Published in final edited form as:
J Gastrointest Surg. 2009 November ; 13(11): 1948–1954. doi:10.1007/s11605-009-1001-9.

Plectin-1 is a Biomarker of Malignant Pancreatic Intraductal Papillary Mucinous Neoplasms

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Abstract

Introduction—Pancreatic intraductal papillary mucinous neoplasms (IPMN) are now identified with increasing frequency. The detection of carcinoma in IPMN is difficult and suffers from high false-positive and false-negative rates, often resulting in inappropriate treatment decisions. Improved detection of malignancy using novel biomarkers may therefore improve diagnostic accuracy. One such promising novel biomarker is Plectin-1 (Plec-1).

Methods—Using immunohistochemistry, Plec-1 expression was assayed in benign (low and moderate dysplasia, n=6) as well as malignant IPMN (high-grade dysplasia and invasive carcinoma, n=31) and lymph node metastases from carcinoma arising in IPMN (n=12). Furthermore, cyst fluids from benign (n=3) and malignant IPMN (n=4) were evaluated for Plec-1 expression.

Results and discussion—Twenty-six of 31 malignant IPMN and all 12 lymph node metastases were Plec-1 positive. In contrast, only one of six benign IPMN expressed Plec-1. The specificity of Plec-1 in distinguishing malignant IPMN from benign IPMN was 83% and its sensitivity 84%. Furthermore, all (four out of four) cyst fluids from malignant IPMN, but none of the three benign IPMN, were Plec-1 positive. These data support Plec-1 as an excellent biomarker for the early detection of carcinoma arising in IPMN.

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Keywords

Plectin-1; Biomarker; Malignant IPMN; Benign IPMN

Introduction

Over the past decades, intraductal papillary mucinous neoplasms (IPMN) of the pancreas have been identified with increasing frequency; they now account for up to 20% of all resected pancreatic specimens in large referral centers.[1–6] IPMNs are thought to progress from low-grade dysplasia (adenoma) to high-grade dysplasia (carcinoma in situ) and invasive carcinoma through moderate-grade dysplasia (borderline malignancy). Invasive carcinoma in IPMN is present in 12.5–57% of all IPMN cases.[3,4,7,8] The 5-year survival rate of all patients with surgically resected malignant IPMN is up to 70%[3,4,7,8] and thus much better than the corresponding rate for pancreatic ductal adenocarcinoma, which is less than 20%.[9–11]

Clinically, IPMNs are classified as lesions of the main pancreatic duct (MD-IPMN), the branch ducts (BD-IPMN), or both (combined-type IPMN).[5] MD-IPMN and combined-type IPMN have a high risk of malignancy (57–92%) and frequently of invasive carcinoma (23–57%), and surgery is therefore recommended.[5,6,12,13] BD-IPMN has a much lower risk of malignancy, 6–46%[5,6,12,13] and indications for surgery are less clear. The only way to stratify the risk of malignancy in IPMNs at present is by clinical symptoms, high-resolution cross-sectional abdominal imaging, endoscopic ultrasound with fine needle aspiration biopsies (FNA), cytology, and cyst fluid analysis for carcinoembryonic antigen and carbohydrate antigen 19.9.[14–19] Current international consensus management guidelines[5] recommend following small BDIPMN (<3 cm) in asymptomatic patients with high-resolution cross-sectional abdominal imaging studies. Mural nodules on radiologic studies, a dilated main pancreatic duct (>6 mm), positive cytology, or a cyst size larger than 3 cm all correlate with malignancy, and resection is therefore recommended when these signs and symptoms are present. However, the predictive value of these guidelines to correctly distinguish benign from malignant cysts remains low, as not all small and asymptomatic BD-IPMN are nonmalignant[20] and up to 85% of surgically treated patients have no malignancy despite the presence of the aforementioned signs and thus undergo unnecessary resection.[21]

Recently, in an effort to improve diagnostic accuracy, analyses of genetic changes in cyst fluid have been used, but these suffer from limited clinical validation and high cost.[22] No other reliable biomarkers are presently available to clearly distinguish malignant from nonmalignant IPMN.

Novel biomarkers that improve the accuracy of detection of malignancy in IPMN, especially in BD-IPMN, are therefore much needed. One such novel and promising biomarker may be Plectin-1 (Plec-1). Plec-1 was initially identified in a phage display screen for unique markers of pancreatic ductal adenocarcinoma[23] and found to be highly specific and sensitive for early and invasive cancer. Plec-1 expression intensity increases during pancreatic carcinogenesis; strong and specific staining has been identified in 60% of PanIN-3 lesions (ductal carcinoma in situ; Bausch et al., manuscript in preparation). Based on these findings, the aim of this study was to evaluate whether Plec-1 is a potential specific biomarker for malignant IPMNs, whether Plec-1 expression can be exploited to identify metastatic foci in lymph nodes, and whether cyst fluid analysis for Plec-1 allows the discrimination of malignant from benign cysts.

Material and Methods

Tissue Samples

All tissues and biologic samples were collected with the approval and in accordance with the requirements of the Institutional Review Board of the Massachusetts General Hospital, Boston, MA, USA. Paraffin-embedded tissue samples were obtained from the files of the Department of Pathology of the Massachusetts General Hospital, Boston, MA, USA. All specimens had an established diagnosis at the time of assessment. A total of 37 cases of IPMN were obtained, six benign cases and 31 malignant IPMN.

The six benign IPMN were low- and moderate-grade dysplasia (adenoma and borderline malignancy). Ten of the malignant cases were high-grade dysplasia (carcinoma in situ); six of the invasive carcinomas arising in IPMN were of colloid and 15 of ductal phenotype. Three colloid and nine ductal adenocarcinomas were noted to have lymph node metastases and were also analyzed for Plec-1 expression. Cyst fluids were from benign (n=3) and malignant (n=4) IPMN and were also analyzed for Plec-1 expression.

Immunostaining

Paraffin-embedded sections were deparaffinized, hydrated with Tris-buffered saline (TBS) and blocked with H_2O_2. Antigen retrieval was achieved by boiling tissue in Retrievit (BioGenex, San Ramon, CA, USA). After blocking with avidin/biotin (Vector Laboratories, Burlingame, CA, USA) and 5% goat serum in TBS, slides were incubated overnight at 4°C with 1:250 Plec-1 antibody (Abcam). Sections were washed three times in TBST, followed by incubation with biotinylated antirabbit goat secondary antibody (Vector Laboratories, Burlingame, CA, USA), then developed using 3,3′-diaminobenzidine tetrachloride (Invitrogen, Carlsbad, CA, USA), and counterstained with hematoxylin.

Histological Assessment

Nerves were noted to have moderate staining intensity for Plec-1 and were present on all slides. Expression of Plec-1 in nerves within each slide was therefore used as a staining control and reference for staining intensity. Staining intensity was recorded by two independent observers and, in case of discrepant results, evaluated by a third observer. Specific focal staining of abnormal epithelial cells was considered positive if it was noted to be at least as strong as nerves.

Immunoprecipitation and Western Blot Analysis of Cyst Fluids

Plec-1 expression was evaluated by Immunoprecipitation and Western Blot analysis of cyst fluids from nonmalignant and malignant IPMN. After the addition of Triton X-100 to a final concentration of 1% (v/v) in combination with a protease inhibitor cocktail (Halt™, Thermo Scientific, Rockford, IL, USA), fluids were incubated at 4°C and cleared by centrifugation. 0.1 to 1 ml of fluid was incubated together with 10 μg mouse monoclonal antibody against human Plec-1 (Santa Cruz Biotechnology, La Jolla, CA, USA) and 50 μl protein G Sepharose (Amersham Biosciences, NJ, USA). The beads were then washed thrice with washing buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1% Triton X-100). Bound protein was eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated via SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Antigen detection was performed using a rabbit monoclonal antibody against human Plec-1 (Abcam, Cambridge, MA, USA). The secondary antibody was an horseradish peroxidase-coupled goat antirabbit polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). Bands were visualized with enhanced chemiluminescence. Rat brain lysate (Santa Cruz Biotechnology, La Jolla, CA, USA) was used as positive control.[24]

Results

Plec-1 was identified in 84% (26 out of 31) of malignant IPMNs. Eighty percent (eight out of ten) of the high-grade dysplasia (carcinoma in situ) samples expressed Plec-1 and 86% (18 out of 21) of the invasive carcinomas were Plec-1 positive. Two distinct types of invasive carcinoma occur in IPMN: colloid carcinoma (CC) and ductal adenocarcinoma (DA). DA is characterized histologically by infiltrating small tubular units and a marked desmoplastic host reaction, while CC is characterized by dissecting nodules of mucin that contain scant numbers of malignant cells. Fifteen of the 21 invasive carcinomas were classified as DA; the remaining six were classified as CC. Fourteen of the 15 DA samples (93%), but only 66% (four out of six) of CC samples, expressed Plec-1 (Fig. 1a, b). In contrast to malignant IPMNs, only one out of six of the benign IPMN was Plec-1 positive (Fig. 1a, b). The positive benign IPMN was identified as moderate-grade dysplasia.

Taken together, the specificity of Plec-1 in distinguishing malignant from benign IPMN was 83% and its sensitivity was 84%. Sensitivity for high-grade dysplasia (in situ carcinoma) was 80% and for invasive carcinoma was 86%.

Plec-1 was also reliably identified in lymph node metastases. Twelve of the 31 malignant IPMN evaluated had metastases to lymph nodes. Nine were of DA and three of CC differentiation. Metastases in all nine lymph node metastases deriving from DA-IPMN and all three lymph nodes from CC-IPMN stained for Plec-1 (Fig. 1a, b).

To determine whether there is sufficient Plec-1 present in IPMN cyst fluids to allow the detection of malignancy, Plec-1 immunoprecipitation of cyst fluids from benign and malignant IPMN was performed. Plec-1 was found in 100% (four out of four) of the cyst fluids from malignant IPMN. Three of the Plec-1-positive cyst fluids were from DA and one was from CC. In contrast, cyst fluid from all three benign IPMN contained no detectable Plec-1 (Fig. 2).

Discussion

Presently, there are no available biomarkers to assist accurately in distinguishing benign from malignant IPMN. Here, we identify Plec-1 as a potential novel biomarker for carcinoma arising in IPMN. Plec-1 was specifically expressed in the vast majority of carcinomas arising in IPMN as well as in its lymph node metastases, whereas most benign IPMN did not express the protein. In addition to these findings, we were able to demonstrate specific Plec-1 detection in cyst fluid from malignant IPMN. This allows the incorporation of Plec-1 expression analysis into the routine clinical analysis of cyst fluid as an additional screening measure for cancer.

Two distinct types of invasive carcinoma occur in IPMN, CC and DA. It is estimated that 25% to 50% of carcinomas arising in IPMNs are CC,[1,4,8,25–27] and in our series, CC accounted for 29% of the invasive carcinomas. In contrast to DA, where Plec-1 was almost always (93%) expressed, CC showed somewhat fewer Plec-1-positive cases (66%). This may be due in part to difficulty in detecting Plec-1 in the abundant intracellular mucin content of CC cells. However, genetic differences between DA and CC may also account for the lower expression rate of Plec-1 in CC compared to DA.

IPMN follow a classical adenoma–carcinoma sequence, progressing from low-grade and moderate-grade dysplasia to carcinoma in situ (high-grade dysplasia) and finally to invasive carcinoma. Based on our data, Plec-1 expression is acquired during the transition from moderate-grade dysplasia to carcinoma in situ (high-grade dysplasia). A small fraction of moderate-grade dysplasia IPMN (17%) expressed Plec-1, while the majority of carcinoma in

situ cases (80%) were Plec-1 positive. Invasive DA had an even higher rate of positive samples (93%). It appears that Plec-1 overexpression may begin to appear at the stage of moderate dysplasia even before histological progression becomes evident.

Overall, Plec-1 expression analysis offers improved specificity over present methods of detecting malignancy in IPMN. In one series of 84 patients, the overall sensitivity of the international consensus guidelines[5] for predicting malignancy in BD-IPMN was 97.3%; however, their specificity was only 29.8%.[14]

FNA with cytology can identify malignancy with high specificity and sensitivity when a relevant tissue sample can be obtained.[17,19] However, the reliability of this diagnostic method depends on an experienced gastrointestinal cytopathologist, and its utility for risk stratification and therapeutic approach is limited.[28]

In contrast, Plec-1 in this study of 37 patients had a sensitivity of 84% and specificity of 83% in distinguishing malignant from benign IPMN and was thus equivalent to or better than other diagnostic approaches. The sensitivity of Plec-1 staining for high-grade dysplasia was a somewhat lower (80%), but it was excellent for invasive carcinoma (86%), especially the more common DA (93%).

Evaluation of cyst fluid from FNA for Plec-1 may greatly improve diagnostic accuracy and add valuable additional information to the clinical diagnostic panel at little additional cost. Additionally, in contrast to cytology, it is technically easy to perform and not operator dependent. The technical feasibility of cyst fluid analysis for Plec-1 was successfully demonstrated in this study, in which the protein was detectable in all cyst fluids from malignant IPMN. Validation in a larger data set is in progress.

We conclude that Plec-1 is a sensitive and specific biomarker for the early detection of malignant IPMN. Plec-1 expression analysis can be easily incorporated into routine clinical cyst fluid analyses and holds promise of contributing substantially to improving diagnostic accuracy for the detection of malignancy arising in IPMN.

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Appendix

Discussant

Dr. Edward Whang (BWU, Boston): Congratulations for a nice study, and congratulations on picking an excellent mentor with whom to work.

I have some tough questions, but I am sure you will be able to handle them.

First, some methodology questions: How did you pick the cases for inclusion in the study? Surely this is a very small subset of IPMNs available to study at MGH.

Also, I noticed the results you presented today are somewhat different from what you wrote in the abstract. In the abstract, you wrote that one of the colloid carcinomas expressed Plectin-1, whereas today, you said four of them expressed Plectin-1. Is there difficulty in interpretation of the immunohistochemistry in the examples that account for that difference?

Now for some philosophical questions: The sensitivity and specificity associated with using Plectin-1 expression status as a basis for distinguishing benign and malignant IPMNs are each less than 85%. Are those performance characteristics sufficient for clinical application?

Lastly, why is it important to differentiate benign from malignant IPMNs preoperatively? Is not the goal of surgery to prevent cancer from developing? Maybe what you really should seek is a biomarker that differentiates benign IPMNs that are destined to become cancer from benign IPMNs that are destined to remain benign for the remainder of the patient's life.

Closing Discussant

Dr. Dirk Bausch: Invasive carcinoma arising in IPMN is a rare occurrence. Therefore, only a limited number of cases were available to us and included in the study. Benign IPMN and noninvasive malignant IPMN are much more common. For the purpose of the study, an

equal number of main duct and branch duct IPMN were assayed. The relatively small number of benign cases compared to malignant cases assayed is a limitation of the study.

Colloid carcinoma cells contain a high amount of mucin, replacing most of the cytoplasm where Plectin-1 is normally identified. This made the evaluation of these samples exceedingly difficult. To accommodate for these difficulties, two independent observers evaluated all slides.

Sensitivity and specificity of Plectin-1 to detect malignant IPMN were about 85%. Currently employed screening methodology to detect malignancy in branch duct IPMNs, such as the international consensus criteria, have a sensitivity of only about 30%. Therefore, the clinical use of Plectin-1 as an additional screening modality may improve the sensitivity to detect malignancy in IPMN substantially.

The distinction between benign and malignant IPMN is important in the case of branch duct IPMNs, which have a relatively low risk of malignancy. Here, the risk associated with surgical therapy can outweigh the risk of malignancy, especially in the elderly population with small IPMNs.

However, it is important that a biomarker for IPMN identifies preinvasive carcinoma in situ, i.e., high-grade dysplasia lesions, whose prognosis is excellent after surgical resection. Plectin-1 identified about 80% of these cases.

Discussant

Dr. Joe Hines (UCLA): Let me ask the goal of this would be to take cyst aspirate to determine if it the cyst benign or malignant?

Closing Discussant

Dr. Dirk Bausch: The aim is to determine if a cyst is benign or malignant by assessing Plectin-1 expression in a cyst fluid aspirate. The goal is to exclude the operator dependency cytology suffers from and to substitute or augment it with an objective assay for Plectin-1.

Discussant

Dr. Joe Hines (UCLA): But my question is, is Plectin-1 shed into the fluid or does the analysis actually require cells? Because, as you said, the ability to access cytologic aspects for these types of lesion is highly unreliable.

Closing Discussant

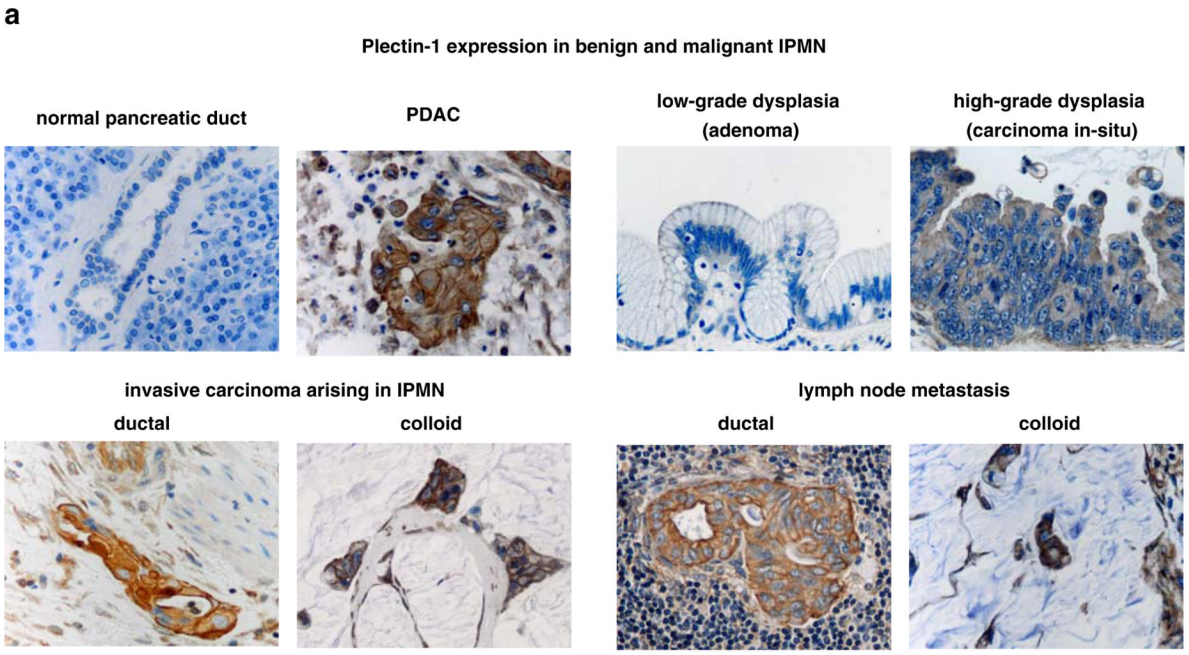
Dr. Dirk Bausch: In this study, we used cyst fluids obtained from surgical specimens. Since all cyst fluids were centrifuged before being assayed, they should not contain cells. Therefore, Plectin-1 is most likely shed into the fluid itself. However, Plectin-1 content is very low in cyst fluid. Therefore, enrichment by immunoprecipitation was required prior to detection.

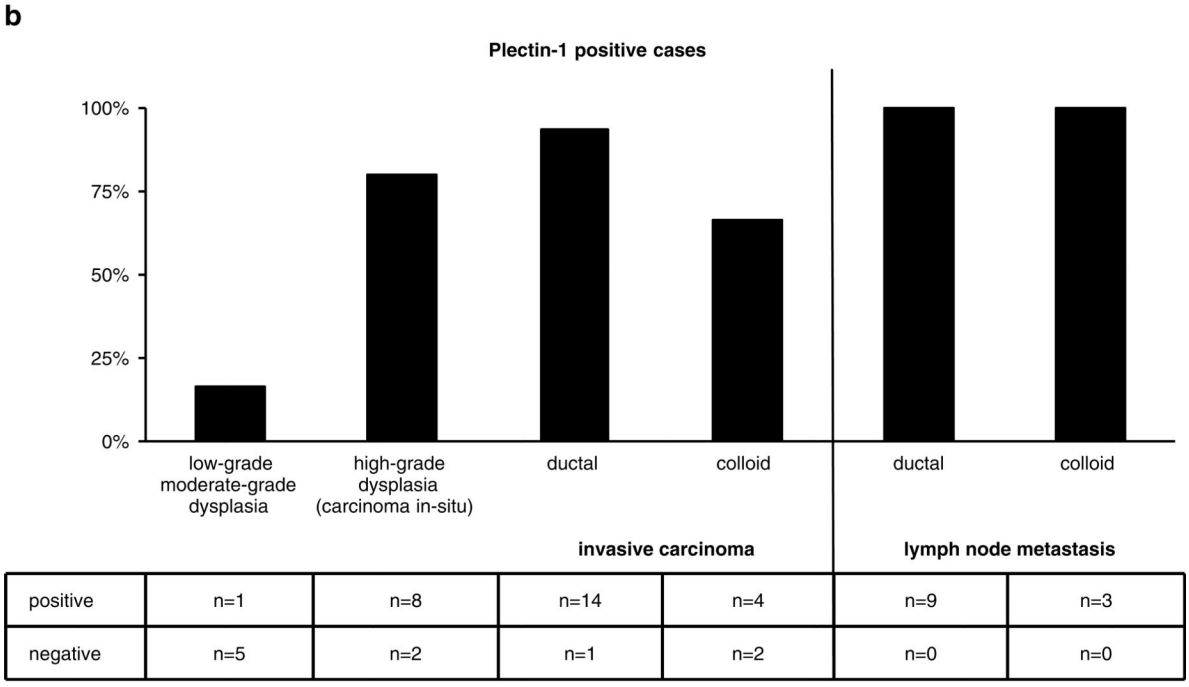
Discussant

Dr. Marc Basson (Michigan State University, East Lansing, MI): Do you think that you would you improve on your sensitivity and specificity if you combined the Plectin-1 reactivity with the other clinical criteria that are already in use. Have you gone back and looked at the numbers? I realize they are small.

Closing Discussant

Dr. Dirk Bausch: No such comparison was made in the current study. Our long-term goal is to improve overall sensitivity and specificity by using Plectin-1 together with clinical criteria.





positive	n=1	n=8	n=14	n=4	n=9	n=3
negative	n=5	n=2	n=1	n=2	n=0	n=0

Figure 1.

Plectin-1 immunohistochemistry. **a** Representative images of evaluated normal pancreata, PDAC, low-grade and high-grade dysplasia IPMN, ductal and colloid carcinoma arising in the background of IPMN as well as lymph node metastases from ductal and colloid carcinoma arising in the background of IPMN. Normal pancreas and the majority of benign IPMN do not express Plec-1. PDAC, as well as most high-grade dysplasia IPMN, ductal and colloid carcinomas arising in the background of IPMN and their lymph node metastases, are Plec-1 positive. **b** Distribution of staining for Plec-1 in the specimens. The majority of benign IPMN are Plectin-1 negative. Most high-grade dysplasia IPMN, ductal and colloid

carcinomas arising in the background of IPMN, as well as their lymph node metastases, are Plec-1 positive.

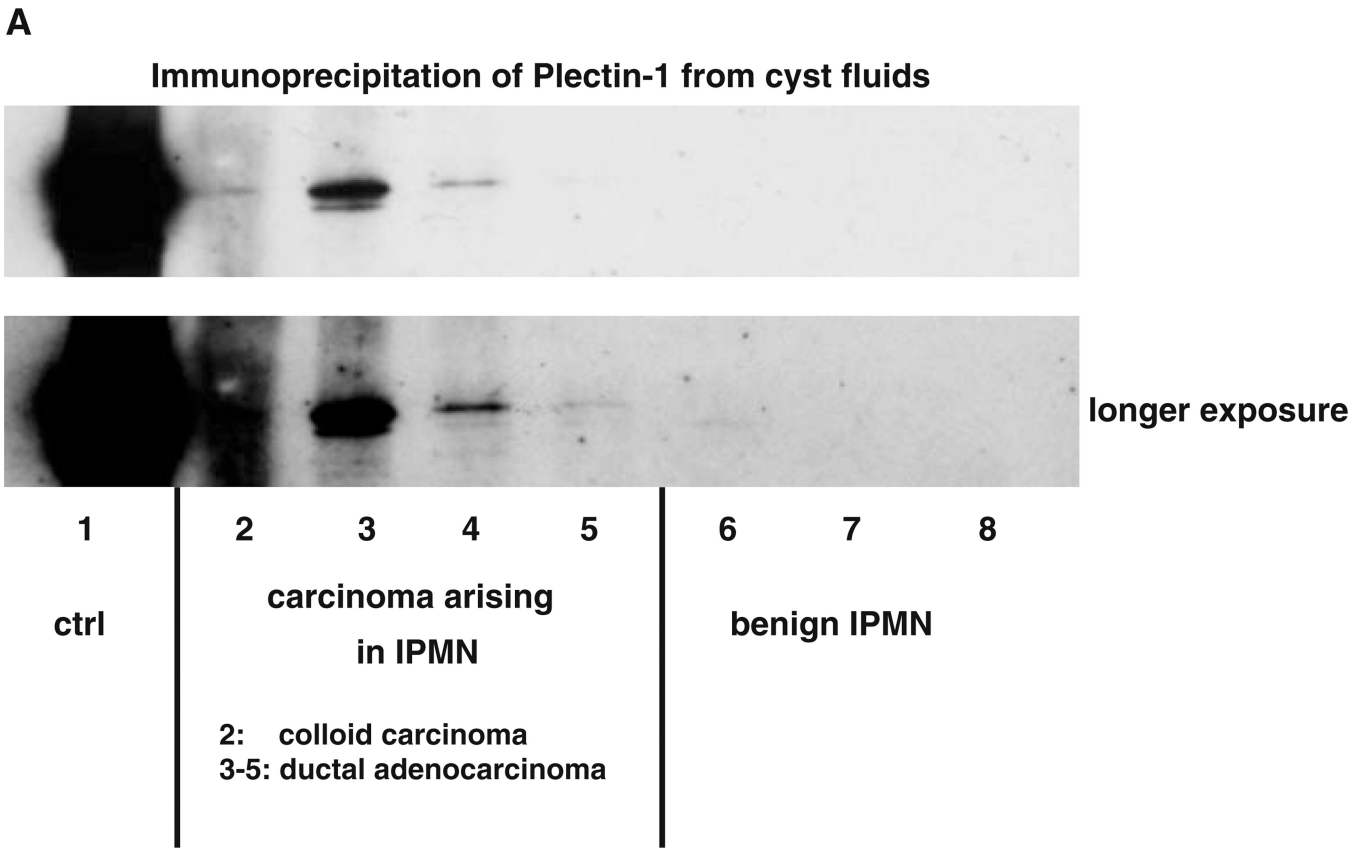


Figure 2.

Plectin-1 immunoprecipitation. **a** Plec-1 in cyst fluid alone is sufficient to distinguish malignant from benign IPMN. Plec-1 was found in 100% (four out of four) of the cyst fluids from malignant IPMN, whereas the cyst fluid from all three benign IPMN did not contain detectable amounts. The Plec-1-positive malignant IPMN cyst fluids were from three ductal adenocarcinomas and one colloid carcinoma.