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SOX9: A useful marker for pancreatic ductal lineage of pancreatic neoplasms

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Abstract

Previous studies showed that SOX9 plays a critical role in pancreatic ductal development. The aim of this study was to evaluate SOX9 as a marker for pancreatic ductal lineage. SOX9 expression was evaluated by immunohistochemistry in 146 benign pancreas (BP), 136 pancreatic ductal adenocarcinomas (PDAC), 47 pancreatic intraepithelial neoplasia (PanIN), 21 intraductal papillary mucinous neoplasms (IPMN), 14 mucinous cystic neoplasms (MCN), 10 serous cystadenomas (SCA), 39 pancreatic neuroendocrine tumors (PET), 9 acinar cell carcinomas (ACC) and 23 solid pseudopapillary neoplasms (SPN). Nuclear expression of SOX9 was detected in the centroacinar cells and ductal cells, but not in acinar or endocrine cells in 100% BP. Focal or diffuse SOX9 expression was detected in 100% PanIN, 100% IPMN, 100% MCN, 100% SCA, 89.0% PDAC, 2.6% PETs, 11.1% ACC and 0% SPN. SOX9 expression was lower in PanIN2 and PanIN3 than PanIN1 lesions ($P < 0.01$). Compared to BP, IPMN had lower SOX9 expression ($P < 0.05$). No correlation between SOX9 expression and other clinicopathologic parameters was identified. Our study showed that SOX9 is expressed in centroacinar and ductal epithelial cells of BP and is a useful marker for pancreatic ductal lineage of pancreatic neoplasms.

Keywords

SOX9; centroacinar cells; pancreatic ductal adenocarcinoma; PanIN; IPMN; MCN; SCA; ACC; PET; SPN

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INTRODUCTION

The transcription factor SOX9 [SRY (Sex determining region Y)-box9] belongs to a family of transcription factors that are characterized by a highly conserved high mobility group (HMG) DNA binding domain, which was initially identified in SRY gene (1). Previous studies from mice and human have demonstrated that SOX9 play a critical role in the development and differentiation of multiple tissues during embryogenesis, including the differentiation of Sertoli cells of the testis (2), the development of prostate, chondrocyte differentiation (3–5), glial and neural crest differentiation (6–8), the formation of hair stem cells in the skin (9) and Paneth cells in the intestine (10–14). Recent studies have also showed that SOX9 plays a key role in the development of pancreas (15–17). *SOX9*, a downstream target of Notch, is expressed from the early stage of pancreatic development and is required for the maintenance of the pancreatic progenitor pool and for establishment of the pancreatic endocrine and exocrine cell fates (15, 17). A dose-dependent expression of Notch has been suggested in this process. Intermediate levels of Notch are required to activate *SOX9*. *SOX9* in turn leads to activation of neurogenin 3 (*Ngn3*), a gene necessary and sufficient for establishment of an endocrine cell fate in the developing pancreas (18–20). Once activated, *Ngn3* subsequently down-regulates *SOX9* expression in the endocrine cell compartment (20). At high-levels of Notch signaling, the *Ngn3* repressor, *Hes1* (*hairly and enhancer of split-1*) is induced. The progenitor cells consequently retain *SOX9* expression and undergo conversion to a ductal fate (20). A recent study using a zebrafish model system has shown that the formation of pancreatic and biliary ductal system is severely impaired in *SOX9* mutants while the endocrine and acinar compartments of the pancreas appear unaffected (21). These data suggest that SOX9 is a key regulator for the development of pancreatic ductal system.

The pancreatic ductal system is composed of the centroacinar cells, the intercalated ducts, intralobular, interlobular and main pancreatic ducts. Located in the middle of the acini, the centroacinar cells are small, inconspicuous, flat to cuboidal cells with minimal pale or lightly eosinophilic cytoplasm and central oval nuclei. These cells constitute the beginning of the ductal system and convey the secretions from acinar cells to the intercalated ducts, which in turn fuse to form the intralobular and subsequently the interlobular pancreatic ducts. A spectrum of non-invasive lesions with mucinous histology of the pancreatic ductal system, including pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms (IPMN) have been determined to be precursors for pancreatic ductal adenocarcinoma (PDAC) (22). However, the cell of origin or lineage for pancreatic ductal carcinogenesis has been a topic of debate in the literature (23–28). A recent study by Kopp et al. showed that *SOX9* accelerates the formation of precursor lesions of PDAC when co-expressed with oncogenic *Kras*. By lineage tracing, their study also suggests that PanIN lesions and subsequently pancreatic ductal adenocarcinoma arise from ductal metaplasia of the pancreatic acinar cells, a phenomenon known as acinar-to-ductal metaplasia (29). *SOX9* has been shown to be expressed in PDAC and IPMNs, however, the expression pattern of *SOX9* in normal pancreas, PanIN lesions, MCN and other types of pancreatic neoplasms has not been examined in detail. We therefore examined the expression of *SOX9* in the various compartments of benign pancreatic tissue (BP) and different types of precursor lesions for PDAC, including PanIN, MCN and IPMN. In addition, we also compared the *SOX9* expression in different types of pancreatic neoplasms and correlated *SOX9* expression with clinicopathologic parameters. Our study showed that *SOX9* is expressed in centroacinar cells, pancreatic ductal cells, PDAC and its precursor lesions, but rarely in other pancreatic neoplasms.

MATERIALS AND METHODS

Case selection

We retrospectively reviewed medical records and tissue specimens of 146 benign pancreatic (BP) tissue (109 normal pancreas and 37 chronic pancreatitis) and 136 paired PDAC, 47 PanIN lesions of different histologic grades, 21 patients with IPMN, 14 patients with MCN, 10 patients with serous cystadenoma (SCA), 39 patients with low-grade pancreatic neuroendocrine tumor (PET), 9 patients with acinar cell carcinoma (ACC) and 23 patients with solid pseudopapillary neoplasm (SPN) who underwent pancreatectomy at the University of Texas MD Anderson Cancer Center between 1990 and 2011. Clinicopathologic parameters such as age, gender, tumor differentiation, tumor stage, and survival data were obtained from medical records. This study was approved by the Institutional Review Board of the University of Texas MD Anderson Cancer Center.

Tissue Microarray construction

To construct the tissue microarrays for PDAC, SPN, ACC, PET and BP cases used in this study, formalin-fixed, paraffin-embedded archival tissue blocks and their matching hematoxylin and eosin-stained (H & E) slides were retrieved, reviewed and screened for representative tumor regions by a pathologist. For each patient, two cores of tumor were sampled from representative areas using a 1.0-mm punch. The tissue microarray was constructed with a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) as described previously (30).

Immunohistochemical analysis for SOX9 and Double immunohistochemical stain for CK19/SOX9

Immunohistochemical staining for SOX9 was performed either on 4- μ m unstained sections from the tissue microarray blocks for PDACs and their paired benign pancreas tissue, ACCs, PETs, SPTs or on 4- μ m whole sections from the formalin-fixed paraffin-embedded archival tissue blocks for IPMNs, MCNs, SCAs, and PanIN lesions using a polyclonal antibody against SOX9 (Millipore, Billerica, MA). Following deparaffinization, antigen retrieval was performed on the tissue sections at 100 °C in a steamer containing 10 mmol citrate buffer (pH, 6.0) for 5 min. The sections were then washed and immersed in anti-SOX9 antibody (1:2000 dilution) at 35°C for 15 min. Subsequently, these sections were immersed in 3.0% hydrogen peroxidase at 35 °C for 5 min to block the endogenous peroxidase activity. A primary enhancer solution was then applied to the slides and incubated at 35°C for 8 minutes. The sections were then incubated with secondary Poly-HRP anti-mouse/anti-rabbit immunoglobulin at 35°C for 8 min. Diaminobenzidine (DAB) was used as a chromogen and DAB enhancer was applied, and hematoxylin was used for counterstaining.

Double immunohistochemical stain for CK19 (RCK108, Dako, Carpinteria, CA) and SOX9 was performed on 4- μ m unstained sections from the formalin-fixed paraffin-embedded archival tissue blocks. The sections were stained for SOX9 first using the conditions described above. The sections were then washed and immersed in anti-CK19 antibody (1:50 dilution) at 35°C for 15 min. A post primary enhancer was applied after to the slides after two washes and incubated at 35°C for 20 minutes. The sections were then washed and 3-amino-9-ethylcarbazole (AEC) was applied at 35°C for 15 min. Hematoxylin was used for counterstaining.

Measurement of SOX9 expression

The immunohistochemically stained slides of tissue microarrays and the formalin-fixed paraffin-embedded archival tissue were examined using standard light microscopy (Olympus BX41). The staining results were independently scored semiquantitatively by two

pathologists (S.S. and H.W.), who were blinded to the clinicopathologic data. Only moderate to strong nuclear staining for SOX9 was considered as positive. The percentage of positive cells was estimated as a ratio between the number of the positively stained nuclei and the total number of tumor cell nuclei. The average percentage of SOX9 positive nuclei from the two pathologists were recorded and used for statistical analyses in all cases. Nuclear expression of SOX9 was categorized as negative (no staining or staining in less than 5% of the tumor cells), focal staining (5–30% nuclear staining in tumor cells) or diffuse staining (>30% nuclear staining in tumor cells).

Statistical analysis

Independent t test was used to compare the mean percentages of positive cells between the different histologic grades of PanINs, IPMNs or MCNs. The immunohistochemical staining results were correlated with the histology type of the pancreatic neoplasms by Fisher's exact tests or Chi-square test. Statistical analysis was performed using Statistical Package for Social Sciences software (for Windows 12.0, SPSS Inc., Armonk, New York). We used a two-sided significance level of 0.05 for all statistical analyses.

RESULTS

SOX9 expression in benign pancreatic tissue

Diffuse and uniform nuclear expression of SOX9 was detected in the centroacinar cells and the epithelial cells of the pancreatic ductal system in all 109 (100%) normal pancreas samples (Figure 1A). Diffuse and uniform nuclear SOX9 expression was also detected in the ductal cells in all 37 (100%) chronic pancreatitis tissue samples (Figure 1B). There was no difference in SOX9 expression in the ductal cells between normal pancreas and chronic pancreatitis (Table 1). To confirm the expression of SOX9 in centroacinar cells and pancreatic ductal cells, double staining for SOX9 and cytokeratin 19, which is a known marker for the pancreatic ductal system including the intercalated ducts and centroacinar cells (31), were performed. The centroacinar and ductal epithelial cells that were positive for cytokeratin 19 co-expressed SOX9 (Figure 1C). No nuclear expression of SOX9 was detected in either pancreatic acinar cells or the pancreatic islet cells. The pancreatic islet cells demonstrated weak cytoplasmic staining for SOX9, which may be due to non-specific cross-reaction (Figure 1A).

SOX9 expression in PanIN, IPMN and MCN

The immunohistochemical staining results for SOX9 in PanIN lesions, IPMN and MCN are summarized in Table 1 and Table 2. Although nuclear expression of SOX9 was noted in all grade 1 (18/18), grade 2 (21/21), and grade 3 (8/8) PanIN lesions (Figure 2A–2C), SOX9 staining was focal in 0 (0%) of PanIN1, 6 (28.6%) of PanIN2 and 2 (25%) of PanIN3 lesions. The mean percentages of SOX9 positive cells were 78.9 ± 11.3 , 54.5 ± 23.9 , and 53.4 ± 25.2 for PanIN1, PanIN2 and PanIN3 respectively (Table 2). PanIN1 had higher SOX9 expression than PanIN2 ($P < 0.001$) and PanIN3 ($P = 0.002$). However, there was no difference in the percentage of SOX9 positive cells between PanIN2 and PanIN3 ($P = 0.94$). All 21 (100%) of IPMN and 14 (100%) of MCN were also positive for SOX9. The nuclear staining for SOX9 was focal and heterogenous in 23.1% (3/13) of IPMN with low-grade dysplasia and 18.2% (2/11) of IPMN with high-grade dysplasia. Compared to normal pancreatic ductal cells, the expression of SOX9 in IPMN was decreased ($p < 0.05$). In the three IPMN cases with both low-grade and high-grade dysplasia, the percentages of SOX9 positive cells were 25%, 50% and 60% respectively in the low-grade areas and 60%, 70% and 25% respectively in the high-grade areas. In contrast, all 14 MCN with low-grade and/or high-grade dysplasia showed diffuse nuclear staining for SOX9 (Figure 3C & 3D). There were no correlation between the percentage of SOX9 positive cells or the frequencies of

SOX9 expression and the degree of dysplasia in IPMNs and MCNs. In addition, strong nuclear staining for SOX9 was also detected in the tumor cells in all 10 (100%) cases of SCA (Figure 3E and Table 1).

SOX9 expression in PDAC, ACC, PET and SPN

The results of the immunohistochemical staining for SOX9 in pancreatic tumors, including PDAC, ACC, PET and SPN, are summarized in Table 3. Diffuse and strong nuclear SOX9 expression was observed in 83.1% (113/136) of the PDAC cases (Figure 3F). In contrast, nuclear SOX9 expression was detected only in 2.6% (1/39) PETs, 11.1% (1/9) ACC, and 0% (0/23) SPTs examined ($P < 0.05$, Figure 4A–4F). Compared to the paired benign pancreatic tissue samples, PDAC has decreased nuclear expression of SOX9 ($P = 0.0001$). Our data suggested that SOX9 may be used as a marker for the ductal lineage of pancreatic neoplasms. However, no correlation between SOX9 expression and the degree of tumor differentiation or grades, lymph node metastasis, other clinicopathological factors or survival was observed in the patients with any types of the above-mentioned pancreatic tumors ($P > 0.05$, data not shown).

DISCUSSION

Several animal studies have demonstrated the key role of *SOX9* in pancreatic development and pancreatic ductal carcinogenesis. In this study, we examined the expression of SOX9 in a large cohort of benign pancreatic tissue, all types of precursor lesions of PDAC, and different types of pancreatic neoplasms, including PDAC, ACC, PET, SPN, and SCA. Our data showed that nuclear expression of SOX9 is present in centroacinar cells and ductal epithelial cells in benign pancreatic tissue and all types of pancreatic lesions/neoplasms of ductal origin, but rare in other pancreatic neoplasms. Although SOX9 expression in benign pancreatic tissue is identical to that of cytokeratin 19, recent studies showed that cytokeratin 19 is expressed in 86% of ACCs and 70% of PETs (32, 33). In addition, SOX9 is a nuclear marker. Thus SOX9 is a useful marker for the epithelial cells of pancreatic ductal system, including centroacinar cells, and for the ductal lineage of pancreatic neoplasms.

In this study, we examine the expression of SOX9 in 109 normal pancreas samples and 37 chronic pancreatitis samples and we observed uniform strong nuclear expression of SOX9 in the centroacinar cells, normal pancreatic ductal cells and the proliferating ductules of chronic pancreatitis in all cases. No nuclear expression of SOX9 was observed in acinar cells and the islet cells of the pancreas. Our findings are consistent with the previous studies which showed that the central acinar cell and ductal cell specific nuclear expression of SOX9 (17, 29). These results support the critical role of SOX9 in the development of pancreatic ductal system.

In a recent study, Tanaka et al. showed that the percentage of cells expressing SOX9 was lower in invasive PDAC than normal pancreatic ducts and that the decrease of nuclear SOX9 expression correlated with the progression of IPMN (34). In this study, we observed significantly lower nuclear expression of SOX9 in PanIN2 and PanIN3 lesions than PanIN1 lesions ($P < 0.01$). Consistent with their results, we also observed decreased nuclear expression of SOX9 in IPMNs with either low-grade or high-grade dysplasia compared normal pancreatic ducts. However, we did not observe any significant difference in the nuclear SOX9 expression between IPMN with low-grade dysplasia and IPMN with high-grade dysplasia. In addition, there were no significant difference in the nuclear expression of SOX9 between PanIN2 and PanIN3 lesions or between the MCNs with low-grade and those with high-grade dysplasia. Furthermore, diffuse and strong nuclear SOX9 expression was observed in 83.1% of 136 invasive PDAC samples examined in this study. Therefore, our findings did not support their observations that there is a progressive loss of nuclear SOX9

expression with the progression of IPMN (34). The difference between ours and their study could be attributed in part to the difference in the antibodies used in these two studies. On the other hand, our findings are consistent with the previous study by Kopp *et al.* which reported SOX9 expression in 82% of MCNs and 95% IPMNs (29). Our observation that nuclear expression of SOX9 in PDAC samples and all precursor lesions of PDAC suggest that SOX9 is a sensitive marker of pancreatic ductal lineage. Since SOX9 is expressed in all cells of the ductal lineage, it cannot be utilized to distinguish between chronic pancreatitis and PDAC.

The expression of SOX9 in PET, SPT, ACC, and SCA has not been reported. In this study, we observe strong nuclear SOX9 expression in 100% of SCAs examined, which was consistent with the ductal origin of this tumor. The cell origin of pancreatic SPN is not clear. None of the 23 SPNs examined in this study were positive for SOX9 expression. Our results would argue against the ductal origin of this tumor. Interestingly, we observed nuclear expression of SOX9 in one of 9 ACCs examined. Careful histologic review and immunohistochemical study did not show unequivocal ductal differentiation in this case. We also observed diffuse nuclear expression of SOX9 in one PET case (2.6%). Although the number of cases for PET and ACC examined in this study is relatively small, our study demonstrated that nuclear SOX9 expression may be used as a marker for ductal lineage, especially on challenging core needle biopsies of pancreatic tumors to differentiate PDAC from acinar cell carcinoma, neuroendocrine tumor and solid pseudopapillary neoplasms of the pancreas.

In summary, our study showed that SOX9 is expressed in the centroacinar and pancreatic ductal cells in benign pancreatic tissue, PDAC and all known types of its precursor lesions, and SCA, but rare in PET, SPT, and ACC. Our study demonstrates that nuclear expression of SOX9 is a useful marker for the pancreatic ductal lineage of pancreatic neoplasms, which may be used in the differential diagnosis of pancreatic tumors.

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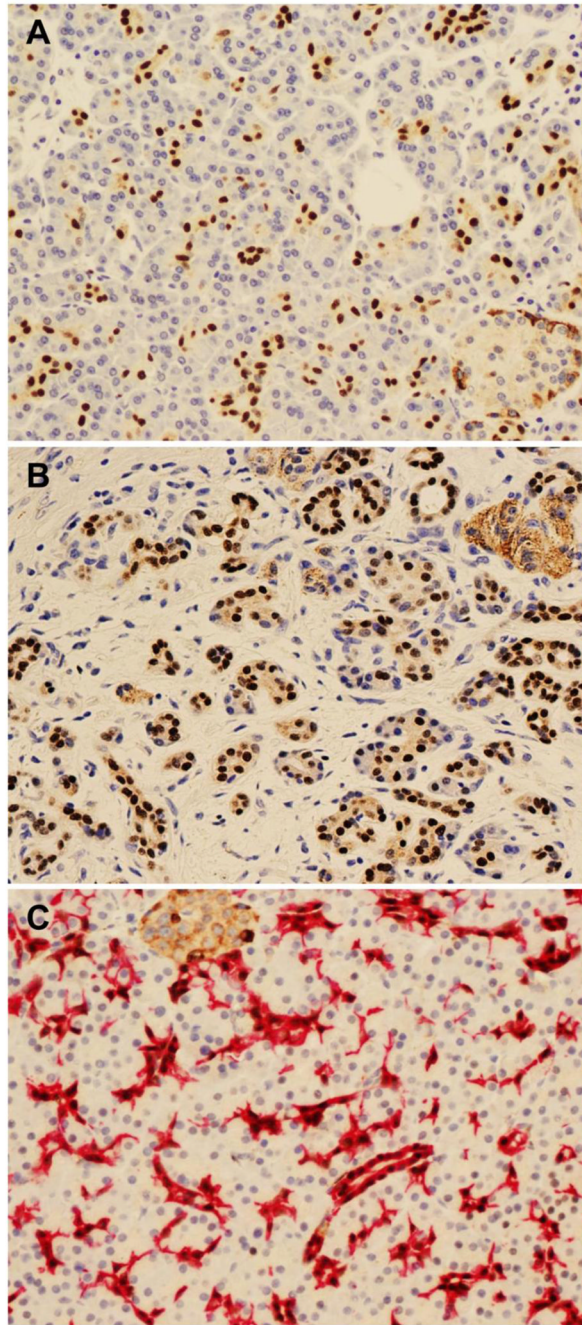


Figure 1. Nuclear SOX9 expression is present in centroacinar cells and pancreatic ductal cells, but not in acinar or islet cells in normal pancreatic tissue (A) and chronic pancreatitis (B). Nuclear expression of SOX9 (brown) is observed in all cells expressing CK19 (red) by double immunohistochemical stain (C). Original magnification, 200x for A–C.

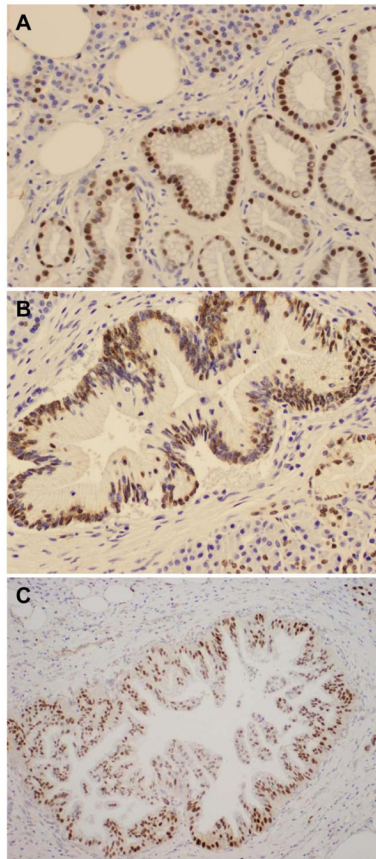


Figure 2. Representative micrographs showing nuclear SOX9 expression in pancreatic intraepithelial neoplasia (PanIN) 1 (A), PanIN2 (B) and PanIN3 lesions (C). Original magnification, 200x for A–B and 100x for C.

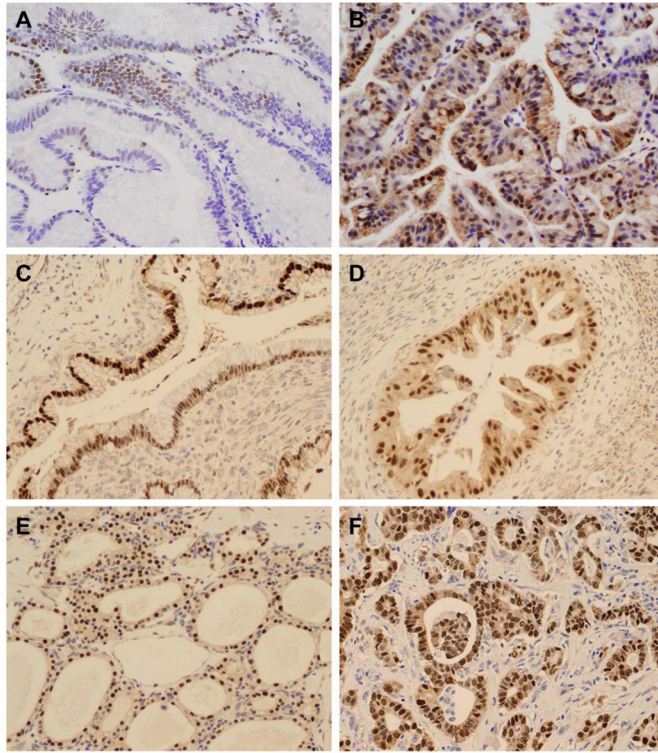


Figure 3. Representative micrographs showing nuclear SOX9 expression in intraductal papillary mucinous neoplasm (IPMN) with low grade dysplasia (A), IPMN with high grade dysplasia (B), mucinous cystic neoplasm (MCN) with low grade dysplasia (C), MCN with high grade dysplasia (D), serous cystadenoma (E) and pancreatic ductal adenocarcinoma (F). Original magnification, 200x for A–F.

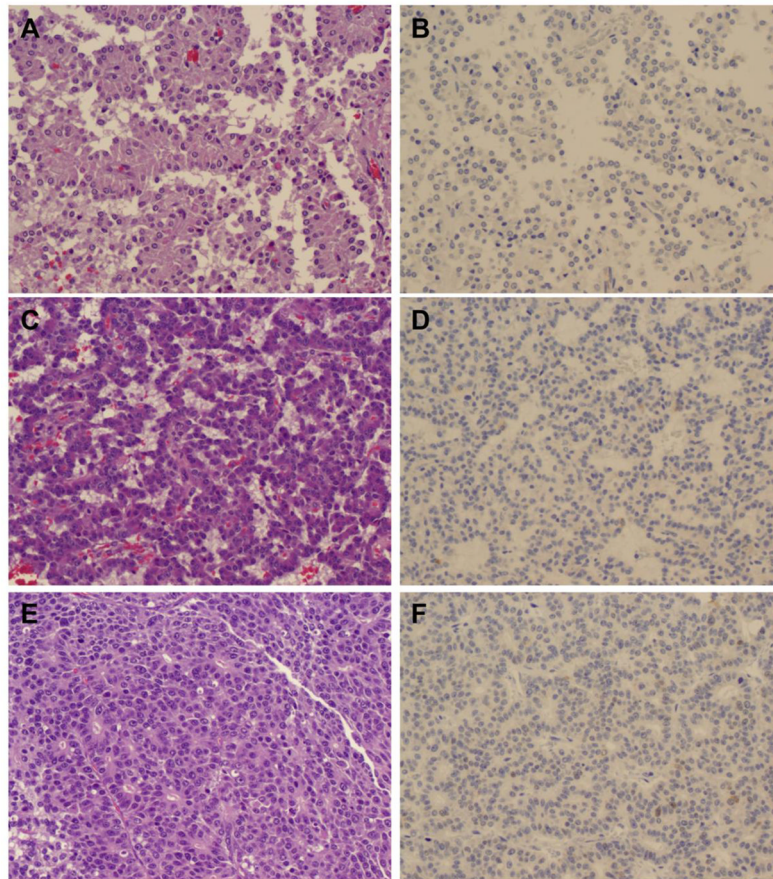


Figure 4. Representative micrographs showing the hematoxylin & eosin (H & E) staining and SOX9 expression in solid pseudopapillary neoplasm (A & B), pancreatic neuroendocrine tumor (C & D) and acinar cell carcinoma (E and F). No nuclear staining was detected in solid pseudopapillary neoplasm (B), pancreatic neuroendocrine tumor (D) and acinar cell carcinoma (F). Original magnification, 200x for A–F.

Table 1

Expression of SOX9 in benign pancreatic tissue, pancreatic intraepithelial neoplasia and cystic neoplasms of the pancreas

Type of tissue	Number of cases	Negative (%)	Focal staining (%)	Diffuse staining (%)	P value ^b
Benign pancreatic tissue	146				
Normal	109	0 (0)	0 (0)	109 (100)	
Chronic pancreatitis	37	0 (0)	0 (0)	37 (100)	1.00
Pancreatic intraepithelial neoplasia					
Grade 1	18	0 (0)	0 (0)	18 (100)	1.00
Grade 2	21	0 (0)	6 (28.6)	15 (71.4)	<0.01
Grade 3	8	0 (0)	2 (25.0)	6 (75.0)	0.004
Intraductal Papillary Mucinous Neoplasm	21				
Low Grade Dysplasia	13 ^a	0 (0)	3 (23.1)	10 (76.9)	0.001
High Grade Dysplasia	11	0 (0)	2 (18.2)	11 (81.8)	0.01
Mucinous cystic neoplasm	14				
Low Grade Dysplasia	14 ^a	0 (0)	0 (0)	14 (100)	1.00
High Grade Dysplasia	3	0 (0)	0 (0)	3 (100)	1.00
Serous cystadenoma	10	0 (0)	0 (0)	10 (100)	1.00

^aIncluding three patients with both low-grade and high-grade dysplasia;

^bP values compared to normal pancreas

Table 2

Percentage of positive tumor cells in pancreatic ductal adenocarcinoma and its precursor lesions

Types of tissue	Number of cases	Percentage of positive cells (%; Mean \pm SD)	P value
Pancreatic intraepithelial neoplasia			
Grade 1	18	78.9 \pm 11.3	
Grade 2	21	54.5 \pm 23.9	<0.01
Grade 3	8	53.4 \pm 25.2	0.002
Intraductal papillary mucinous neoplasm	21		
Low Grade Dysplasia	13*	50.8 \pm 18.1	
High Grade Dysplasia	11	58.2 \pm 19.0	0.34
Mucinous cystic neoplasm	14		
Low Grade Dysplasia	14*	78.6 \pm 12.2	
High Grade Dysplasia	3	70.0 \pm 20.0	0.33
Pancreatic ductal adenocarcinoma	136	68.0 \pm 31.6	NA

* Including three patients with both low-grade and high-grade dysplasia

Table 3

Expression of SOX9 in different types of pancreatic neoplasms

Types of tumor	Number of cases	Negative (%)	Focal staining (%)	Diffuse staining (%)	P values
Pancreatic ductal adenocarcinoma	136	15 (11.0)	8 (5.9)	113 (83.1)	NA
Pancreatic endocrine neoplasm	39	38 (97.4)	0 (0)	1 (2.6)	<0.001
Acinar cell carcinoma	9	8 (88.9)	0 (0)	1 (11.1)	<0.001
Solid pseudopapillary neoplasm	23	23 (100.0)	0 (0)	0 (0.0)	<0.001