RESEARCH ARTICLE

Expression Profile and Potential Roles of EVA1A in Normal and Neoplastic Pancreatic Tissues

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Abstract

Background: EVA1A (eva-1 homolog A) is a novel gene that regulates programmed cell death through autophagy and apoptosis. Our objective was to investigate the expression profiles and potential role of EVA1A in normal and neoplastic human pancreatic tissues. Materials and Methods: The expression pattern of EVA1A in normal pancreatic tissue was examined by indirect immunofluorescence and confocal microscopy. Protein levels in paraffin-embedded specimens from normal and diseased pancreatic and matched non-tumor tissues were evaluated by immunohistochemistry. Results: EVA1A colocalized with glucagon but not with insulin, demonstrating production in islet alpha cells. Itwas strongly expressed in chronic pancreatitis, moderately or weakly expressed in the plasma membrane and cytoplasm in pancreatic acinar cell carcinoma, and absent in normal pancreatic acinar cells. Although the tissue architecture was deformed, EVA1A was absent in the alpha cells of pancreatic ductal adenocarcinomas, intraductal papillary mucinous neoplasms, mucinous cystadenomas, solid papillary tumors and pancreatic neuroendocrine tumors. Conclusions: EVA1A protein is specifically expressed in islet alpha cells, suggesting it may play an important role in regulating alpha-cell function. The ectopic expression of EVA1A in pancreatic neoplasms may contribute to their pathogenesis and warrants further investigation.

Keywords: EVA1A - islet cells - pancreatic neoplasms - pancreas - expression profile

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Introduction

The pancreas is a mixed gland with exocrine and endocrine functions. Endocrine dysfunction is associated with diabetes, whereas abnormal exocrine function can lead to pancreatitis. There are various types of pancreatic neoplasms, these include pancreatic ductal adenocarcinoma (PDAC), intraductal papillary mucinous neoplasm (IPMN), solid papillary tumor (SPT), mucinous cystadenoma (MCN), pancreatic neuroendocrine tumor (pNET) and pancreatic acinar cell carcinoma (pACC). However, the pathogenesis of pancreatic cancer is unclear. PDAC has a very poor prognosis with a 5-year survival rate under 5% (Poruk et al., 2013); IPMN, SPT and MCN have the potential for malignant change, therefore early detection can be difficult due to hidden pathogeneses. Although patients with pNET and pACC have a better prognosis than those with PDAC, the rates of metastasis and recurrence after surgery are high (Kuo et al., 2013; Sumiyoshi et al., 2013)

Pancreatic islet cells secrete various hormones, including the counter-regulatory hormones insulin and glucagon which maintain normal blood glucose levels. Glucagon is produced in alpha cells and insulin is produced by beta cells. Type 1 and type 2 diabetes are characterized by a deficiency in islet beta cells and an increase in beta-cell apoptosis.

Autophagy is one of the processes involved in lysosomal degradation of endogenous protein substrates. Dysregulated autophagy has been implicated in the pathogenesis of diverse diseases, including diabetes (Jung et al., 2008). Rivera et al showed that increased expression of human islet amyloid polypeptide (IAPP) could impair autophagy, but suggested that scaffold protein p62, which delivers polyubiquitinated proteins to autophagy, may play a protective role against human-IAPP-induced apoptosis (Rivera et al., 2011; 2014). Quan et al reported that beta-cell-specific ATG7-knockout mice developed hyperglycaemia and glucose intolerance (Quan et al., 2013). As a deficiency in autophagy is a proinflammatory condition in beta cells, it may contribute to the development of diabetes by promoting the recruitment of inflammatory cells and enhancing inflammatory activity (Jung et al., 2008).

EVA1A (eva-1 homolog A), which is also known as *TMEM166* (trans-membrane protein 166) or *FAM176A*

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(Family with sequence similarity 176, member A), is a novel human gene that was originally characterized by Peking University Health Science Center (Beijing, China) (Wang et al., 2007). It is expressed in most normal human tissues and organs in a cell-type and tissue-type specific manner and is involved in cell autophagy and apoptosis (Sun et al., 2012; Chang et al., 2013; Xu et al., 2013; Xie et al., 2014). EVA1A is strongly expressed in the glomerular zona of the adrenal cortex, chromophil cells of the pituitary gland, pancreatic islet cells, the squamous epithelium and esophageal mucosa, the fundic glands, and hepatocytes (Xu et al., 2013). In contrast, underexpression of EVA1A has been reported in several types of human cancer, including gastric cancer and esophageal cancer (Sun et al., 2012; Xu et al., 2013). Restoring EVA1A expression has been shown to significantly inhibit the proliferation of tumor cells through autophagic and apoptotic mechanisms (Wang et al., 2007; Chang et al., 2013; Xie et al., 2014). Together, these reports suggest that EVA1A is a novel positive regulator of programmed cell death.

We previously reported that EVA1A is expressed in pancreatic islet cells (Xu et al., 2013), however its precise location in the islets was unclear, and its role in human pancreatic neoplasms remained to be investigated in detail. In this study, we examined the protein expression profiles of EVA1A protein in normal human pancreatic tissue and tissues from different pancreatic diseases, including PDAC, IPMN, SPT, MCN, pNET, pACC and chronic pancreatitis (CP). We further characterized the location of EVA1A in islet alpha cells. Our findings have provided a foundation for further studies on the specific functions of EVA1A in normal and neoplastic pancreatic tissues.

Materials and Methods

Human pancreatic tissue samples

Paraffin-embedded pancreatic tumor tissues and adjacent non-tumor tissues were obtained from patients who had undergone surgery at the Peking University Third Hospital, Beijing, China, and had been diagnosed by an experienced pathologist. They included five cases of PDAC, five cases of IPMN, five cases of SPT, five cases of pNET, four cases of pACC and two cases of CP. This study complied with the ethical standards of the Chinese Medical Association (IRB00001052-08044) and national legislation.

Immunofluorescence analysis

The paraffin-embedded sections of normal pancreatic tissues (5 μm thick) were deparaffinized and rehydrated following standard procedures. Antigen retrieval was performed using a pressure cooker at 100°C for 2 min in 0.01 M sodium citrate (Zhongshan Golden Bridge Biotechnology; Beijing, China). After nonspecific blocking in 1% goat serum, the sections were incubated with monoclonal anti-glucagon (Sigma-Aldrich; St. Louis, MO, USA), anti-insulin (Sigma-Aldrich), or polyclonal anti-EVA1A at 4°C overnight. The slides were washed three times in phosphate-buffered saline (PBS) before being incubated with Alexa Fluor 594-conjugated AffiniPure anti-rabbit IgG or Alexa Fluor 488-conjugated AffiniPure anti-mouse IgG (Zhongshan Golden Bridge Biotechnology) for 1 h at room temperature. Nuclei were counterstained with 4, 6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich). After the slides had been washed three times in PBS, they were sealed with coverslips and visualized under a Zeiss confocal system (LSM 510 META; Jena, Germany).

Immunohistochemistry (IHC)

The paraffin-embedded sections of pancreatic tumor tissues and adjacent normal tissues were deparaffinized and rehydrated following standard procedures. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and nonspecific binding was blocked with 1% goat serum. The sections were incubated with anti-human EVA1A polyclonal antibody at 4°C overnight. After washing three times in PBS, the slides were incubated with EnVision peroxidase/DAB, rabbit/mouse detection kit (Dako Diagnostics; Glostrup, Denmark). The nuclei were counterstained with ammonia water. The specimens were dehydrated and sealed with coverslips. Hematoxylin and eosin (H and E) staining was also performed, and control samples were prepared. The slides were visualized under a Leica microscope (Wetzlar, Germany).

Results

EVA1A *is specifically localized in islet alpha cells*

We have previously reported that EVA1A protein is strongly expressed in pancreatic islet cells (Xu et al., 2013). In this study, its location was examined in different types of islets cells. Immunofluorescent staining revealed that the fluorescence from EVA1A completely overlapped that from glucagon (Figure 1A), demonstrating that EVA1A colocalized with glucagon. In contrast, EVA1A was not colocalized with insulin (Figure 1B). Furthermore, EVA1A fluorescence was primarily observed in cytoplasm of alpha cells (Figure 1C). These findings suggested that constitutive expression of EVA1A in islet cells may be essential to maintain the architecture and function of

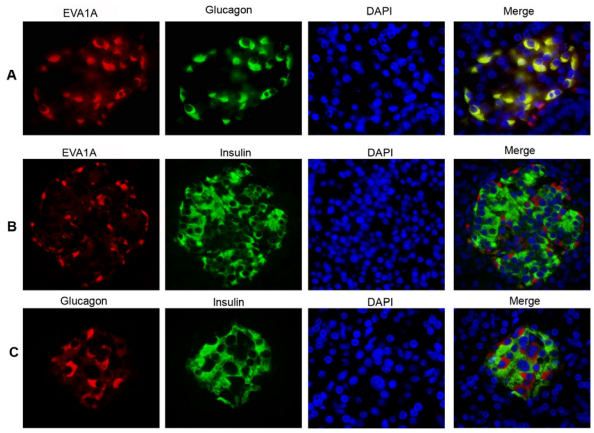

Figure 1. Localization of EVA1A in Pancreatic Islet Alpha Cells. Indirect immunofluorescence analysis by confocal microscopy was performed on the paraffin-embedded sections of normal pancreatic tissues, and nuclei were stained with DAPI as indicated. **A)** Co-localization of EVA1A and glucagon. **B)** staining of EVA1A and insulin. **C)** staining of glucagons and insulin

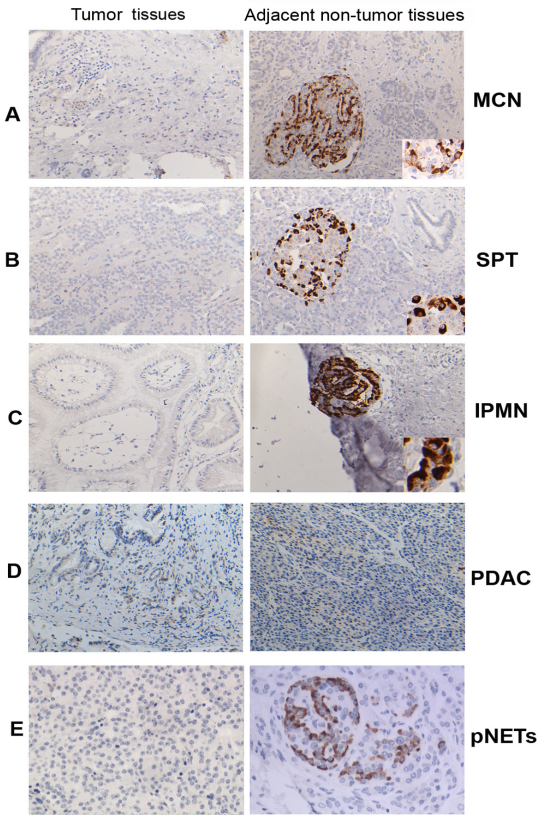

Figure 2. Immunohistochemical Staining for EVA1A in Human Pancreatic Tumor Tissues and Adjacent Non-Tumor Tissues. Strong EVA1Aexpression was observed in adjacent non-tumor tissues in **A)** MCN, **B)** SPT, **C)** IPMN and **E)** pNETs. Negative EVA1A expression was observed in pancreatic tumor tissues detected and adjacent non-tumor tissue in **D)** PDAC

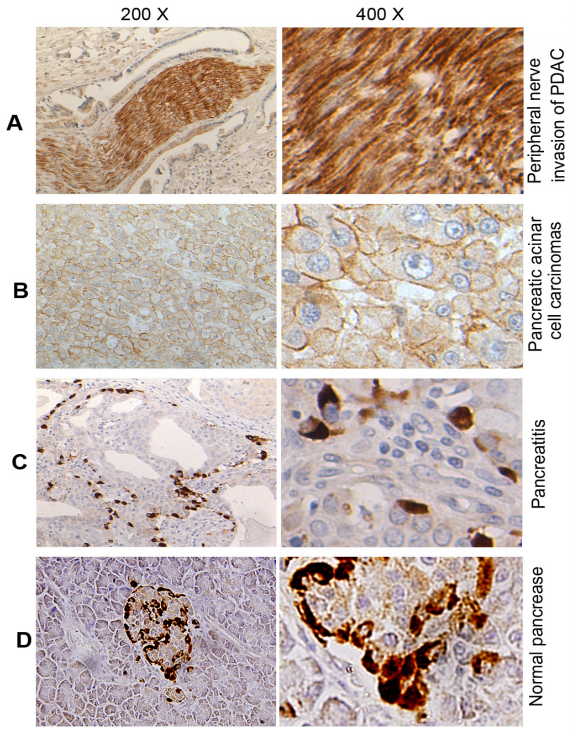

Figure 3. EVA1A Immunostaining was Observed in Invaded Peripheral Nerves of PDAC. A), pancreatic acinar cell carcinoma **B)**, pancreatitis tissue **C)** and normal pancrease **D)**

normal alpha cells. Consistent with our earlier report (Xu et al., 2013), we found that EVA1A expression was negative in normal pancreatic acinar and duct cells.

The distribution of EVA1A *in pancreatic neoplasms*

The expression patterns of EVA1A protein in a variety of human pancreatic tumor tissues and adjacent non-tumor tissues were examined by IHC. A breakdown in the architecture of the tumor tissues was observed, however the staining patterns showed that EVA1A was not expressed in the islet cells of MCN, SPT, IPMN and pNET (Figure 2, left panel). Conversely, it was expressed in their adjacent non-tumor islet cells (Figure 2, right panel). In the PDAC specimens, EVA1A was not detected in either the tumor or adjacent non-tumor islet cells. It was also not detected in pancreatic insulinoma tissues (data not shown).

We also detected EVA1A staining distributed throughout PDAC tissue that invaded the peripheral nerves, suggesting that EVA1A was expressed in neural cells (Figure 3A). However, this observation requires more samples for further investigation.

The specimens of pancreatic acinar cell carcinoma displayed a different staining pattern (Figure 3B) to those in normal pancrease (Figure 3D) and other tested pancreatic tumor tissues (Figure 2), as moderate or weak EVA1A immunostaining was detected in the plasma membranes (Figure 3B). The biological significance of this difference in pACC requires further exploration.

The expression of EVA1A in pancreatitis tissue

Our observations had revealed that EVA1A was expressed in the adjacent normal tissues of both benign and malignant pancreatic tumors. Furthermore, the extent of EVA1A staining in the islets of adjacent normal tissues appeared greater than that in normal pancreatic tissue.

Patients with pancreatic neoplasms often present with preoperative pancreatitis or diabetes, both of which can lead to abnormal islet cell morphology and function. This could also account for the absence of islet cells in the tissue specimens observed by H and E staining. Therefore, we examined EVA1A expression in pancreatitis tissue specimens (Figure 3C). Our results showed that although the islet morphology was altered, the alpha cells displayed strong EVA1A immunoreactivity.

Discussion

EVA1A is a lysosomal and endoplasmic reticulum-associated protein that has been associated with the regulation of autophagy and apoptosis (Wang et al., 2007; Chang et al., 2013; Xie et al., 2014), however, the precise role of autophagy in alpha-cell function is unknown. Therefore, the aim of this study was to evaluate the expression and function of EVA1A in pancreatic cells. Our results showed that the expression profiles of EVA1A protein in normal and diseased human pancreatic tissues had the following characteristics: EVA1A was specifically expressed in islet alpha cells in normal pancreatic tissue; it was strongly expressed in chronic pancreatitis; it was absent in acinar cells in both tumor and non-tumor pancreatic tissues; however, it was moderately or weakly expressed in acinar cells in pACC tissue; EVA1A was not expressed in IPMN, MCN, SPT and pNET tumor tissues,

but was expressed in the islet alpha cells of their adjacent normal tissues; in contrast, it was not expressed in tumor or matched non-tumor tissues in PDAC, but was detected in the perineural invasion of PDAC.

The overexpression of EVA1A, and its specificity in islet alpha cells, suggested it may be implicated in blood glucose dysregulation in diabetes and several types of pancreatic neoplasms. However, some preoperative patients with pancreatic neoplasms also have diabetes, which can cause an imbalance in the transformation between islet alpha and beta cells, leading to an increase in the proportion of alpha cells. Chronic systemic inflammation plays an important role in the pathogenesis of insulin resistance and type 2 diabetes (T2DM). The histology of islets in patients with T2DM exhibits many typical features associated with tissue inflammation, including immune cell infiltration, decreased insulin staining and beta-cell apoptosis (Donath, 2013; Wu et al., 2013). Recurrent pancreatitis is also known to disrupt pancreatic endocrine function. Our results revealed strong cytoplasmic expression of EVA1A and nuclear EVA1A immunoreactivity in islet cells from patients with pancreatitis. The imbalance of alpha-beta cells implied that EVA1A might be involved in the inflammatory reaction.

PACC is a rare form of pancreatic cancer and its pathogenesis is unclear. Pathological diagnosis of pACC is generally performed by trypsin and chymotrypsin staining, which lacks sensitivity and specificity as pACC cells do not always secrete these two enzymes leading to errors in diagnosis (Armstrong et al., 2011; La et al., 2012). Therefore, the expression of EVA1A in the plasma membrane of pACC cells may be a potential marker for the diagnosis of pACC.

The specificity of EVA1A expression in pancreatic endocrine cells, nervous tissue, pituitary adenoma and pheochromocytoma has indicated that EVA1A protein is expressed in cell-type or tissue-type-specific manner (Xu et al., 2013). We found that EVA1A was strongly expressed in the perineural invasion of PDAC. The protective role of EVA1A in cerebral ischemic injury was reported by Li et al who demonstrated that cell loss could be prevented by blocking EVA1A activity with siRNA (Li et al., 2012). ARX/Arx is a homeodomain-containing transcription factor that is essential for brain development and necessary for the specification and early maintenance of islet alpha cells (Wilcox et al., 2013). The similarity between ARX/Arx and EVA1A in the development of islet alpha cells and the nervous system, suggested that EVA1A may also have a role in nervous system development and play a role in the neuroendocrine system. However this requires further investigation. It is believable that the development of EVA1A-knockout mice will further understand the role of EVA1A in pancreatic islet alpha cells.

In conclusion, this study has provided compelling evidence that EVA1A is specifically expressed in human pancreatic islet alpha cells. The overexpression of EVA1A protein in the pancreatic alpha cells in matched non-tumor tissues and pancreatitis tissues implied that EVA1A might be involved in the inflammatory response. The specificity of EVA1A expression in the cytoplasm membrane of pACC and perineural invasion of PDAC suggested that EVA1A could be a potential diagnostic and pathogenic marker in these neoplasms. Further studies of EVA1A expression and function in the islet cells could provide valuable insights into the pathophysiology of the pancreas and its disorders.

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