

The evaluation of SOX9 expression in pancreatic ductal adenocarcinoma and intraductal papillary mucinous neoplasm

Takayuki Tanaka, MD¹

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Tamotsu Kuroki, MD, PhD¹

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Tomohiko Adachi, MD, PhD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Shinichiro Ono, MD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Masataka Hirabaru, MD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Akihiko Soyama, MD, PhD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Amane Kitasato, MD, PhD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Mitsuhsa Takatsuki, MD, PhD¹,

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

Tomayoshi Hayashi, MD, PhD²,

Department of Pathology, Nagasaki University Graduate School of Biomedical
Sciences, Nagasaki, Japan

Susumu Eguchi, MD, PhD¹

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan

¹ Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences,
1-7-1 Sakamoto, Nagasaki 852-8501, Japan

² Department of Pathology, Nagasaki University Graduate School of Biomedical

Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

Running title: SOX9 in pancreatic ductal adenocarcinoma and IPMN

Correspondence to: Takayuki Tanaka

Department of Surgery

Nagasaki University Graduate School of Biomedical Sciences

1-7-1 Sakamoto, Nagasaki, 852-8501, Japan

Phone: +81-95-819-7316

Fax: +81-95-819-7319

Email: pay-it-forward.197675@hotmail.co.jp

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Abstract

[Objectives]

Sex-determining region Y (SRY) box 9 (SOX9) is an important transcription factor required for development and has been implicated in several types of cancer. SOX9 has never been linked to pancreatic ductal adenocarcinoma (PDAC) and intraductal papillary mucinous neoplasm (IPMN) of the pancreas. The aim of this study is to investigate the relation between SOX9 and PDAC, as well as SOX9 and IPMN.

[Methods]

Surgical specimens were obtained from 55 patients with PDAC and 68 patients with IPMN and were investigated using SOX9 immunohistochemical analysis.

[Results]

The rate of SOX9-positive cells to total pancreatic duct epithelial cells in a normal pancreas was 82.7%. On the other hand, the SOX9-positive rate in PDAC was 0.8%. There was a significant difference between the normal pancreas and PDAC ($p=0.0002$). In IPMN, the SOX9-positive rate gradually decreased according to tumor progression, with the following rates observed: intraductal papillary mucinous adenoma (IPMA) (66.3%); noninvasive intraductal papillary mucinous carcinoma (NI-IPMC) (46.3%); and minimally invasive intraductal papillary mucinous carcinoma (MI-IPMC) (30.5%); and

invasive carcinoma originating in intraductal papillary mucinous carcinoma (IC-IPMC) (2.3%). There were significant differences between each group ($p < 0.05$).

[Conclusion]

Our data suggested that SOX9 might contribute to carcinogenesis in PDAC and IPMN.

Key words: SOX9, pancreatic ductal adenocarcinoma, intraductal papillary mucinous neoplasm

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignant diseases and has a very poor prognosis¹⁾. Due to the absence of specific symptoms and the lack of early detection, PDAC is usually diagnosed at an advanced incurable stage²⁾³⁾. Thus, the median overall survival is only 5-6 months after conventional therapies for locally advanced and metastatic disease¹⁾. Consequently, the 5-year overall survival rate is less than 5%¹⁾²⁾. Such a shorter survival rate is primarily due to late diagnosis and to the intrinsic and extrinsic drug resistance, which contributes to tumor recurrence and metastasis.

Intraductal papillary mucinous neoplasm (IPMN) is characterized by intraductal proliferation of neoplastic mucinous cells, which usually form papillae and lead to cystic dilation of the pancreatic ducts, forming clinically and macroscopically detectable masses⁴⁾. As in adenoma-carcinoma sequences such as that in colon cancer⁵⁾, IPMN progresses from an intraductal papillary mucinous adenoma (IPMA) to an intraductal papillary mucinous carcinoma (IPMC), and eventually to an invasive adenocarcinoma of the pancreas⁶⁻⁸⁾. IPMC is classified by the Japan Pancreatic Society (JPS) as either a noninvasive IPMC (NI-IPMC) or an invasive IPMC (I-IPMC)⁹⁾. I-IPMC is classified into two categories: a minimally invasive IPMC (MI-IPMC) and an

invasive carcinoma originating in IPMC (IC-IPMC), the latter being more advanced.

Sex-determining region Y (SRY) box 9 (SOX9) is a member of a highly conserved family of transcription factors defined by their similarity to the high mobility group DNA-binding domain of SRY¹⁰. SOX9 plays a key role in sex determination, in chondrogenesis during development, and in regulating the differentiation of normal melanocytes¹¹⁻¹³. Several recent studies have demonstrated that SOX9 plays active roles in adult tissues as well¹⁴⁻¹⁶. Recently, several studies have also demonstrated that SOX9 has an important role in normal pancreatic differentiation¹⁷⁾¹⁸. Moreover, the formation of organs during normal development requires the activation and silencing of gene expression. Inappropriate or reactivated expression of genes, which is a key regulator of developmental processes, can cause several neoplasms. For example, Wilms' Tumor-1 (WT-1) gene, which plays a role in the differentiation of the kidney, mutates and forms Wilms' tumor¹⁹, and a novel RING finger protein (LUN) gene, which plays a role in the differentiation of the lung, mutates and forms lung cancer²⁰⁾²¹. However, there are no reports that SOX9 is concerned with PDAC and IPMN. In this study, we revealed the role of SOX9 in PDAC and IPMN by immunohistochemical analysis.

Patients and Methods

Patients

The normal pancreases of gastric cancer patients were used as controls. The subjects comprised five patients who had undergone total gastrectomy and distal pancreatectomy for gastric cancer for controls from October 2010 to July 2011, 55 patients who had undergone surgery for PDAC from April 2003 to December in 2010, and 68 patients who had undergone surgery for IPMN from April 1995 to December 2010 at Nagasaki University Hospital. The characteristics of the patients are given in Tables 1 and 2.

Immunohistochemical Analysis

All the surgical specimens were fixed in 10% formalin. The specimens were then sectioned, and serial sections were cut from paraffin blocks. Each section was carefully made from the paraffin-embedded blocks and stained with hematoxylin and eosin. Immunohistochemical examinations were performed as follows. Sections were deparaffinized in various ethanol concentrations and washed in phosphate-buffered saline (PBS). The sections were then treated with hot water at 95 °C for antigen retrieval (Target Retrieval Solutions pH 9.0, Dako, Japan) for 20 min. After being washed, the samples were treated with 0.03% hydrogen peroxide in methyl alcohol. Thereafter, they

were treated with 0.25% casein in PBS, containing stabilizing protein and 0.015 mol/L sodium azide (Protein Block, Serum-Free, Dako, Japan) at room temperature for 20 min and covered with a mouse monoclonal antibody to SOX9 (Abcam plc, Cambridge, UK) at a dilution of 1:500 in PBS at room temperature for 30 min. After the samples were rinsed with PBS, the sections were treated with a rabbit polyclonal antibody against mouse IgG, IgA, and IgM at room temperature for 30 min and washed with PBS. The peroxidase/antiperoxidase complex was allowed to react with the rabbit antibody, and the sections were stained with 3, 3-diaminobenzidinetetrahydrochloride (DAB) containing 0.03% hydrogen peroxide (Envison kit / HRP, DAB, Dako, Japan). The sections were counterstained with Mayer's Hematoxylin.

The expression of SOX9 was evaluated by the percentage of positive cells in each tumor specimen and in corresponding normal epithelium of the pancreatic duct. The percentage was analyzed by counting using 500 cells at x200 magnification in four views.

Statistical Analysis

Two blinded investigators (TT and TH) evaluated the immunohistochemical stain of specimens and all specimens were blinded to the stage of diseases. Continuous variables are expressed in terms of the median \pm SD. Continuous variables were

compared using a Mann Whitney-U test. We assigned statistical significance at $< .05$.

The calculations were performed with the help of Excel statistics version 2009 (Social Survey Research Information Co., Ltd, Japan).

Results

SOX9 expression in the normal pancreas and PDAC

SOX9 positivity was nuclear and was observed in normal pancreatic duct epithelium. The positive cells were expressed in almost all normal pancreatic duct epithelium (Figure 1a,b), and the positive rate was $82.7\% \pm 5.0\%$ (mean \pm SD). On the other hand, SOX9 expression was very low in PDAC compared with the normal pancreatic duct (Figure 1c, d), and the positive rate was $0.8\% \pm 1.6\%$ (mean \pm SD). SOX9 expression showed a significant difference between normal pancreas and PDAC ($p=0.0002$) (Figure 2).

SOX9 expression in IPMN

We classified IPMN into 4 groups: IPMA, NI-IPMC, MI-IPMC, and IC-IPMC. Moreover, considering that SOX9 expression in PC was extremely low, we investigated IPMN by the same means of analyzing for normal pancreases and PC pancreases. SOX9 expression gradually decreased according to IPMN progression including IPMA, NI-IPMC, MI-IPMC, and IC-IPMC (Figure 3). The positive rates of IPMA, NI-IPMC, MI-IPMC, and IC-IPMC were, in order, $66.3\% \pm 12.9\%$, $46.3\% \pm 8.2\%$, $30.5\% \pm 5.9\%$, and $2.3\% \pm 1.7\%$ (mean \pm SD). There were significant differences between each group ($p<0.05$) (Figure 4).

Discussion

This study suggested that the expression of SOX9 in PDAC was extremely low, and that in IPMN gradually decreased according to IPMN progression. It is well known that PDAC is one of the high potential malignancies and IPMN is characteristic of the adenoma-carcinoma sequence such as that in colon cancer. SOX9 was originally known as a chondrogenic transcription factor involved in bone formation and testis development, whereas its mutation has been linked to campomelic dysplasia and autosomal sex reversal¹¹⁾²²⁾. Subsequently, SOX9 was shown to be a multifaceted transcription factor indispensable for the development of many other organs/ tissues, such as prostate²³⁾²⁴⁾, intestine²⁵⁾, and pigment cells²⁶⁾. Recently, Furuyama et al.²⁷⁾ analyzed adult SOX9 expression by immunolabeling of a human pancreas and detecting SOX9 in the pancreatic duct. Given the scenario in which developmentally required genes are also likely oncogenes upon their deregulation, it is not surprising that SOX9 is associated with cancers. In fact, abnormal expression of SOX9 has been reported to be linked to mesenchymal chondrosarcoma²⁸⁾, prostate cancer²⁵⁾²⁹⁾, colorectal cancer³⁰⁾³¹⁾, cutaneous basal cell carcinoma³²⁾, and melanoma¹³⁾³³⁾. In particular, Passeron et al.¹³⁾ analyzed SOX9 expression in melanoma and discovered that SOX9 expression gradually decreased according to disease progress from normal skin to nevi, primary

melanoma, and metastatic melanoma. However, there was only one report about the relation between SOX9 and benign pancreatic tumors³⁴⁾. To our knowledge, no reports have shown a relation between SOX9 and pancreatic malignant tumors. The therapy for PDAC and IPMN will require the identification of molecular biomarkers for prognosis or novel targets for therapeutic intervention. In this study, we suggested that SOX9 was a potent candidate for the new therapeutic strategy by showing its low expression and its association with an adverse prognosis in PDAC and IPMN. The components of the prostaglandin D synthase (Pgds) / SOX9 pathway were expressed in ovarian cancer cell lines and the treatment of these cell lines, with prostaglandin D2 (PGD2) could inhibit their growth and induce apoptosis³⁵⁾. Calcineurin inhibitors are able to increase the phosphorylation of SOX9 and thus its translocation to the nucleus³⁶⁾. Interestingly, a new calcineurin inhibitor without immunomodulatory effects has shown promising results in treating melanomas³⁷⁾. Histone deacetylase inhibitors can increase SOX9 expression in sarcomas and induce growth arrest and apoptosis in clear cell sarcoma cells³⁸⁾³⁹⁾. Recently, microRNA-145 was reported to regulate chondrogenic differentiation of mesenchymal stem cells by targeting SOX9, and over-expression of microRNA-145 was reported to have decreased expression of SOX9 only at the protein levels, and microRNA-145 inhibition significantly elevated SOX9 protein levels⁴⁰⁾, so

that microRNAs such as microRNA-145 might be an important therapy for PDAC and IPMN. SOX9 was reported to regulate the Notch effector HES1, which suggests a Notch-dependent mechanism and establishes a possible genetic link between SOX factors and Notch¹⁹⁾⁴¹⁾⁴²⁾. The Notch pathway was also reported to play an important role in cell fate determination in both embryonic development and organ homeostasis¹⁹⁾⁴¹⁾⁴²⁾. Moreover, the activation of Notch-1 was reported to be associated with the development and progression of human malignancies including PDAC⁴²⁾⁴³⁾. Actually, Mazur PK et al.⁴²⁾ reported that SOX9 and Notch in the biliary tract cancer expressed abnormally and there was the significant relationship between SOX9 and Notch. These findings suggested that SOX9 had an important role as the main target of Notch in the condition of both normal organ and cancer development. Considering that significant differences between the normal pancreas, PDAC, and IPMN, we suggested that SOX9 might contribute to carcinogenesis of PDCA and IPMN and be the target of the therapy or the molecular biomarker for PDAC and IPMN.

In summary, it was found that SOX9 is a novel promising marker for the evaluation of PDAC and IPMN. This result implies that SOX9 could be a potential therapeutic target in PDAC and IPMN. However, further studies should be conducted on the molecular and biological effects of SOX9 in PDAC and IPMN.

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Figure Legends

Figure 1: The result of pathological and immunochemical tests in a normal pancreas and PDAC. SOX9 positivity was nuclear and was observed in normal pancreatic duct epithelium. The positive cells in the normal pancreatic duct were expressed in almost all pancreatic duct epithelium. SOX9 expression in PDAC was extremely low compared with the normal pancreatic duct.

Figure 2: Comparison of SOX9 positive rate between a normal pancreas and PDAC.

The positive cells in the normal pancreatic duct were expressed in almost all pancreatic duct epithelium, and the SOX9 positive rate was $82.7\% \pm 5.0\%$ (mean \pm SD).

SOX9 expression was very low in PC compared with the normal pancreatic duct. The SOX9 positive rate was $0.8\% \pm 1.6\%$ (mean \pm SD). There was a significant difference in the SOX9 positive rate between the normal pancreatic duct and PDAC ($p=0.0002$).

Figure3: The result of pathological and immunochemical tests in IPMN.

SOX9 expression gradually decreased in IPMN as the degree of malignancy went up in the following order: IPMA (3a, b), NI-IPMC (3c, d), MI-IPMC (3e, f), and IC-IPMC (3g, h).

(Figure 3a, c, e, g: HE \times 200, 3b, d, f, h: SOX9 \times 200)

Figure 4: Comparison of the SOX9-positive rate between a normal pancreas, PC, and IPMN. SOX9 expression gradually decreased in IPMN compared with the normal pancreatic duct. The positive rates of IPMA, NI-IPMC, MI-IPMC, and IC-IPMC were $66.3\% \pm 12.9\%$, $46.3\% \pm 8.2\%$, $30.5\% \pm 5.9\%$, and $2.3\% \pm 1.7\%$ (mean \pm SD), respectively. There were significant differences between all groups ($p < 0.05$).

Table 1: Characteristics of pancreatic cancer patients.

Variable	Patients (n=55)
gender (Male : Female)	26 : 29
age	70 ± 10
operative method*	PD : 7
	PpPD : 26
	DP : 14
	SSPPD : 5
tissue-type(well/mod/poor/tub/pap)	TP : 3
stage (0/1/2/3/4a/4b)	16/28/4/5/2
	1/1/3/20/22/8

*PD: pancreaticoduodenectomy, PpPD: pylorus-preserving pancreaticoduodenectomy, DP: distal pancreatectomy,

SSPPD:subtotal stomach-preserving pancreaticoduodenectomy, TP: total pancreatectomy

Table 2 Characteristics of IPMN patients.

Variable	Patients (n=68)				
	IPMA (n=37)	NI-PIMC (n=15)	MI-IPMC (n=7)	IC-IPMC (n=9)	
gender (Male : Female)	25: 12	8 : 7	2 : 5	5 : 4	
age	70±10	68±7	66±10	76±6	
size of cyst (mm)	30±12	40±15	33±9	60±26	
type	main duct type	4	2	5	3
	mixed type	12	11	2	6
	branch type	21	2	0	0
operative method*	PD	1	1	1	2
	PpPD	8	10	2	2
	DP	7	2	3	2
	PHRSD	6	1	0	0
	DPPHR	5	0	1	1
	SDR	6	0	0	0
	Segmentectomy	4	1	0	1
	TP	0	0	0	1

*PD: pancreaticoduodenectomy, PpPD: pylorus-preserving pancreaticoduodenectomy, DP: distal pancreatectomy,

PHRSD: pancreatic head resection with segmental duodenectomy, DPPHR: duodenum preserving pancreas head resection,

SDR: segmental duodenal resection, TP: total pancreatectomy

Figure 1

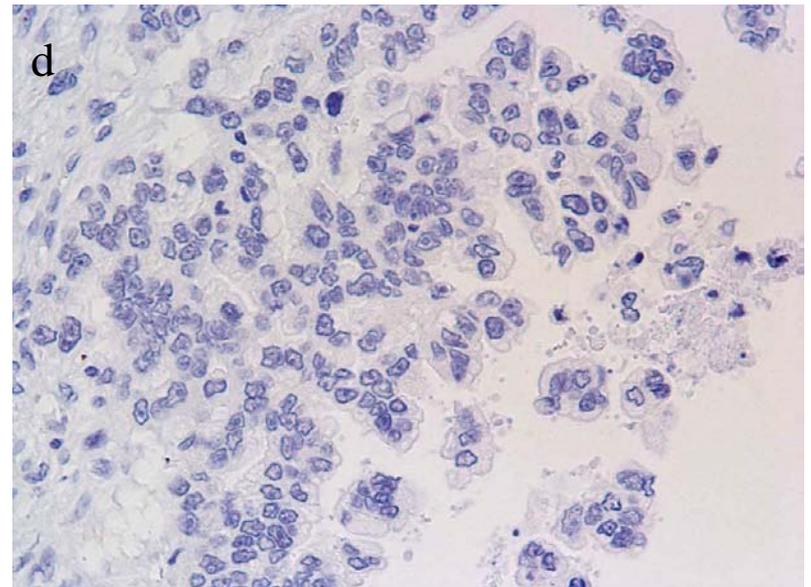
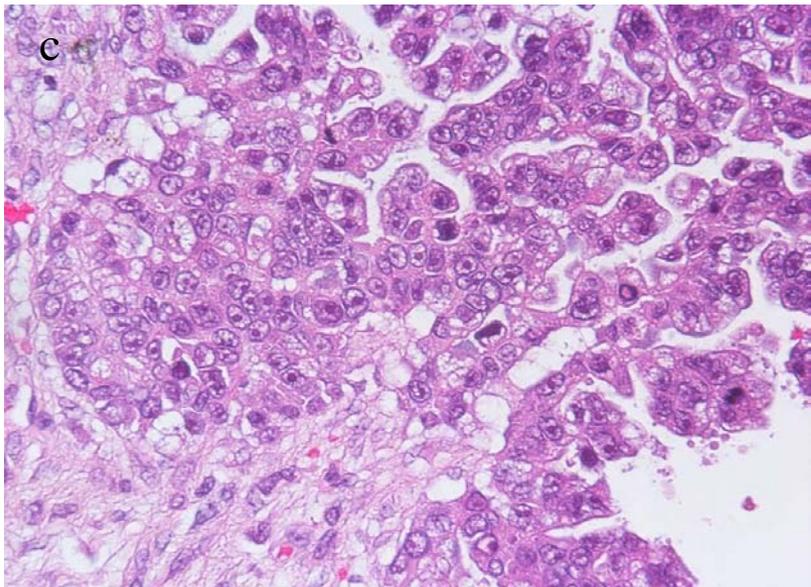
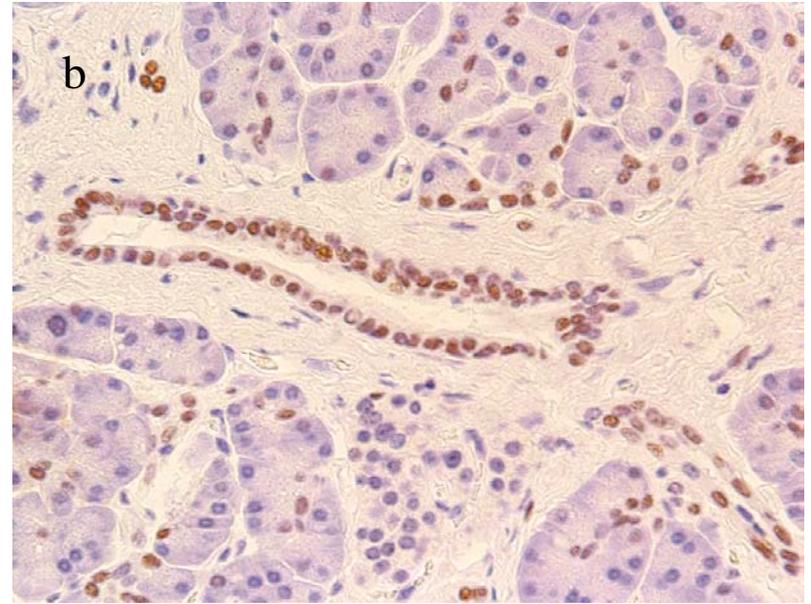
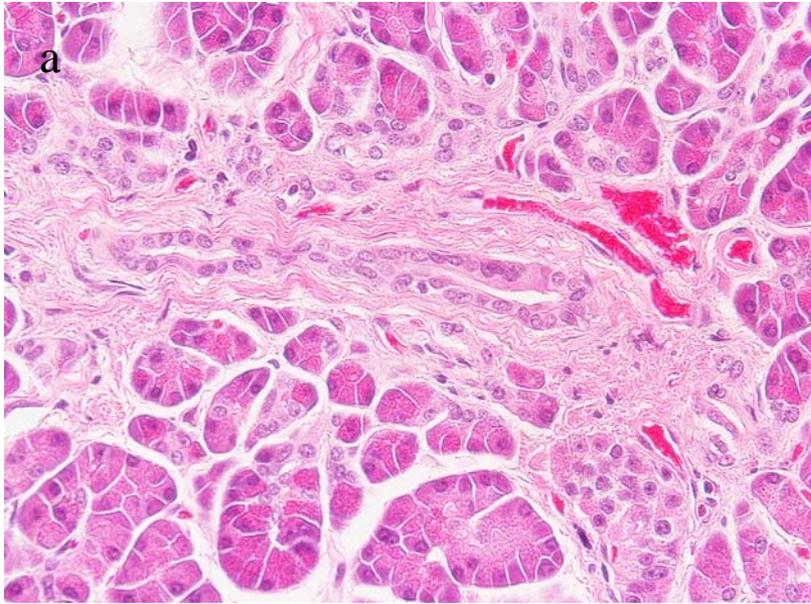


Figure 2

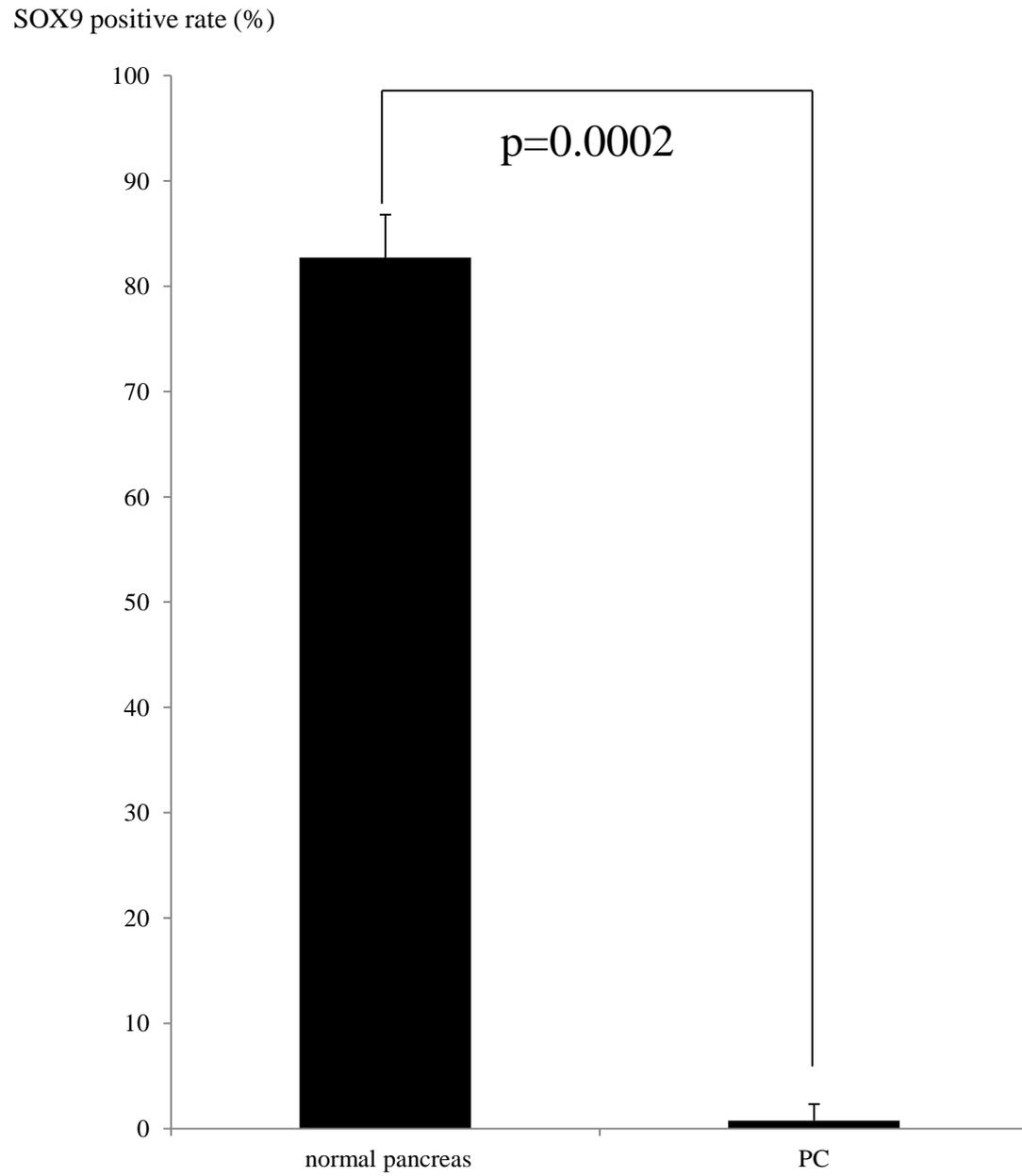


Figure 3

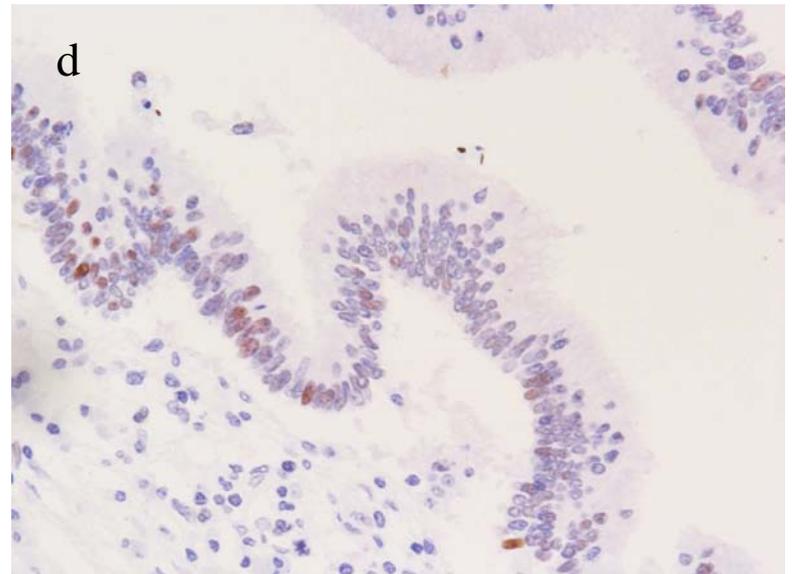
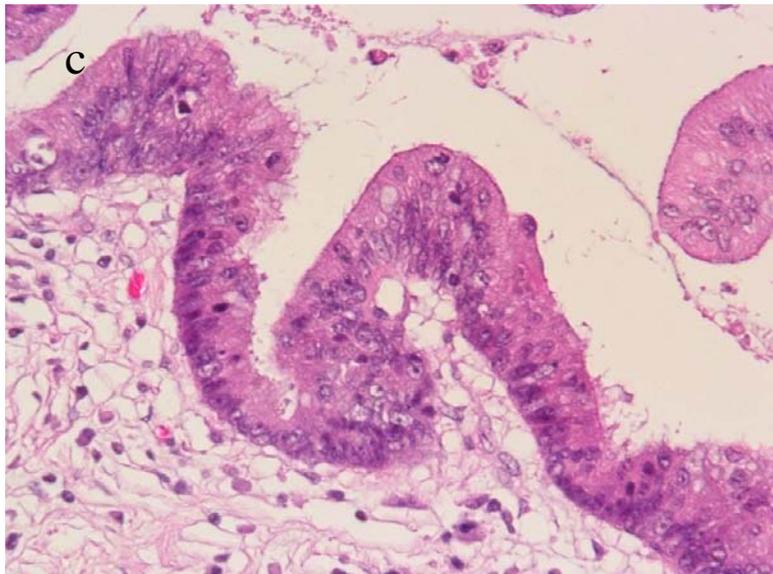
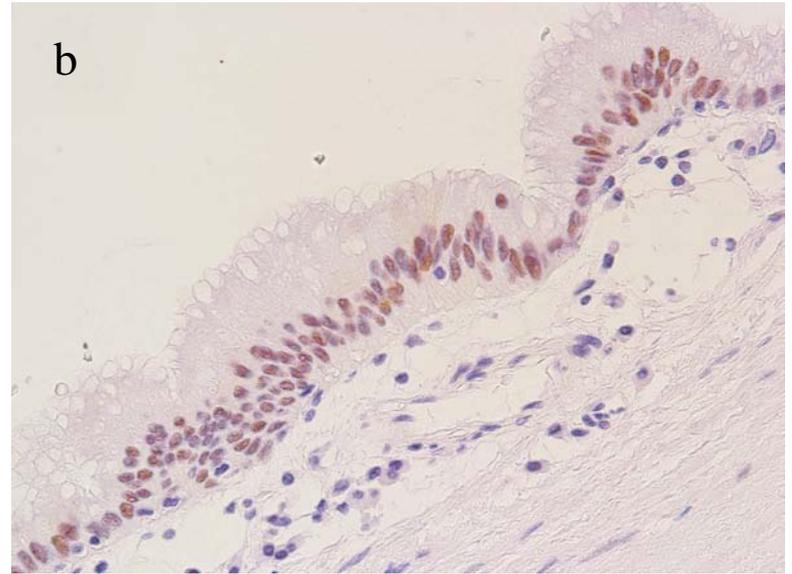
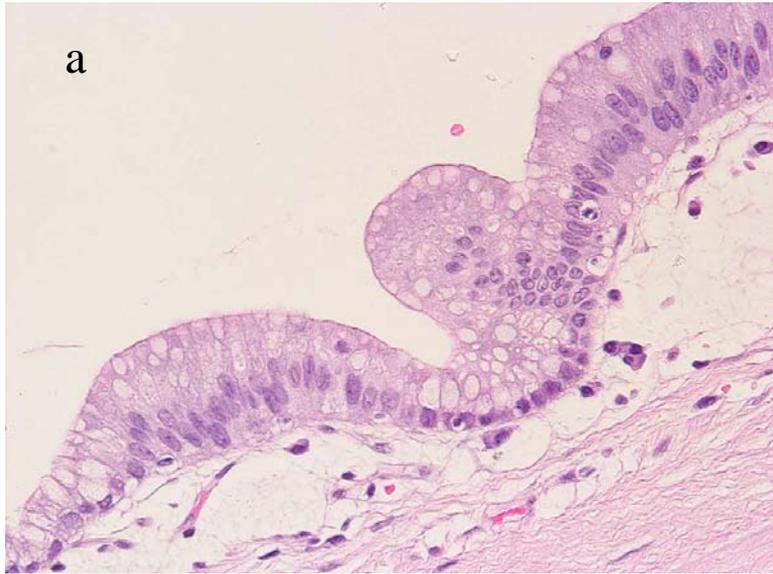


Figure 3

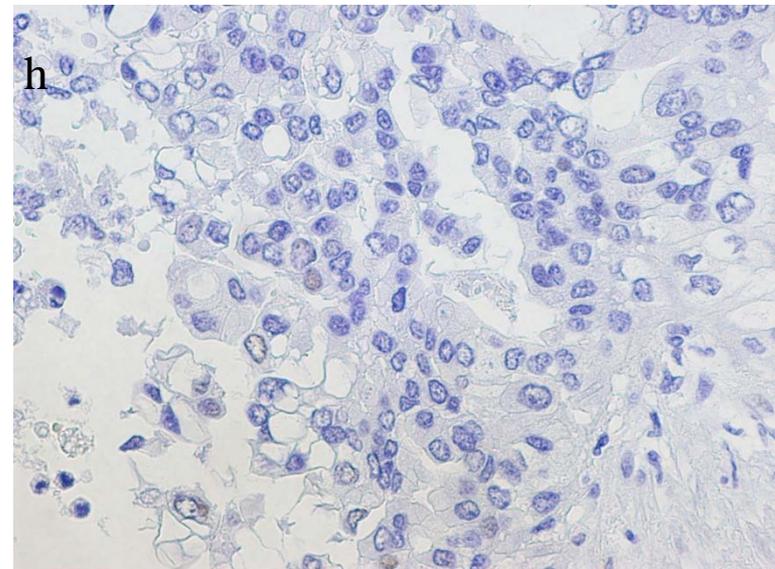
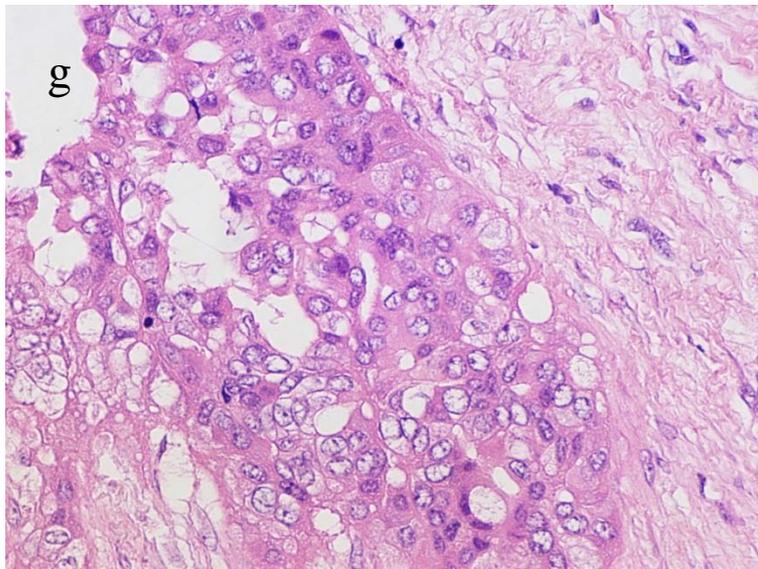
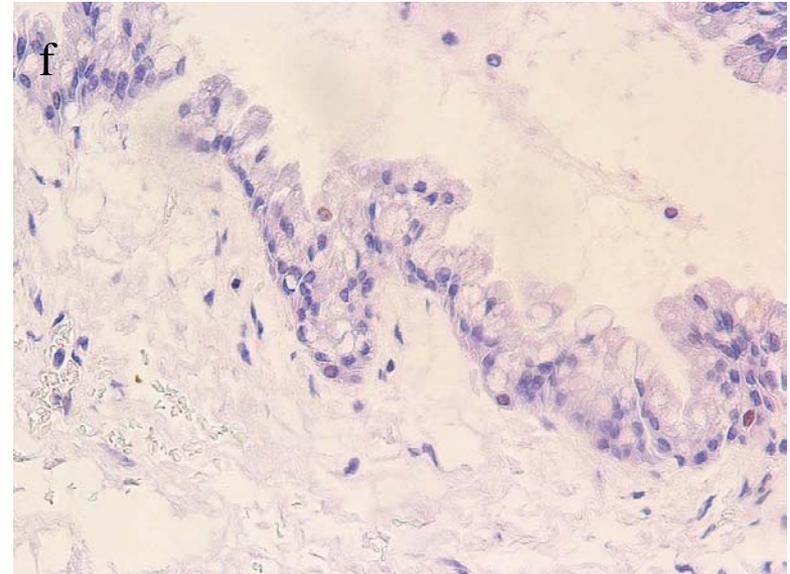
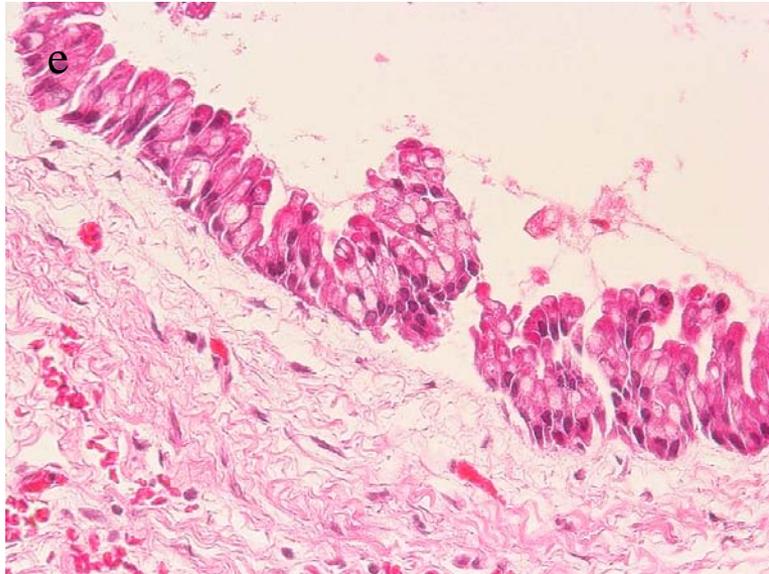
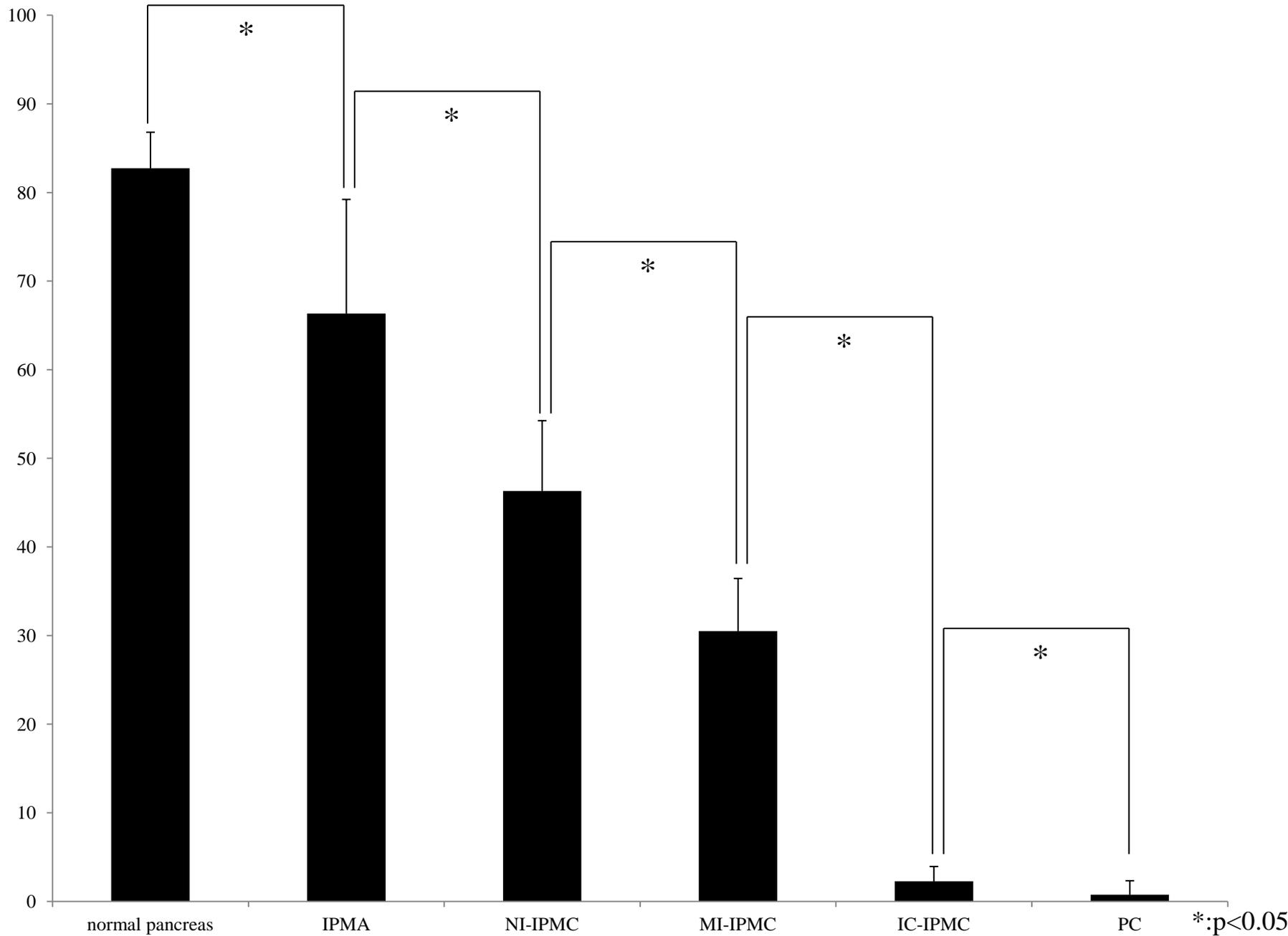


Figure 4

SOX9 positive rate (%)



*:p<0.05