

## Design of an immunohistochemistry biomarker panel for diagnosis of pancreatic adenocarcinoma

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### ARTICLE INFO

#### Article history:

Received 5 July 2019

Accepted 12 August 2019

Available online 13 August 2019

#### Keywords:

Biomarkers

Pancreatic cancer

Immunohistochemistry

Panel

### ABSTRACT

**Background:** Challenges still exist in differentiating pancreatic adenocarcinoma from benign disease. The use of adjuvant testing of tissue biopsies has demonstrated potential diagnostic value. We designed a proof of concept study to first validate four individual immunohistochemistry biomarkers and then combine them into a panel to boost overall diagnostic sensitivity.

**Methods:** Malignant and benign pancreas from 27 pancreaticoduodenectomy specimens underwent immunohistochemistry staining with VHL, IMP3, S100A4, S100P. Using ROC curve analysis, threshold criteria for number of cells staining were chosen for each biomarker. Biomarkers were then evaluated as a panel for their ability to discriminate malignant from benign specimens.

**Results:** Diagnostic sensitivity of VHL, IMP3, S100A4, and S100P were 75.0%, 79.2%, 45.8%, and 0%. When VHL, IMP3, and S100A4 were grouped into a panel, they were able to distinguish cancer from normal tissue with a sensitivity of 100% and a specificity of 96%.

**Conclusions:** The high diagnostic value of an IHC panel consisting of VHL, IMP3, and S100A4 on surgical specimens suggests the need for future prospective studies of these biomarkers on biopsy specimens.

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### Introduction

Pancreatic cancer represents a major morbidity and mortality in the US. For 2019, it is estimated that there will be over 56,000 new diagnoses of pancreatic cancer with an associated 45,000 deaths [1]. With current improvements in imaging techniques and tissue sampling, a definitive diagnosis of pancreatic adenocarcinoma can be made in majority of patients. However, there still exists certain challenges in distinguishing pancreatic malignancy from benign disease. This is particularly true for chronic pancreatitis, which can resemble pancreatic malignancy both on imaging and in elevation

of CA 19-9 [2,3]. As a result, patients sometimes undergo major hepatopancreaticobiliary (HPB) surgical resections for conditions that turn out to be benign. This is concerning due the morbidity and mortality associated with such procedures. For instance, patients that undergo a pancreaticoduodenectomy for a benign condition are reported to experience a 17% drop in long-term survival at 10 years [4]. Thus, a great deal of focus has been put on the advancement and development of diagnostic techniques for pancreatic cancer.

Due to a low complication rate and the ability to obtain a tissue specimen, endoscopic ultrasound with fine needle aspiration (EUS-FNA) has gained favor as the diagnostic modality of choice for pancreatic cancer [5]. The use of EUS-FNA has been supported by a high sensitivity and specificity, with one large meta-analysis demonstrating a pooled sensitivity of 86.8% and a pooled specificity of 95.8% [6]. Nonetheless, difficulties still remain in obtaining adequate samples for diagnosis. Factors such as tumor size, tumor location, needle gauge size, technique used, endoscopist experience, and the presence of a cytopathologist on-site all contribute to

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the ability to collect an adequate biopsy [7,8]. Accordingly, multiple studies have been devoted to exploring new techniques and adjunctive tests to help mitigate inadequate sampling. One such method that is currently being investigated due to its widespread availability is the use of immunohistochemical (IHC) markers.

Despite the identification of numerous IHC markers for pancreatic adenocarcinoma, no marker has been shown to have high enough diagnostic value to be used in isolation. As a result, several studies have focused on combining IHC markers within a panel in order to increase the sensitivity and specificity of the test. The panel is able to achieve this result by having the additional biomarkers capture the malignancies that were missed by the individual tests. Utilizing a previous review of the literature, we selected the following biomarkers to examine as potential markers for our panel: insulin-like growth factor 3 mRNA binding protein 3 (IMP3), S100 Calcium-Binding Protein P (S100P), S100 Calcium-Binding Protein A4 (S100A4) and von Hippel Lindau (VHL) [9].

The goal of this study was to determine whether the selected biomarkers could successfully be combined into a panel to identify pancreatic adenocarcinoma at a high sensitivity and specificity. To achieve this goal, we designed a proof of concept study that was split into two parts. First, we would determine the ideal diagnostic staining thresholds for the individual IHC markers by comparing stained pancreatic adenocarcinoma samples with stained normal pancreas. Following this portion of the study, we performed an analysis to determine which combination of the biomarkers produced the highest diagnostic value.

## Methods

### Literature review

We previously performed a search of the literature identifying all studies which reported sensitivity of immunohistochemistry biomarkers VHL, IMP3, S100A4, and S100P for diagnosis of HPB malignancies in ERCP brushing specimens, EUS-FNA, or core needle biopsies [9]. While certain serum markers such as miRNAs have

been shown to be highly sensitive [10], we chose IHC analysis for this study as it is a common technique already in use by most pathology labs. Each study was analyzed for total number of individuals in the study (N), the true positive (pathology positive) results, and the test positive (number of patients with positive test results who have disease) for each biomarker [11–19]. The combined overall sensitivity for each biomarker was then calculated as the test positive patients divided by the true positive patients.

### Patient selection and immunostaining

After IRB approval was obtained, a total of 27 whipple specimens from patients with pancreatic adenocarcinomas treated between 2011 and 2015, were retrieved from the surgical pathology archives at University Hospital, Rutgers New Jersey Medical School. Archival tissue blocks were obtained and new 5 um sections were cut and immunohistochemistry staining was carried out in the standard fashion according to our lab protocol.

Briefly, specimens were deparaffinized and antigen retrieval was performed using the 2100 Antigen Retriever (Aptum Biologics, South Hampton, UK) and 0.01 M citrate buffer, at Ph- 6.0. Endogenous peroxidases were blocked with 3% hydrogen peroxide, and endogenous biotin was blocked with avidin. Immunohistochemical labeling was performed at 4C overnight using primary antibodies for Von Hippel-Lindau gene product (VHL, rabbit polyclonal, FL-181, Santa Cruz Biotechnology, Santa Cruz, CA, 1:400 dilution), insulin-like growth factor 2 mRNA-binding Protein 3 (IMP3, mouse monoclonal, Clone 69.1, Dako Agilent, Santa Clara, CA, 1:400 dilution), EF-hand Calcium 2 + binding S100 subfamily member A4 (S100A4, rabbit polyclonal, A5114, Dako Agilent, Santa Clara, CA, 1:2000 dilution), EF-hand Calcium 2 + binding S100 subfamily member P (S100P, mouse monoclonal, Clone 16, BD Biosciences, San Diego, CA, 1:800 dilution).

Depending upon the species of the primary antibody used, each section was incubated with either a biotinylated Goat anti-Rabbit or Goat anti-Mouse secondary antibody (Vector Labs, Burlingame, CA) at a dilution of 1:200 for 30 min at room temperature. Negative

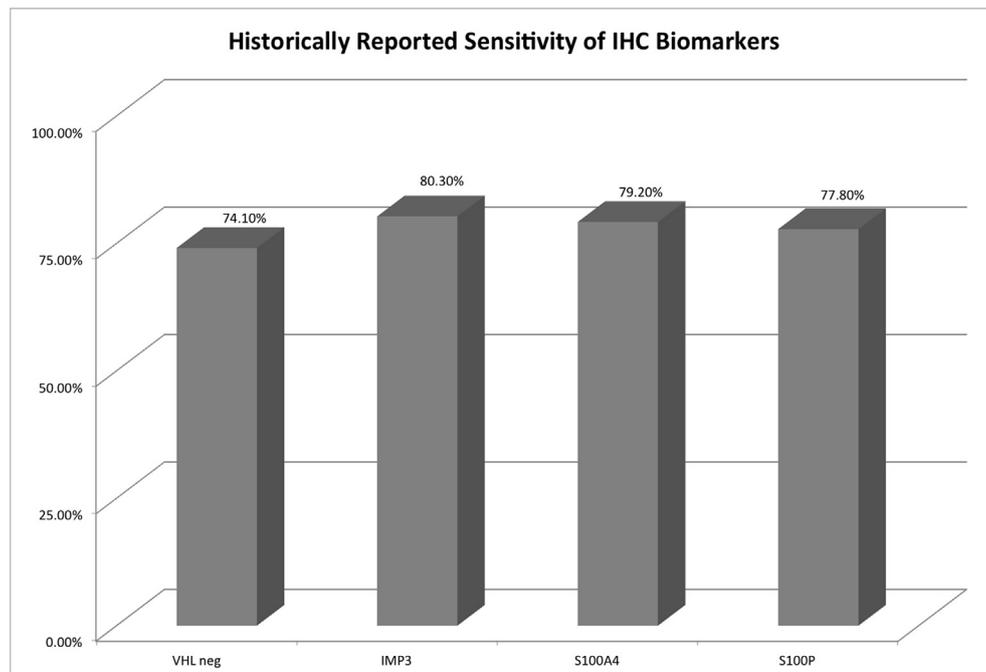


Fig. 1. Historically reported sensitivity of IHC biomarkers.

Control slides for the rabbit primary antibodies were incubated with a concentration matched Normal Rabbit Immunoglobulin Fraction (rabbit polyclonal, X0903, Dako Agilent, Santa Clara, CA) followed by secondary antibody. Negative control slides for the mouse antibodies were incubated with only the secondary antibody. The sections were then incubated with Vectastain Elite Avidin-Biotin Complex conjugated to horseradish peroxidase and detected using the NovaRed substrate solution (Vector Labs, Burlingame, CA) for 5 min at RT. Sections were counterstained using Lillie's Modified Mayer's Hematoxylin solution. Given previously described staining in the literature, positive controls for staining were established using pilot experiments with tumor (IMP3, S100A4, S100P) and normal pancreas (VHL).

Areas with tumor and areas with normal pancreas tissue were graded in all specimens by an experienced GI pathologist for intensity of staining and the percent of cells stained. Separate counts were taken for the normal major ducts, minor ducts, acini, islets, and tumor. Comparisons were performed between staining of major ducts and tumor unless otherwise specified.

### Statistical analysis

A histogram was created for each biomarker to facilitate in determining the correct cutoff criteria for the percent of cells staining, to use in order to call a specimen malignant. On the x-axis, the percent of cells in the specimen that stained for the indicated biomarker was recorded. On the y-axis, the number of specimens (as a percent of all specimens stained) for each biomarker was graphed. The cutoff points for each biomarker were chosen so that greater than 95% of normal pancreas specimens were consistently classified as negative by all biomarkers. The expectation, and primary hypothesis, was that any positive specimen that was missed by one biomarker would be caught by one of the other three biomarkers. Test parameters were calculated by standard formulas: sensitivity (true test positive/all pathology positive), specificity (true test negative/all pathology negative), negative predictive value (true test negative/all test negative), positive predictive value (true test positive/all test positive).

## Results

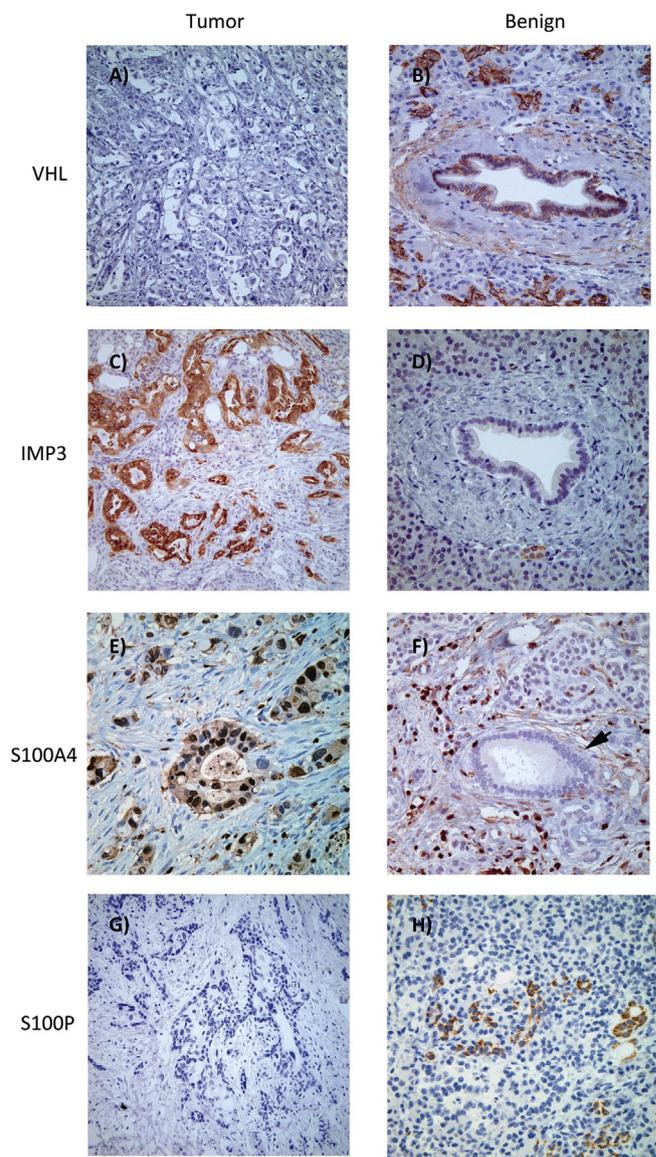
### Historical reports of biomarker sensitivity

Combined historical reports for the sensitivities of all 4 biomarkers are illustrated in Fig. 1. Historically, the literature reports the combined sensitivity of VHL for diagnosis of HPB malignancies as 74.1% ( $n = 43/58$ ) from two studies [11,13]. Both of these studies required less than 1% of cells staining in order to be consistent with malignancy. Combined IMP3 sensitivity in the literature is reported as 80.3% ( $n = 295/367$ ) from 9 studies. Four studies required staining to be at least 1% of cells or more [11,13,14,19], two studies required greater than 5% [12,15], 1 study required at least 10% or more [16], and one study require greater than 75% of cells staining to be considered malignant [17]. Information on the criteria used was not available for one study [18]. Sensitivity for S100A4 staining was reported as 79.2% ( $n = 19/24$ ) in one study which required staining of greater than 5% of cells to be considered malignant [12]. Finally, combined sensitivity for S100P has been reported as 77.8% ( $n = 98/126$ ) in three studies which all required staining of 1% of cells or more [11,13,14].

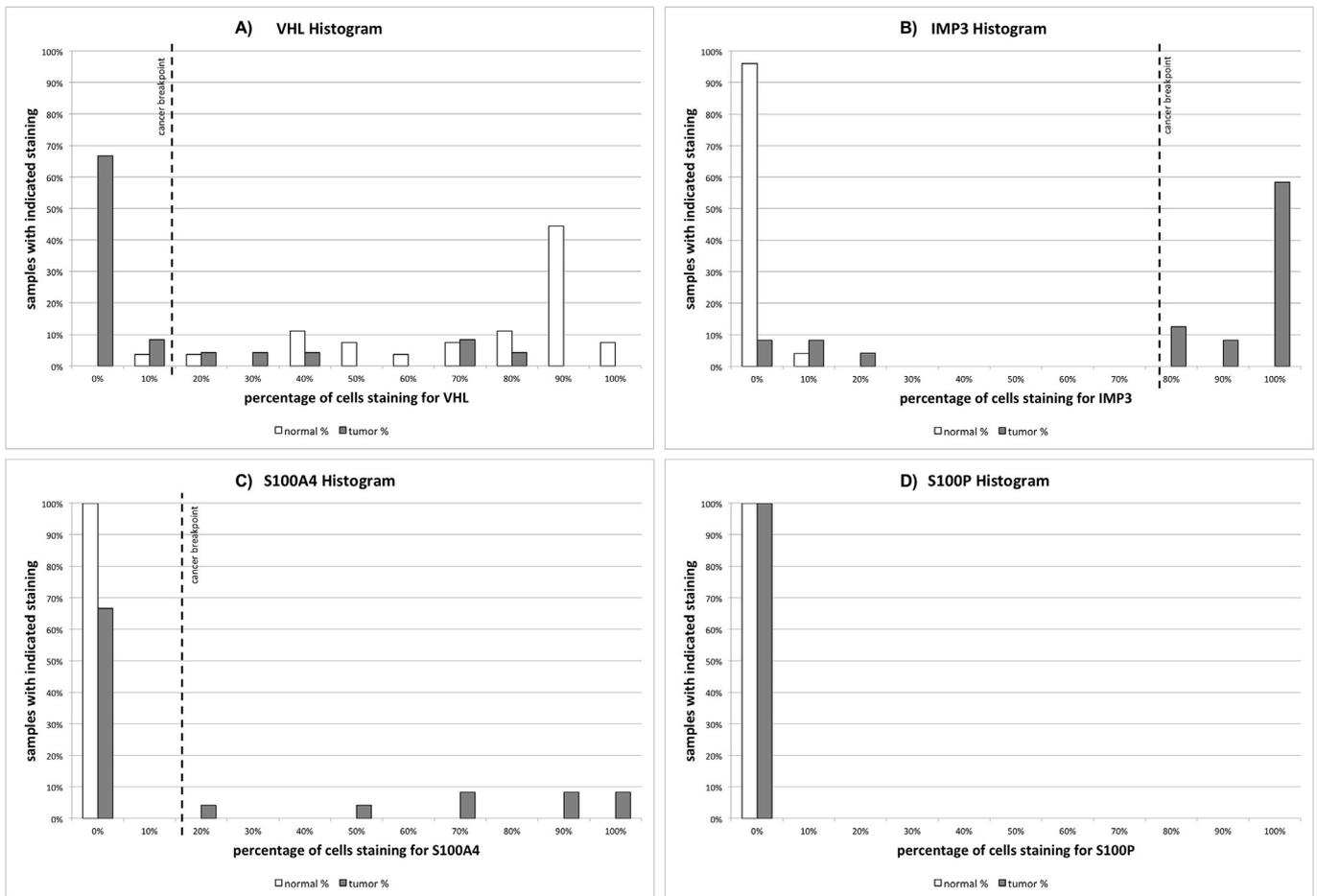
### Immunostaining of pancreatic cancer specimens

In order to validate these reports and to subsequently assess the function of these biomarkers as a combination panel, we first

performed immunostaining of 27 pancreatic adenocarcinoma Whipple specimens and their associated normal pancreas. Representative sections illustrate robust VHL staining in normal pancreas duct cells, but a dramatic loss of expression of VHL in pancreatic adenocarcinomas (Fig. 2B and A). IMP3 showed a complete lack of staining in pancreatic duct cells while the pancreatic adenocarcinoma specimens demonstrated strong staining (Fig. 2C–D). S100A4 also showed a complete lack of staining in pancreatic duct cells, but strong staining in pancreatic adenocarcinoma cells (Fig. 2E–F). S100P did not stain normal pancreas duct cells nor did it stain pancreatic adenocarcinoma cells (Fig. 2G–H). It was clear this was not due to a failure of the antibody because the S100P antibody had moderate staining of the smaller intercalating pancreatic ducts (2–60% of cells, Fig. 2H). Our negative control, Rabbit IgG, did not stain any specimens (data not shown), pancreatic acini did not stain with any of the biomarkers (data not shown), and pancreatic islet cells stained with VHL and IMP3 (data not shown).



**Fig. 2.** Immunohistochemistry in representative benign and malignant specimens. VHL stained A) tumor and B) benign specimens. IMP3 stained C) tumor and D) benign specimens. S100A4 stained E) tumor and F) benign specimens. S100P stained G) tumor and H) benign specimens.



**Fig. 3.** Distribution of staining for each biomarker. Histograms showing the percent of cells staining (x-axis) in each specimen, and number of samples with each level of staining (percent, y-axis). Histograms for A) VHL, B) IMP3, C) S100A4, D) S100P are shown with a line drawn at the chosen cutoff value.

**Table 1**  
Diagnostic criteria for biomarker panel.

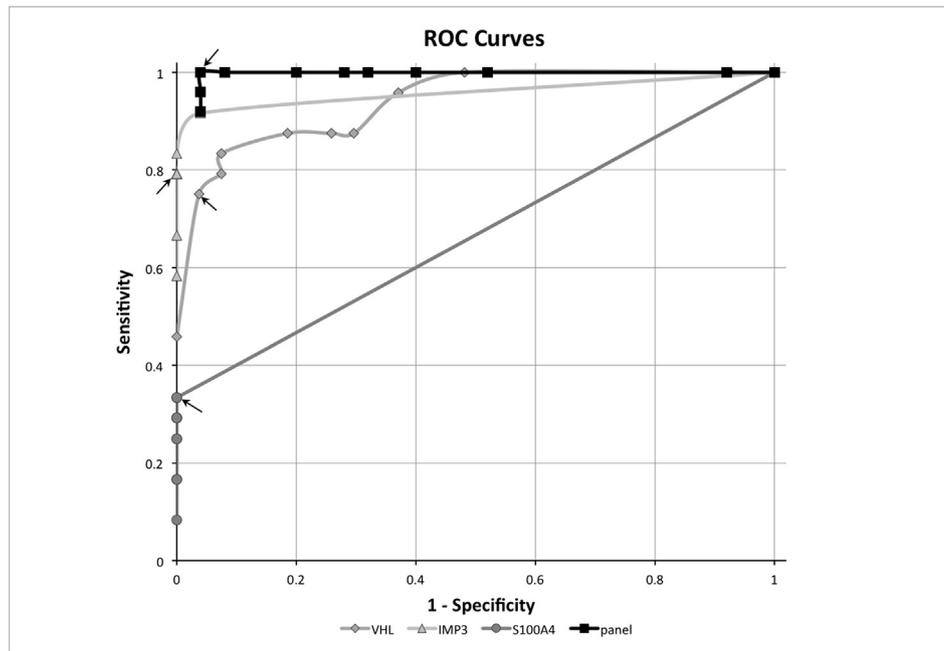
Value	Treatment
VHL neg	malignancy $\leq$ 10% cells staining
IMP3	malignancy $\geq$ 80% cells staining
S100A4	malignancy $\geq$ 20% cells staining
minimum malignancy criteria	any of the above tests being positive
minimum benign criteria	all tests negative for malignancy
indeterminate	some tests missing data but all other tests benign

Histogram analysis of VHL staining revealed that the majority (75.0%) of the adenocarcinoma specimens had 10% or fewer cells staining while the majority (96.3%) of normal duct cells had greater than 20% VHL staining (Fig. 3A). Therefore, we defined VHL staining of 10% or fewer cells as being consistent with malignancy (Table 1). Histogram analysis of IMP3 staining revealed that the majority (79.2%) of adenocarcinoma specimens had 80% or more cells staining while all (100%) normal duct cells examined had 10% or fewer cells staining for IMP3 (Fig. 3B). Therefore, we defined IMP3 staining of 80% or more cells to be consistent with malignancy (Table 1). Histogram analysis of S100A4 revealed that a modest 33.3% of adenocarcinoma specimens had 20% or greater cells staining. However, S100A4 staining of all (100%) normal tissues was limited to less than 10% of cells (Fig. 3C). Therefore, we defined S100A4 staining of 20% or greater as being consistent with

malignancy (Table 1). Major duct cells had no staining for S100P which was indistinguishable from the staining pattern of normal cells and therefore this marker could not be used to identify tumor cells (Fig. 3D). If any of the 3 biomarkers were positive for malignancy, this alone was sufficient to call the specimen malignant (Table 1). However, if biomarker staining were negative for malignancy but 1 or more biomarkers were inconclusive, we considered the biomarker panel non-diagnostic for that specimen (Table 1).

*Receiver Operator Curve Analysis*

In order to further determine the appropriateness of our cutoff values, receiver operator curves were created for each test separately and for the test panel as a whole (Fig. 4). The biomarker panel is able to discriminate tumors with a superior sensitivity (100%) compared with the individual tests, and an acceptably high specificity (96%), (Fig. 4, black squares, small arrow). The small arrows illustrate our selected cutoff values. As noted earlier, individual cutoff values with high specificities were chosen in order to preserve specificity in the final combined panel analysis. While this decision resulted in slightly lower individual sensitivities, it did not present a problem as the combined panel was able to compensate and find the tumors that were missed by the single biomarker test, thus proving our hypothesis.



**Fig. 4.** Receiver Operator Curve Analysis for Biomarkers and Combined Panel. ROC curves were drawn for VHL (medium grey diamonds), IMP3 (light grey triangles), S100A4 (dark grey circles), and the combined panel (black squares). The small arrows illustrate the cutoff values (percent of cells staining) chosen for each test and show where on the ROC curve they fall.

#### Biomarkers used as a panel

Using the criteria defined in Table 1, and validated by our ROC curves in Fig. 4, all the pancreatic adenocarcinoma specimens and normal pancreas specimens were scored for each biomarker. Sensitivity of each biomarker separately and in combination was calculated. We found that VHL loss of expression had a sensitivity of 75.0%, IMP3 had a sensitivity of 79.2%, and S100A4 had a sensitivity of 45.8% in detecting malignancy (Fig. 5A). However, in combination, the sensitivity of the biomarker panel was 100% (Fig. 5A). Cutoff values were chosen to maintain specificity of VHL (96.3%), IMP3 (100%), and S100A4 (100%). When the biomarkers were combined as a panel, the specificity was maintained at 96.0% (Fig. 5B). We examined the other parameters of our biomarker panel and found the negative predictive value was 100% and the positive predictive value was 96.2% (Table 2). The biomarker panel produced a non-diagnostic result 7.4% of the time based on our criteria.

#### Discussion

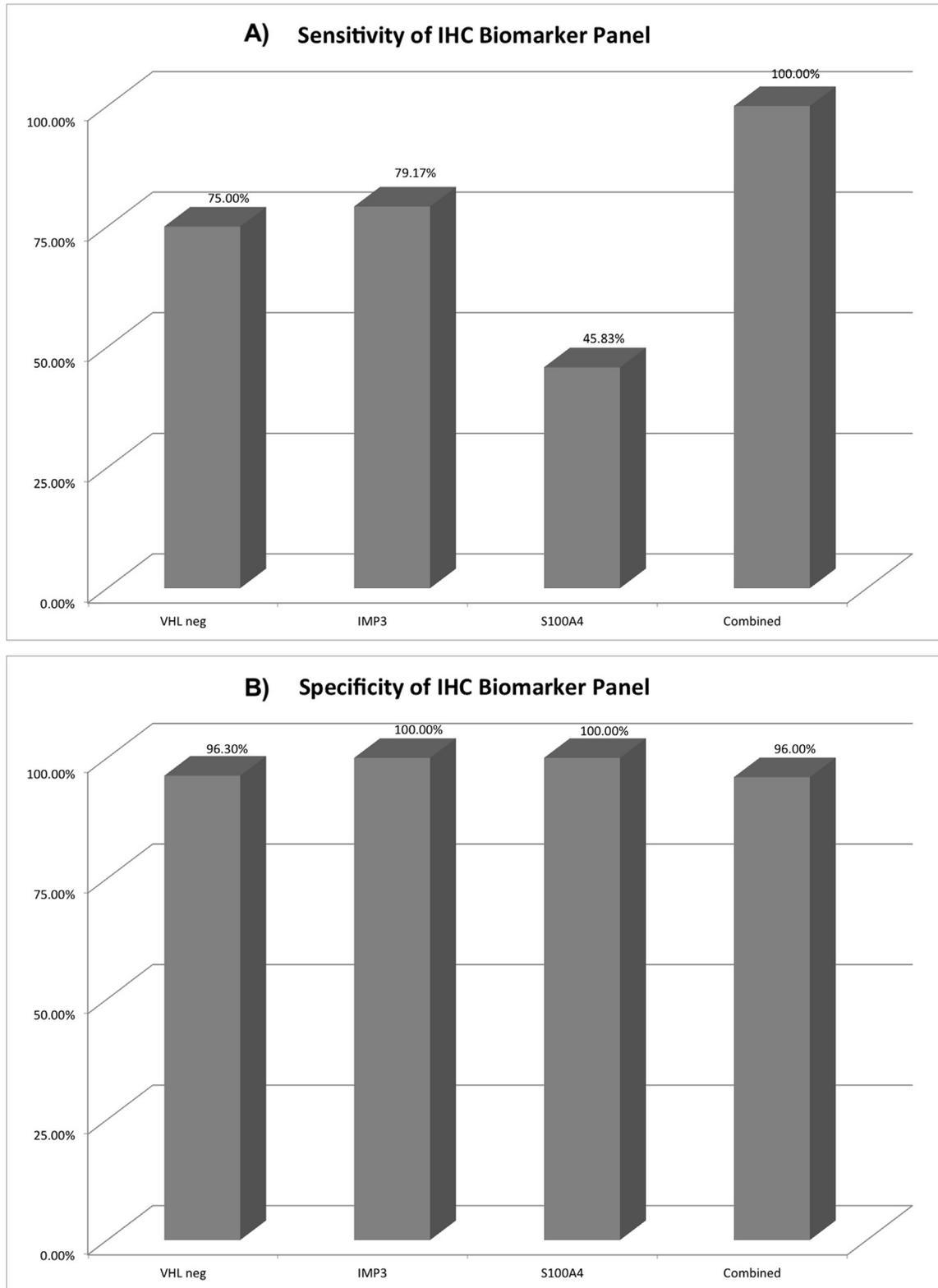
Although the ability to diagnose pancreatic adenocarcinoma has improved over time, there are still challenges with differentiating malignancy from benign disease. Application of EUS-FNA has helped to reduce these issues by providing a tissue sample for cytological review, however, an inadequate biopsy can diminish its diagnostic value [20]. As a way to lessen the need for an adequate biopsy, researchers have begun to utilize adjuvant testing for biomarkers on the tissue sample. One such method that is currently being evaluated is IHC staining. Due to the lack of any singular biomarker having a high diagnostic value, studies have implemented the use of biomarker panels [21]. Identification of the particular combination of IHC markers that yields the highest sensitivity and specificity will be instrumental in further strengthening our ability to diagnose pancreatic adenocarcinoma.

In this study we sought to identify biomarkers with high

sensitivities that when used in a panel might boost sensitivity to 100% by allowing the complimentary biomarkers to find the malignancies missed by the individual tests. Following literature review, we decided on IMP3, S100P, S100A4, and VHL as our biomarkers of interest. After the determination of the proper staining thresholds for each IHC marker to indicate malignancy, we were able to achieve a sensitivity of 100% and a specificity of 96% when utilizing a panel of VHL, IMP3 and S100A4. A search of the published literature revealed that we were the first to use this specific combination of markers within a panel. However, several other recent studies have incorporated two of these markers within their own panels [22,23].

Each of above-mentioned studies examined a different set of biomarkers for pancreatic adenocarcinoma. Liu et al. investigated 26 different IHC markers (S100A4 not included) and identified that VHL, Maspin, S100P, and IMP-3 would yield the panel with the best diagnostic value [22]. This conclusion was based upon the percentage of staining seen between ductal adenocarcinoma versus normal pancreatic tissue, with Maspin, S100P and IMP-3 being positive in 90% of malignant cases and VHL being negative in 100% of malignant cases. Sensitivity and specificity were not calculated for the panel. Sweeney et al. found that a panel of S100P, IMP3 and mothers against decapentaplegic homolog 4 (SMAD4) resulted in a sensitivity of 91.89% and specificity of 100% when at least two of the three markers were positive [23]. Our study differed from these two investigations in two major ways. First, both of these studies reported false-positive staining of S100P, as did we for intercalating (small) ducts, which made use of this biomarker difficult to justify. Secondly, our study examined the ideal cutoff values to signify malignancy for each corresponding biomarker, something of which we have found to be severely underreported within the literature.

Several previous studies have also examined the utility of these biomarkers within cholangiocarcinoma [10–13]. Due to the similar morphology seen in extrahepatic cholangiocarcinomas, it is not unanticipated that these biomarkers were used successfully within our study for pancreatic adenocarcinoma. Each of the above-



**Fig. 5.** Sensitivity and Specificity of Biomarkers and Combined Panel. The A) sensitivity and B) specificity of each biomarker separately and the test panel as a whole was calculated based on our chosen cutoff values.

**Table 2**  
Parameters of biomarker panel.

Parameter	Result
Sensitivity	100.00%
Specificity	96.00%
Negative Predictive Value	100.00%
Positive Predictive Value	96.15%
Indeterminate Rate	7.41%

mentioned studies used a portion of the biomarkers we reviewed for our panel. Ligato et al. reported sensitivity of 100% and specificity of 95% using only IMP3 and S100A4 [11]. Within our study, this combination would have led to a sensitivity of only 85% (data not shown); we required the extra information from VHL to achieve a 100% sensitivity. Kawashima et al. were able to achieve a sensitivity of 90% using only IMP3 and S100P as a biomarker panel, however, they reported 50% of their benign samples being positive for S100P [13]. S100P posed a similar problem for Levy et al. and Schmidt et al. as well, who both used a panel of S100P, VHL, and IMP3 [10,12]. In addition, both of these studies had very small sample sizes and neither study was able to combine the results of these biomarkers to achieve 100% diagnostic sensitivity. As seen with the other pancreatic adenocarcinoma studies, our panel differed in that we ruled out the use of S100P and optimized our panel by determining the staining thresholds prior to diagnostic analysis.

Our study is not without limitations. As a retrospective, non-randomized, single institution study, the opportunity for well-described selection biases arises. In addition, we identified several specific points that potentially limit the robustness of our analysis. First, our design used 27 malignant and 27 benign-appearing pancreas areas as a training set, but we did not obtain an entirely different set of specimens as a validation set. We sought to mitigate this limitation by determining our selected cutoff values using both the histogram and ROC curve analyses. Secondly, resected normal pancreas surrounding a tumor may not be entirely normal as we did not compare the samples to control tissues for pancreatitis or other inflammatory conditions. Within our study, tissue was classified as benign so long as no tumor cells were in the field. Third, our panel relies on quantification of staining by pathologist by counting cells in a field. While this is a routine task for most pathologists, it might be time consuming if multiple fields need to be counted. Development of commercial IHC automation devices is ongoing [24].

The next step in our analysis will be to see if the findings presented here with surgical specimens translates to those obtained by EUS-FNA cytology. For purposes of our study, using resected specimens posed an advantage as our retrospective design allowed us to obtain tissue from old paraffin blocks. Furthermore, it provided us with enough tissue to study the ideal cutoff values for malignancy diagnosis for each biomarker. As mentioned previously, we consider this aspect of our study to be one of the notable reasons we were able to identify a biomarker panel with high diagnostic value. The lack of histological architecture will create challenges in translating these results into a prospective study using biopsy specimens. Nevertheless, several studies have demonstrated success using IHC on EUS-FNA biopsies, suggesting that such a technique is feasible [23,25].

To summarize, the risk of a misdiagnosis of pancreatic adenocarcinoma in the presence of benign disease has led to a focus on adjuvant tests for inconclusive tissue biopsies. We designed a proof of concept study to evaluate the use of S100A4, IMP-3, S100P and VHL within a biomarker panel for purposes of diagnosing pancreatic adenocarcinoma. By first identifying the staining thresholds

then performing ROC curve analysis, we were able to determine that the combination S100A4, IMP-3 and VHL yielded a sensitivity of 100% and a specificity of 96%. This study provides the basis for future prospective randomized evaluation of these biomarkers in biopsy specimens.

## Funding source

Rutgers University Foundation Grant 202194.

## Disclosure

There are no financial conflicts of interest to disclose for any of the authors.

## Presentations

ASCO GI as a poster Jan 2018; SSO as a poster March 2018 (Best Poster).

## Author contributions

All authors contributed to the conception and design, acquisition of data, analysis and interpretation of data, editing, and final approval. PLQ, AB, and DA contributed to the drafting of the article. Revision of the article was performed by SP, SA, OM, and RJC.

## Acknowledgement

Rutgers NJMS core facilities manager Luke Fritzy for his help with specimen staining. The authors acknowledge the funding from the Rutgers Foundation Grant.

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