cognate antigen expressed by LS174T in this ELISA format. In contrast, MUC5AC expressed by lung A549 tumor cells was not detected in the ELISA. Thus, a surrogate standard MUC5AC-related antigen reagent was generated, frozen, and used for all subsequent ELISA tests. The NPC-1C antibody immunoreactive antigen is reported here in units of LS174T cells/well equivalent.

The proof-of-concept to demonstrate that the NPC-1C antibody ELISA test could be utilized to detect the variant MUC5AC antigen in human serum specimens was demonstrated with a small number of serum samples from colorectal cancer patients. Figure 3 shows the results from five serum specimens collected from colorectal cancer patients compared to pooled AB serum from healthy donors. The results demonstrate a range of variant MUC5AC antigen shed into the blood of these colorectal cancer patients. In contrast, pooled AB serum from healthy donors did not yield a significant signal and was similar to the background levels for the ELISA. Following this, and other preliminary tests, an optimum serum dilution of 1:24 was routinely used in subsequent testing.

A larger number of serum samples were procured to test the utility of the serum-based ELISA in detecting the variant MUC5AC antigen. A sampling of 41 colorectal or pancreatic cancer patient sera was compared with sera collected from 28 normal healthy blood donors. In this population of cancer patients, blood was collected serially during an approximately 3-month period for several of the patients while they were undergoing various treatment regimens with a medical oncologist. For multiple reasons, blood was not collected from all patients at all three timepoints. Thus,

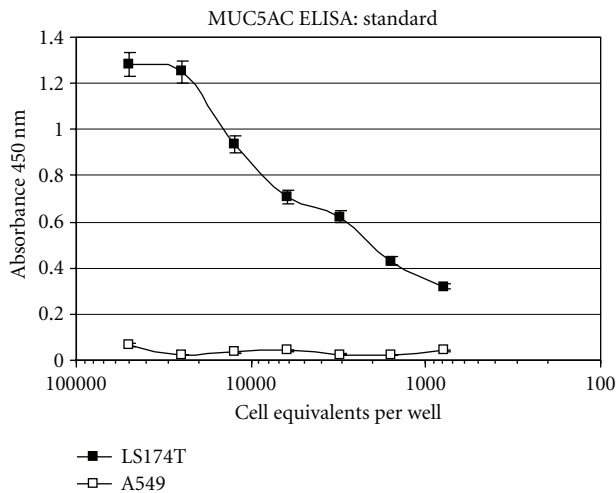


FIGURE 2: Development of a surrogate MUC5AC-related antigen standard. Protein extracted from human LS174T colorectal tumor cells was prepared to generate a standard curve in the NPC-1C antigen-capture ELISA. Unlabeled NPC-1C was used to coat a 96-well microtiter plate. Following a blocking step, a detergent extract made from human LS174T colorectal tumor cells or human A549 lung tumor cells were incubated on the plates. Biotin-labeled NPC-1C was then applied to the plates to detect the bound antigen, followed by development with streptavidin-peroxidase and TMB incubation steps.

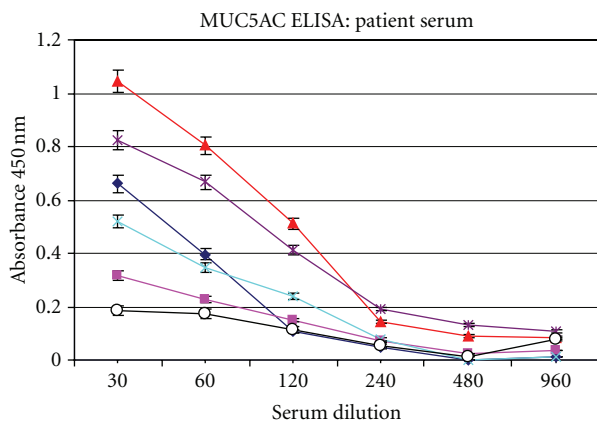


FIGURE 3: NPC-1C antigen detection in colorectal cancer patient serum. The NPC-1C antigen sandwich ELISA was used to test control serum pooled from normal donors (AB serum, shown in open circles with a thick connecting line), in comparison with serum from five individuals diagnosed with colorectal cancer (shown in various symbols and colors). Serum specimens were tested at the dilutions indicated on the x-axis.

there were 41 patients that donated blood at their first evaluation by the medical oncologist, followed by 33 patients that donated their blood at the second visit, and 25 patients who completed all three blood donations at the third visit. The majority of specimens tested in this preliminary study were from patients diagnosed with Stage III or IV disease. Figure 4 shows the results of testing this larger panel of colorectal and pancreatic cancer patient serum specimens,

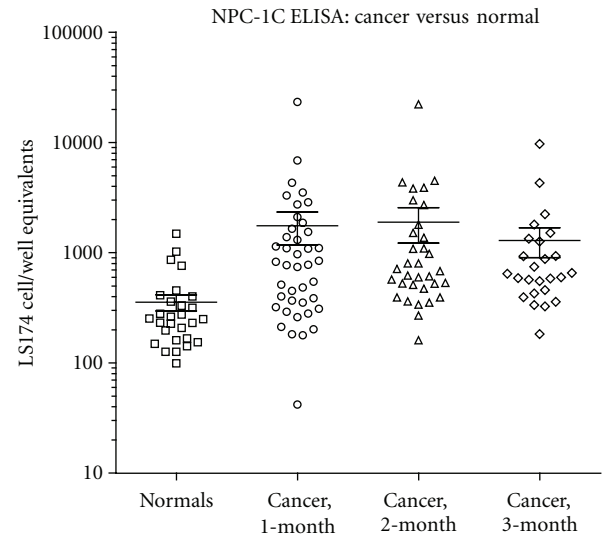


FIGURE 4: NPC-1C antigen detection in patients undergoing treatment. Serial blood draws of cancer patients over an approximate 3-month period were tested. The NPC-1C sandwich ELISA was performed at a 1:24 serum dilution as described in Section 2. Results are presented as a scatter plot of each experimental group, with the mean and standard error of the mean. There were 28 normal sera, 41 colorectal/pancreatic cancer sera at 1-month, 33 colorectal/pancreatic cancer sera at 2-month, and 25 colorectal/pancreatic cancer sera at 3-month.

compared to a group of normal healthy blood donors. Analysis of the results demonstrated approximately a 0.7 log difference between the cancer patients and the healthy donors at each of the three blood draws. The mean and standard error of the mean for each control group for the assays are Normals (355 ± 60), Col/Pan Ca: 1-month ($1,757 \pm 580$), Col/Pan Ca: 2-month ($1,894 \pm 671$), Col/Pan Ca: 3-month ($1,293 \pm 390$). Using the unpaired *t*-test (2-tailed) method to evaluate the difference between the Normal sera group and the cancer sera groups, the differences for each comparison were Normal versus 1-month: $P = .0511$; Normal versus 2-month: $P = .0397$; Normal versus 3-month: $P = .0153$. Furthermore, using a cut-off value of 355 cells/well derived from the Normal sera average, 73% of Col/Pan Ca, 1-month sera were above the cutoff (30 of 41 samples), and 88% were above the cutoff in each of the 2-month (29 of 33 samples), and 3-month (22 of 25 samples) in those groups. Overall, the samples represent an average of 82% positive above the cutoff established for the assay. These results show that the NPC-1C antibody ELISA can distinguish differences between serum from normal donors and colorectal or pancreatic cancer patients, with a promising level of confidence.

The cancer patient population tested in this study was further stratified by disease type. Figure 5 shows that there was no difference distinguished by the mean NPC-1C antibody ELISA results among those patients diagnosed with colorectal cancer ($n = 36$) from those patients diagnosed with pancreatic cancer ($n = 5$). Both groups separately

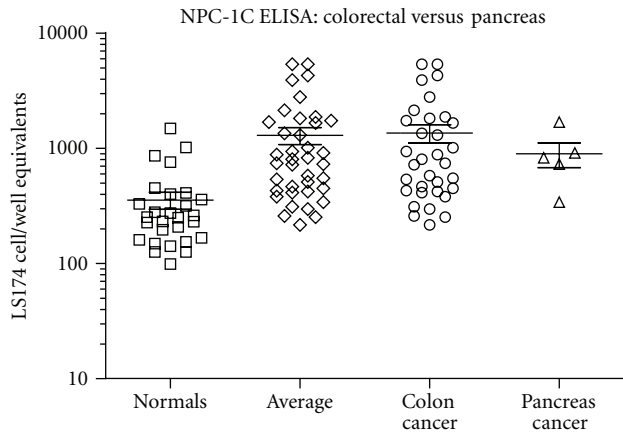


FIGURE 5: Colorectal and pancreatic cancer sera are detected similarly by NPC-1C. Serum specimens were sorted according to patients diagnosed with either colorectal ($n = 36$) or pancreatic cancer ($n = 5$). These were compared to the average of all cancer specimens and the normal serum specimens.

demonstrated approximately 0.7 log units higher variant MUC5AC antigen expression levels compared to the group of healthy donors.

From patients that donated multiple serum samples, the amount of variant MUC5AC biomarker detected in the assay was plotted versus the time of the blood draw. As shown in Figure 6, some patients appeared to express similar amounts of the variant MUC5AC antigen during the 2- or 3-month period when blood was drawn (subjects 5, 14, 15, 19, 25, 28, 29), whereas some patients appeared to experience an increase in this tumor-specific antigen expression (subjects 1, 2, 7, 33, 39) or a decrease in the biomarker expression (subjects 18, 22, 23, 28, 34, 36, 40). The significance of these shifts over time are presently unclear, but may be related to the tumor burden of the patient at the time the blood was drawn, which may be directly related to the specific treatment regimen of individual patients. Thus, the NPC-1C antibody ELISA may be a useful tool as a treatment monitoring biomarker assay. It was not possible within the 3-month time period of the blood collections to determine whether a correlation exists between the rate of variant MUC5AC increase or decrease and the outcome of the disease in these patients. This will be the subject of a future study.

4. Immunohistochemical Analysis

A number of tumor tissue specimens were procured to examine the level and incidence of variant MUC5AC antigen expression in colorectal and pancreatic cancer patients. Biotin-labeled NPC-1C antibody was used at $10\mu\text{g/mL}$, detected with streptavidin-horseradish peroxidase conjugate, and mounted on glass slides. A positive staining scale ranging from +1 to +5 was applied to the staining results, evaluated by light microscopy. Representative examples of the staining results to detect the variant MUC5AC antigen are shown in Figure 7. Tissues from normal pancreas and colon showed

no cross-reactive staining with NPC-1C antibody (panels (a) and (c) resp.). In contrast, tissues from pancreatic and colorectal tumor biopsies demonstrated specific staining of both cytoplasmic and membrane associated antigen (panels (b) and (d) resp.). In the colorectal cancer tissues, frequent staining of secreted variant MUC5AC antigen was observed in the luminal spaces of the tissues (panel (d)). The tissue specificity of NPC-1C binding was demonstrated by the lack of positive staining of lung adenocarcinoma tissue (panel (f)), whereas a commercially available anti-MUC5AC antibody shows that the lung tumor tissue expresses MUC5AC (panel (e)). Thus, while MUC5AC is known to be expressed by lung cells, the NPC-1C antibody does not react with the MUC5AC expressed by lung tumor tissue.

Tissues stained with NPC-1C antibody were considered positive (+1 to +5) for 79% of the tumor samples procured and stained (30 of 38). These staining results are similar to results from several other studies completed with NPC-1C antibody using tissue array slides, and both frozen and paraffin-embedded surgical specimens.

5. Discussion

The proof of concept has been established for the value of the NPC-1C antibody in the detection of the tumor-associated MUC5AC antigen recognized by NPC-1C antibody. This development suggests a new effective, scalable serum biomarker ELISA for the potential diagnosis and immunoregulatory monitoring of patients with colorectal and pancreatic cancer. These results also support further development and large-scale early Noninvasive diagnostic screening of healthy populations for colorectal and pancreatic cancer.

The preliminary results described here demonstrate that the NPC-1C antibody can distinguish normal/healthy serum from serum derived from patients with colorectal or pancreatic cancer. A better defined cohort of healthy serum donors may permit improved comparisons regarding assay specificity and sensitivity. Interestingly, should the assays be predictive in diagnosing colorectal and/or pancreatic cancer, some of the “normal” donors tested in the assays described here may be predisposed to developing cancer, and the potential utility of these ELISAs may be underestimated, if they could detect cancer in asymptomatic persons. Indeed, while the patient population studied in this report was predominantly from Stage III and IV cancer patients, we are currently procuring serum specimens from earlier stage colorectal and pancreatic cancer patients (Stage I and II) as well as serum from asymptomatic persons at risk for developing these types of cancer.

Several research laboratories have demonstrated the association of aberrantly expressed MUC5AC in colorectal and pancreatic cancers [11, 12]. Many monoclonal antibodies that target MUC5AC have been generated [13–17]. However, none of these appear as specific as the NPC-1C antibody in defining the variant MUC5AC antigen expressed in colorectal and pancreatic patient serum as compared with

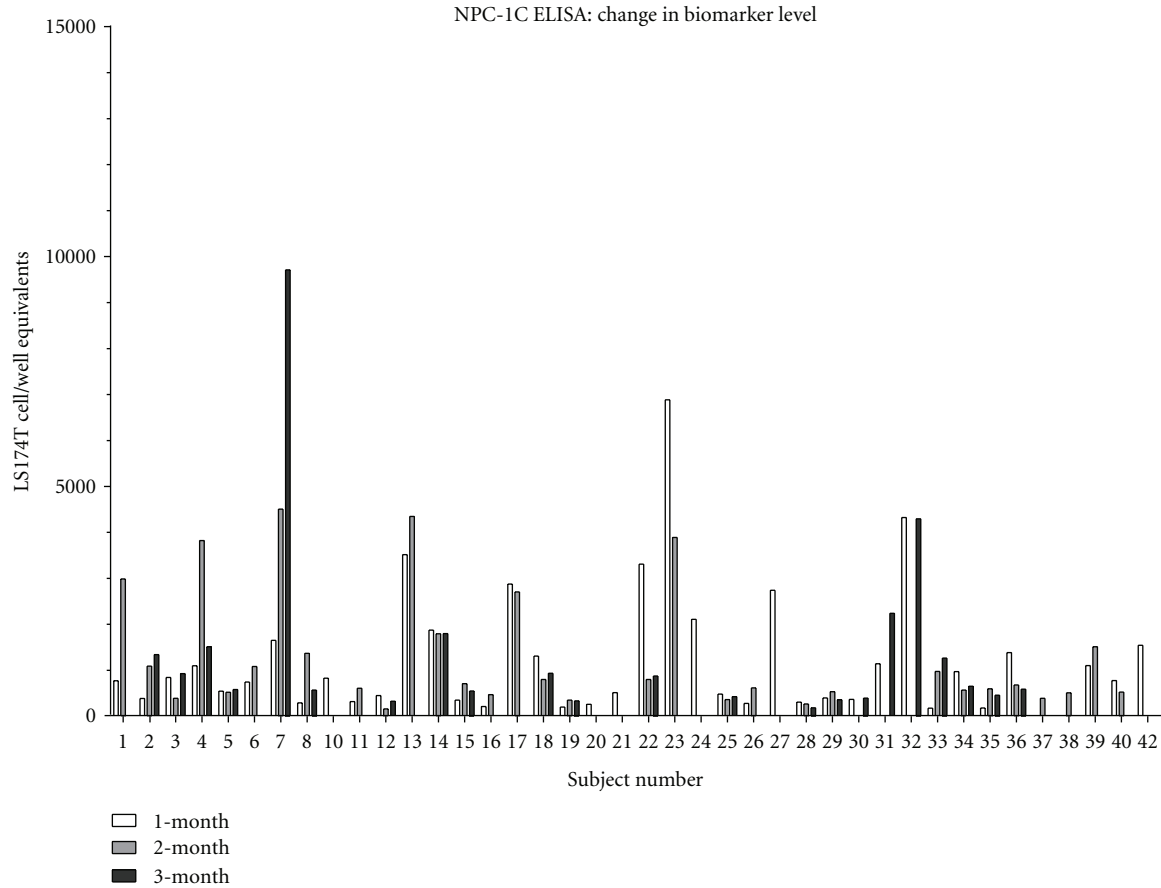


FIGURE 6: NPC-1C antigen detection in serum during treatment over 3 months. Serum was tested in the NPC-1C sandwich ELISA monthly from colorectal and pancreatic cancer patients over approximately 3 months during which they were being treated for their disease. Trends of increases, decreases, and stable biomarker levels were evaluated in each patient.

normal healthy donors, or as compared with MUC5AC antigen expressed by other tumor types. The reason for this difference may be due, in part, to the specific epitope that the NPC-1C antibody recognizes, which is currently an area of active investigation.

The assay specificity using the normal serum samples presented in this interim report are 71% (8/28 normal samples above the mean cutoff). The sensitivity for the NPC-1C antibody ELISA was 82% (18/99 cancer samples below the mean normal cutoff). Future testing and data analysis with new serum from healthy donors should increase the specificities of both serum assays. Importantly, testing earlier stage cancer patient (Stage I-II) will shed light on the application of this ELISA to detect cancers earlier, which will allow earlier interventions and improve treatment outcomes.

Reviewing the IHC results for each antibody, the tumor biopsy specimens were collected from patients diagnosed pathologically with Stage III and IV colorectal and pancreatic cancer. The NPC-1C antibody had a sensitivity of 79% (30 of 38 positive). The chimeric NPC-1C therapeutic antibody (Ensutuximab) is currently being tested in a Phase I dose escalation clinical trial. As part of the eligibility criteria for the study, patients with advanced pancreatic

or colorectal cancers must have their tumor biopsy stain positive for the NPC-1C antigen to be considered eligible for treatment. The preliminary immunohistochemical data presented here suggests that approximately 80% of patients may be treated with the therapeutic biological NPC-1C. This truly represents the new frontier of theranostics, where a biomarker can act as a companion to a specific therapeutic product.

The serum ELISA described here may have utility in monitoring colorectal or pancreatic cancer patients during the course of a treatment regimen. Patients with pancreatic cancer are typically treated with gemcitabine, whereas the treatment options for colorectal cancer patients can include chemotherapies (5-fluorouracil, FOLFOX, or FOLFIRI) or biologics such as cetuximab and bevacizumab. The NPC-1C antibody ELISA may be useful to aid in monitoring the patient responses to such therapies. The results shown in Figure 6 demonstrate trends for certain patients that may reflect cancer regression, progression, or stable disease. Once these data are coupled with the disease status in patients, the correlation may become apparent. Serum-based detection of colorectal and pancreatic cancer biomarkers will improve the chances for early detection

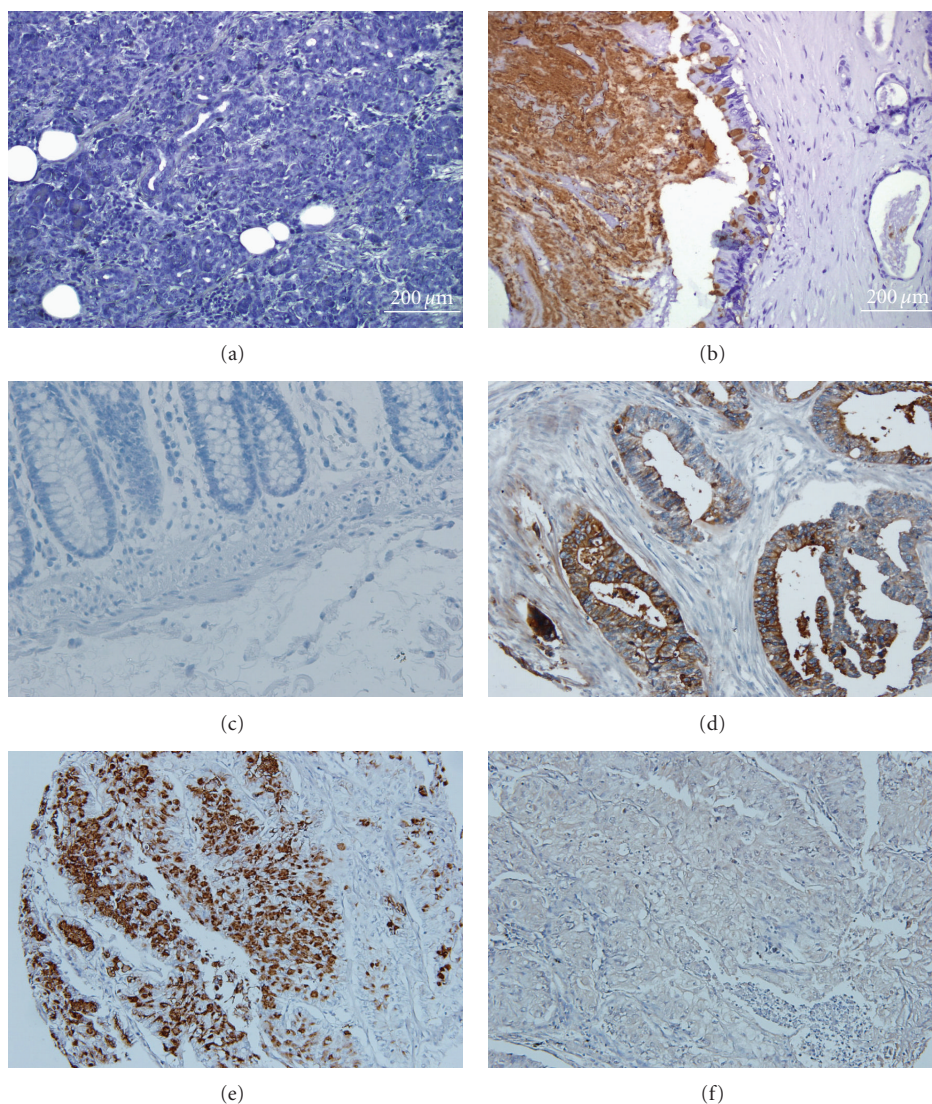


FIGURE 7: Immunohistochemical staining using NPC-1C. NPC-1C staining of normal human pancreatic (a), normal human colorectal (c), pancreatic cancer (b), and colorectal cancer (d). The brown stained areas indicate immunoreactivity of NPC-1C with the cancer-specific MUC5AC antigen. Lung adenocarcinoma stained with a commercially available anti-human MUC5AC antibody (e) or with NPC-1C (f), demonstrating that the NPC-1C antibody does not recognize the lung adenocarcinoma-associated MUC5AC.

of these deadly diseases. Current diagnostic methods are invasive, expensive, and often inconclusive. The preliminary results with the NPC-1C ELISA to specifically detect tumor-associated MUC5AC may improve the diagnosis of these solid tumors as well as aid in the immune monitoring and prognosis of patients undergoing treatment of their disease.

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