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ORIGINAL ARTICLE – PANCREATIC TUMORS

Increased SOX9 Expression in Premalignant and Malignant Pancreatic Neoplasms

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ABSTRACT
Background. SOX9, a progenitor cell marker, is important for pancreatic ductal development. Our goal was to examine SOX9 expression differences in intraductal papillary mucinous neoplasms (IPMNs) and ductal adenocarcinoma (PDAC) compared with benign pancreatic duct (BP).
Methods. SOX9 expression was evaluated by immunohistochemistry performed on 93 specimens: 37 BP, 24 low grade (LG) IPMN, 12 high grade (HG) IPMN, and 20 PDAC. A linear mixed-effects model was used to compare the percentage of cells expressing SOX9 by specimen type. A separate linear mixed-effects model evaluated differences in SOX9 expression by staining intensity in pancreatic epithelial cells.
Results. Nuclear SOX9 expression was detected in the epithelial cells of 98% HG IPMN, 93% LG IPMN, 83% PDAC, and 60% BP. Compared with BP, SOX9 was expressed from a significantly greater percentage of cells in LG IMPN, HG IMPN, and PDAC ($p < 0.001$ for each). BP and PDAC showed greater variability in SOX9 expression in epithelial cells compared with IPMNs which showed strong, homogenous SOX9 expression in almost all cells. Compared with BP, both LG and HG IPMN showed significantly greater SOX9 expression ($p < 0.001$ for each), but there was no significant difference in SOX9 expression between LG and HG IPMN ($p > 0.05$). PDAC had significantly higher expression of SOX9 compared with BP but significantly lower SOX9 expression compared with LG or HG IPMN ($p < 0.001$ for each).
Conclusions. IPMNs demonstrated the highest expression levels of SOX9. SOX9 expression in BP and PDAC demonstrated much more heterogeneity compared with the strong, uniform expression in IPMN.

Intraductal papillary mucinous neoplasms (IPMNs) represent premalignant cystic dilations of the pancreatic ducts, with the potential to evolve into pancreatic ductal adenocarcinoma (PDAC). Recent research efforts have looked into identifying molecular markers involved in the progression from IPMN to PDAC. Epithelial-mesenchymal transition (EMT) is one such molecular process that has been shown to have a role in pancreatic cancer.[1–6] EMT is a normal and necessary process during embryogenesis, allowing for epithelial cells to acquire mesenchymal features by losing polarity and gaining motility leading to the formation of mesoderm and neural crest in early development.[7–10] EMT also plays a major role in inflammation, fibrosis, and wound healing through its multipotent progenitor activity to rebuild tissue and re-epithelialize a wound.[11–14] Thus, EMT plays an important role in the developmental process and tissue regeneration.

In malignancy, EMT involves the loss of rigid cellular support structures transitioning to biological features associated with invasive and metastatic behavior.[10,15,16] Increasing evidence has shown that changes in the expression of transcription factors involved in epithelial-mesenchymal transitions are correlated with higher grade and poorer prognosis in various cancers, including pancreatic cancer.[3,4,17] For premalignant lesions, targets, such as the expression levels of E-cadherin, vimentin, and ZEB-1, the mutational status of ATM and KRAS, and loss of

tumor suppressor genes, such as p16 and p53, have all been studied to identify IPMNs with high malignant potential.[3,4,18,19] Due to the complex tumor characteristics and the numerous transcription factors involved in EMT, no reliable marker has been found that can guide clinicopathological decisions in managing patients.

One EMT transcription factor, sex-determining region Y (SRY) box 9 (SOX9), is a fundamental element of normal organogenesis, including neural crest development, sex determination, and normal pancreatic differentiation.[20–24] Changes in SOX9 expression also are associated with tumor progression in a number of different organ systems, including breast, colon, stomach, lung, and prostate cancers.[25–30] In addition, recent studies have linked SOX9 expression to malignant and premalignant pancreatic tissue, including IPMNs.[31–33] Ectopic induction of SOX9 has even been shown to play an integral role in the induction of acinar cell-to-ductal cell reprogramming, potentiating the formation of PDAC precursor lesions.[31] These findings demonstrate that SOX9 may play an integral part in the malignant conversion of benign intraductal papillary mucinous neoplasms.

The role that SOX9 signaling contributes to the progression of IPMN to invasive adenocarcinoma is just beginning to be elucidated. Few studies have looked at SOX9 expression in human pancreatic tissue to evaluate whether SOX9 expression changes with progression of IPMN to PDAC. Our goal for this study was to examine the differences in expression of SOX9 in IPMNs and pancreatic ductal adenocarcinoma compared with benign pancreas.

METHODS

Patients

The study was conducted after approval from the institutional review board at Loyola University Medical Center. Pathology files were searched for surgical specimens that contained IPMNs with or without associated PDAC between August 2009 and April 2015. Forty-four patients were identified as having sufficient tissue available for additional staining. The pancreatic tissue collected from these 44 patients included 37 BP, 24 low grade (LG) IPMN, 12 high grade (HG) IPMN, and 20 PDAC, resulting in 93 tissue samples for examination. The pancreatic tissue from each patient was examined for these four ductal types; therefore, a given surgical specimen likely had more than one ductal epithelium evaluated. IPMN was classified into two groups, low-grade IPMN and high-grade IPMN, as defined by the World Health Organization classification. All slides were reviewed, and the diagnoses were

confirmed by two pathologists (X.D. and H.C.). Demographic and tumor characteristics were obtained retrospectively from the medical records.

Immunohistochemical Analysis

Immunohistochemical (IHC) staining to evaluate SOX9 expression was performed on formalin-fixed, paraffin-embedded pancreas tissue. The excision specimen was fixed in 10% neutral buffered formalin and embedded in paraffin. IHC stains were performed on 5-μm tissue sections. IHC analyses were performed on the Benchmark XT automated immunostaining module (Ventana Medical Systems, Tucson, AZ). An anti-SOX9 monoclonal antibody was purchased from Abcam (1:200 dilution, Cambridge, MA). The unstained slides were incubated sequentially with the primary anti-SOX9 antibody, secondary anti-mouse antibody, and DAB detection system.

The expression of SOX9 was determined by two criteria: the percentage of cells staining for SOX9 and the intensity of immunostain. The percentage of positive staining cells for SOX9 was evaluated by positive nuclear stain based on $40 \times$ magnification. For staining intensity, the immunostain was graded as 0 (no expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression). Both the percentage of cells staining positive for SOX9 expression and the intensity of the stain were independently scored by two pathologists (X.D. and H.C.) who were blinded to the clinical patient data.

Statistical Analysis

A linear mixed effects model estimated the percentage of positive staining cells for SOX9 by pancreatic tissue type (BP, LG IPMN, HG IPMN, PDAC) and included a random intercept to account for possible within-patient correlation. Similarly, a second linear mixed effects model estimated the percentage of positive staining cells with pancreatic tissue type, staining intensity, and an interaction term as independent predictors. A Sidak adjustment was applied to p values for multiple comparisons. Statistical results with a $p < 0.05$ were considered to be statistically significant.

RESULTS

Patient Population

Between August 2009 and April 2015, 44 patients met the inclusion criteria at our institution and their pancreatic tissue could be utilized for further staining. Of the 44 patients, 24 were male and 20 were female, with a median

age of 71 years (range, 39–87) for the entire study population. The majority of the patients had pathology in the head of their pancreas; 33 patients underwent a Whipple, 9 patients had distal pancreatectomies, and 2 received total pancreatectomies. The pancreatic tissue specimens collected from these 44 patients included 37 BP, 24 low-grade (LG) IPMN, 12 high-grade (HG) IPMN, and 20 PDAC, for a total of 93 specimens. Of note, one patient (male, age 57 years old) only had benign pancreas on sectioning after a Whipple, and seven patients had concurrent IPMN and PDAC as shown in Table 1. Patients who had invasive cancer were more likely to have concurrent chronic pancreatitis and have pathology in the head of the pancreas compared with patients with IPMN alone (Table 1).

Expression of SOX9 in Benign Pancreatic Tissue

First, SOX9 nuclear expression was profiled in terms of cell type in the benign pancreatic tissue (Fig. 1). Cellular staining profiles of acinar cells and islet cells did not demonstrate nuclear expression of SOX 9 (0% staining). Cellular staining for epithelial (ductal) cells demonstrated heterogeneous expression of SOX9, but a majority were positive (60.4%). Therefore, SOX 9 expression appeared to be a marker of epithelial origin. In addition, centroacinar cells, a progenitor cell located in the center of acini and known to have stem-cell like activity, showed 100% of cells staining positive for SOX9.

Percentage of Cells Staining Positive for SOX9 in Pancreatic Neoplasms

After establishing the normal staining pattern for SOX9 in benign pancreatic specimens, the pancreatic neoplasms were examined to determine differences in the percentage of SOX9 positive cells. Nuclear SOX9 expression was detected in the epithelial cells of 98% HG IPMN, 93% LG IPMN, 83% PDAC, and 60% BP (Table 2). Compared with BP (60.4% positive), SOX9 was expressed from a significantly greater percentage of cells in LG IMPN (32.6% greater), HG IMPN (37.5% greater), and PDAC (22.2% greater; $p < 0.001$ for each; Table 2). HG IPMN demonstrated statistically significantly increased expression compared with PDAC as well (15.3% greater, $p < 0.05$). However, there was no difference in the percentage of cells staining positive between LG and HG IPMNs ($p = 0.91$).

As shown in Fig. 2, IPMNs have a similar percentage of cells staining positive for SOX9 but with different expression patterns. Low-grade IPMNs show strong, uniform staining of SOX9 (Fig. 2a). High-grade IPMNs also show strong, uniform nuclear SOX9 expression but with a loss of cell polarity compared with low-grade IPMNs (Fig. 2b). Conversely, PDAC demonstrated a more heterogeneous staining profile compared with both LG and HG IPMN (Figs. 2c, d).

**TABLE 1** Patients and tumor characteristics from the 44 patients included in the study

Characteristic	IPMN $n = 23$	PDAC $n = 20$	p value
Age, median (IQR)	72 (66–78)	69 (62–79)	0.708
Female, gender, n (%)	12 (52.2)	7 (35.0)	0.258
Tumor size (mm), median (IQR)	20 (16–31)	34 (29–37)	0.015
Dysplasia, n (%)[a]			
Low grade	22 (95.7)	2 (10.0)	–
High grade	5 (21.7)	7 (35.0)	
Invasive cancer	0 (0.0)	20 (100.0)	
Type of surgery, n (%)			
Whipple	13 (56.5)	19 (95.0)	0.010
Distal pancreatectomy	8 (34.8)	1 (5.0)	
Total pancreatectomy	2 (8.7)	0 (0.0)	
Presence of chronic pancreatitis, n (%)[b]	5 (22.7)	19 (100.0)	< 0.001

Patient characteristics not included for 1 patient whose tissue sample only demonstrated benign pancreatic duct ($n = 44$ total included)

IPMN intraductal papillary mucinous neoplasm, *PDAC* pancreatic ductal adenocarcinoma, *IQR* interquartile range

[a]Numbers do not add up to total in columns as patients may have had more than one type of dysplasia

[b]$n = 2$ missing values for chronic pancreatitis
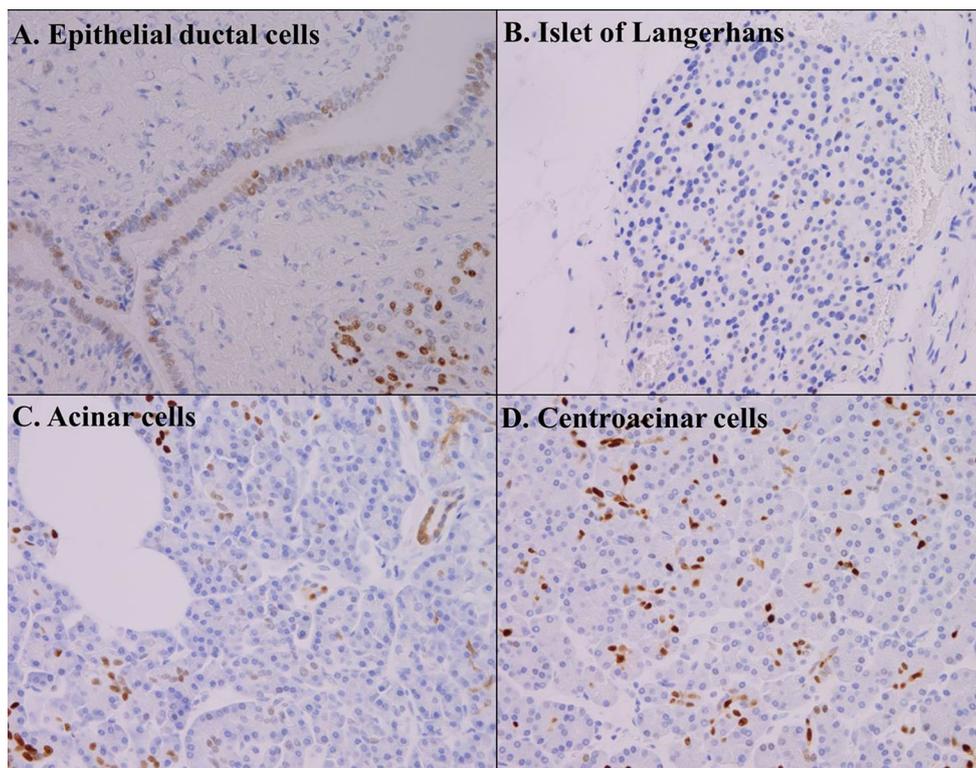
FIG. 1 SOX9 expression in diverse pancreatic tissue. **a** Benign pancreatic epithelial (ductal) cells show heterogeneous expression of SOX9. **b** Cells comprising the islets of Langerhans do not demonstrate nuclear SOX9 expression. **c** Pancreatic acinar cells also do not show significant SOX9 expression. **d** Centroacinar cells, specialized ductal cells demonstrating progenitor activity, show 100% strong SOX9 staining in the acini. Magnification, × 400

TABLE 2 Least squares (LS) mean for percentage of SOX9 positive cells in benign and neoplastic pancreatic tissue

| Percent positive cells	LS mean (SE)	p value
BP	60.4% (2.4%)	< 0.001
LG IPMN	93.0% (3.0%)	
HG IPMN	97.9% (4.2%)	
PDAC	82.6% (3.3%)	

Comparisons	Differences (SE)	Adjusted p value[a]
HG IPMN–LG IMPN	4.9% (5.0%)	0.909
HG IPMN–PDAC	15.3% (5.1%)	0.023
HG IPMN–BP	37.5% (4.7%)	< 0.001
LG IPMN–PDAC	10.4% (4.4%)	0.120
LG IPMN–BP	32.6% (3.6%)	< 0.001
PDAC–BP	22.2% (3.8%)	< 0.001

BP benign pancreatic duct, *LG* low grade, *HG* high grade, *IPMN* intraductal papillary mucinous neoplasm, *PDAC* pancreatic ductal adenocarcinoma, *LS* least squares, *SE* standard error

[a]Sidak adjustment for multiple comparisons

Differences in SOX9 Expression Determined by Staining Intensity

Besides a distinction in the percentage of cells staining positive for SOX9, there was a notable difference in staining intensity between the various pancreatic tissues (Table 3). BP and PDAC epithelial cells showed greater variability in SOX9 staining intensity (BP: 5% strong, 29% moderate, 22% weak staining; PDAC: 22% strong, 48% moderate, 12% weak staining) compared with IPMNs, which showed strong, homogenous SOX9 expression in almost all cells (HG IPMN: 99% strong staining; LG IPMN: 94% strong staining). Compared with BP, both LG and HG IPMN showed significantly greater SOX9 expression ($p < 0.001$ for each in strong staining intensity), but there was no significant difference in SOX9 staining intensity between LG and HG IPMN ($p = 0.99$). PDAC had significantly higher expression of SOX9 compared with BP, driven primarily by differences in moderate staining intensity ($p = 0.02$) but significantly lower SOX9 expression compared with LG or HG IPMN ($p < 0.001$ for strong staining intensity).
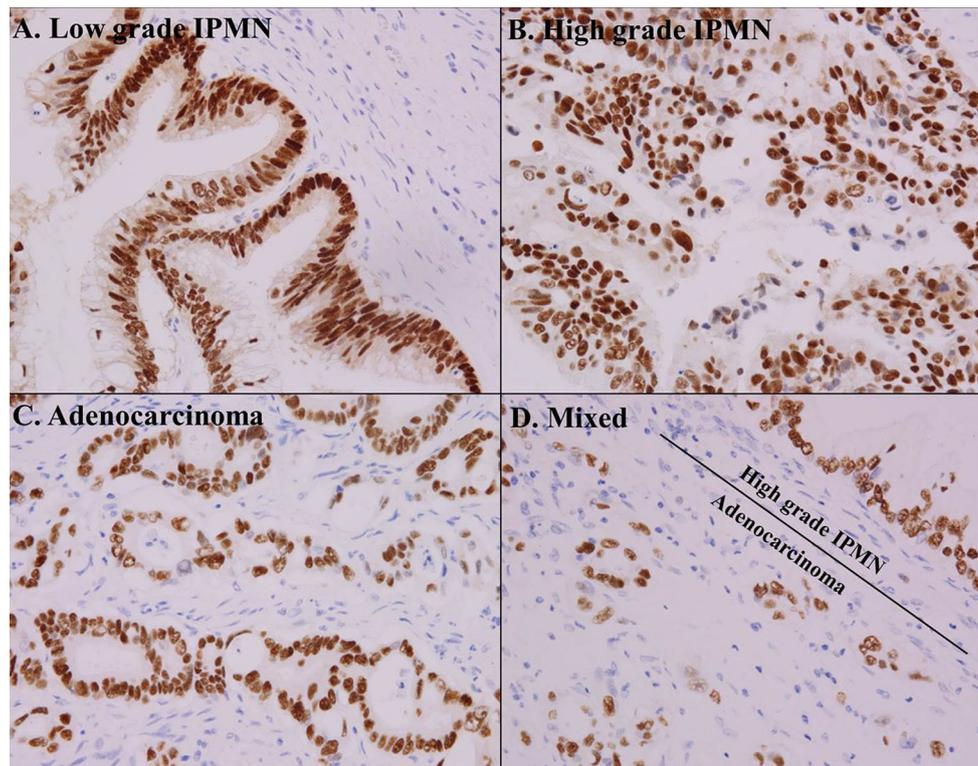
FIG. 2 Variability of SOX9 expression in different pancreatic neoplasms. **a** Low-grade IPMN shows strong, uniform staining of SOX9. **b** High-grade IPMN also shows strong, uniform nuclear SOX9 expression but with a loss of cell polarity compared with low grade IPMN. **c** Pancreatic ductal adenocarcinoma has higher levels of SOX9 expression compared with benign pancreatic ducts but lower levels of SOX9 expression compared with IPMNs. **d** Focus of pancreatic ductal adenocarcinoma adjacent to high grade IPMN. Magnification, × 400

TABLE 3 Means for SOX9 staining intensity in benign and neoplastic pancreatic tissue

	Strong expression	Moderate expression	Weak expression	No expression
Mean percent positive cells				
BP	4.6%	28.6%	21.8%	45.0%
LG IPMN	94.2%	0.0%	0.0%	5.8%
HG IPMN	99.2%	0.0%	0.0%	0.8%
PDAC	21.5%	48.0%	11.5%	19.0%
Adjusted p value for comparisons[a]				
HG IPMN–LG IMPN	0.999	0.999	0.999	0.999
HG IPMN–PDAC	< 0.001	< 0.001	0.726	0.054
HGIPMN–BP	< 0.001	0.002	0.017	< 0.001
LG IPMN–PDAC	< 0.001	< 0.001	0.378	0.168
LG IPMN–BP	< 0.001	< 0.001	0.002	< 0.001
PDAC–BP	0.070	0.016	0.821	< 0.001

BP benign pancreatic duct, *LG* low grade, *HG* high grade, *IPMN* intraductal papillary mucinous neoplasm, *PDAC* pancreatic ductal adenocarcinoma, *LS* least squares, *SE* standard error

[a]Sidak adjustment for multiple comparisons

DISCUSSION

Epithelial-mesenchymal transition (EMT) is a process that occurs when epithelial cells acquire mesenchymal features, leading to loss of adherence, increased motility, and potentially invasiveness.[7–10] Transcription factors involved in EMT are just starting to be studied in various cancers, including pancreatic neoplasms, and have been implicated in malignant transformation.[2,3,5,17,34] Cates et al.[2] evaluated a couple of transcription factors involved

in EMT and found decreased nuclear expression of Twist in malignant pancreatic epithelium. Lahat et al.[3] found that expression of E-cadherin was significantly decreased in malignant versus low-grade IPMN, whereas expression of vimentin was increased in malignant IPMNs. Moreover, studies have shown that expression of certain EMT markers is associated with worse outcomes, including increased lymph node metastasis and decreased survival.[3,17] Therefore, these abundant EMT factors have the potential to be utilized as novel biomarkers to predict malignancy and prognosis in pancreatic neoplasms.

EMT markers encompass a broad range of transcription factors, but this study focused on SOX9 because of the key role that SOX9 plays in normal pancreatic development as well as maintaining the pancreatic progenitor cell pool. In this study, we looked at the spectrum of pancreatic tissue on the sequence of benign pancreas to adenocarcinoma through the dysplastic changes of IPMN. We first confirmed that in the normal pancreatic tissue, nuclear SOX9 staining is not seen in acinar or islet cells, while the epithelial ductal cells showed SOX9 expression confirming the role SOX9 plays in pancreatic ductal development as seen in previous studies.[24,32,35] Not surprisingly, centroacinar cells that exhibit stem cell-like behavior and have progenitor cell activity show strong SOX9 expression in the benign pancreatic tissue.[24]

Strong SOX9 expression also was seen in the dysplastic epithelial cells involved in IPMNs. Although there was no significant difference in staining intensity between low- and high-grade dysplasia, nor did there seem to be a loss or gain of SOX9 expression in higher-grade dysplasia, there was a loss of cell polarity as dysplasia progressed. The loss of polarity in the ductal epithelium was clearly noted in the IPMNs with high-grade dysplasia because nuclear SOX9 expression did not localize to the basal portion of the papillary structures as was seen in low-grade dysplasia (Fig. 2b). Our findings are similar to the study performed by Meng et al.[33] that focused on SOX9 expression patterns in IPMNs. Their results showed that SOX9 expression was confined to the lower portions of the papillary structure in low-grade dysplasia, but that expression pattern was lost and SOX9 was expressed in the entire epithelium as lesions progressed to high-grade dysplasia and invasive cancer.[33] Therefore, it could be hypothesized that changes in the expression pattern of SOX9 may lead to transformation of the pancreatic epithelium and malignant progression.[33]

In a recent study by Kopp et al.[31], SOX9 expression was reported in 95% of IPMNs and 68% of PDAC, which is comparable to our study showing SOX9 in 93% (LG) IPMNs, 98% HG IPMNs, and 83% of PDAC. This increase in SOX9 expression in IPMNs followed by a weaker expression in PDAC reflects a change in the SOX9 expression pattern with progression of malignancy, suggesting that SOX9 may be associated with PDAC initiation. Through a murine model, Kopp et al.[31] was able to test this hypothesis and discovered that the initiation of pancreatic cancer relied on an acinar-to-ductal cell reprogramming through ectopic induction of the ductal gene SOX9. Besides showing that SOX9 can induce a duct-like state in acinar cells, Kopp et al.[31] also showed that SOX9 is a critical mediator of Kras-mediated malignant transformation by accelerating the formation of premalignant lesions when co-expressed with the Kras oncogene. Their study suggests that SOX9 plays an important role in the formation of acinar-derived premalignant lesions.

While our findings support robust SOX9 expression in IPMNs similar to the studies previously mentioned, our data conflicts with other research. Tanaka et al.'s[36] study suggested that the percentage of SOX9 positive cells actually decrease with progression of IPMNs and invasiveness, a finding completely opposite to ours. Although we did identify a weaker and more heterogeneous SOX9 expression in PDAC, the expression of SOX9 did not correlate with dysplasia. Shroff et al.[32] also reported that SOX9 expression was significantly lower in IPMNs compared with benign pancreatic ductal cells. However, although Shroff et al. found that IPMNs had lower expression of SOX9, they did not observe a difference in SOX9 expression between LG and HG IPMNs. This is consistent with our results and thereby does not support the theory that there is a loss of SOX9 expression as IPMNs progress.[32] While differences in staining methods and antibodies used in IHC are one of the main limitations in being able to definitively compare these studies and their results, the actual pattern of SOX9 expression should not be affected by variations in IHC techniques.

Even with that limitation, one thing remains certain: SOX9 expression is modified in the progression of IPMN to invasive malignancy. Our study is unique because we not only investigated the percentage of cells staining positive for SOX9, but also evaluated the staining intensity of the cells and heterogeneity of expression within the epithelium, allowing us to glean more information on SOX9 expression and how it relates to the transformation of the epithelium. Our study contributes to this literature by showing that nuclear SOX9 expression is robust and definitively expressed in IPMNs and that expression persists, but in an altered and weaker pattern, in PDAC. We have contested that SOX9 expression correlates with progression of dysplasia; instead, there is a loss of cell polarity in high grade dysplasia so that nuclear SOX9 expression in no longer confined to the basal portion of the cell. Future studies should focus on how SOX9 can be utilized as a biomarker to predict malignancy and prognosis of IPMNs and PDAC.

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