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PACAP and PAC1 receptor expression in pancreatic ductal carcinoma

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Abstract. Pancreatic carcinoma is one of the most malignant diseases and is associated with a poor survival rate. Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide that acts on three different G protein-coupled receptors: the specific PAC1 and the VPAC1/2 that also bind vasoactive intestinal peptide. PACAP is widely distributed in the body and has diverse physiological effects. Among other things, it acts as a trophic factor and influences proliferation and differentiation of several different cells both under normal circumstances and tumourous transformation. Changes of PACAP and its receptors have been shown in various tumour types. However, it is not known whether PACAP and its specific receptor are altered in pancreatic cancer. Perioperative data of patients with pancreas carcinoma was investigated over a five-year period. Histological results showed Grade 2 or Grade 3 adenocarcinoma in most cases. PACAP and PAC1 receptor expression were investigated by immunohistochemistry. Staining intensity of PAC1 receptor was strong in normal tissues both in the exocrine and endocrine parts of the pancreas, the receptor staining was markedly weaker in the adenocarcinoma. PACAP immunostaining was weak in the exocrine part and very strong in the islets and nerve elements in non-tumourous tissues. The PACAP immunostaining almost disappeared in the adenocarcinoma samples. Based on these findings a decrease or lack of the PAC1 receptor/PACAP signalling might have an influence on tumour growth and/or differentiation.

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Introduction

Pancreas carcinoma is one of the most malignant diseases, associated with late and difficult diagnosis and really short survival after diagnosis. Although in most countries effective radiological and other examination methods can be reached, the early diagnosis of pancreas cancer is difficult (1-4). Pituitary adenylate cyclase activating polypeptide (PACAP) was first isolated as a hypothalamic neuropeptide acting on the pituitary cAMP release (5,6). The peptide is composed of 38 amino acid residues (PACAP38) and has a shorter form, with only 27 amino acids (PACAP27) (7). Subsequent studies have shown that PACAP is distributed in the entire body, with highest concentrations in the central nervous system and endocrine glands, but it is also present in the cardiovascular, urogenital and gastrointestinal systems (8-13). PACAP has a diverse array of functions via specific PAC1 receptor and VPAC1 and 2 receptors shared with vasoactive intestinal peptide, as well as non-receptorial mechanisms (13,14).

PACAP and its receptors have also been shown in several exocrine glands. The lacrimal gland is innervated by a rich PACAP-ergic fiber plexus (15) and PAC1 receptors are responsible for the activation of tear secretion (16,17). Mammary and salivary glands are also innervated by PACAP-ergic nerves (18-20). In the salivary glands, PACAP induces secretion (21), and enhances protein production while inhibits Ca^{2+} channels (22-24). The exocrine pancreas is histologically similar to serous salivary glands, and the presence of PACAP has also been shown in the exocrine pancreas, where it stimulates acinar lipase secretion (25). Endocrine pancreas, composed of the islets of Langerhans, expresses very high levels of the peptide, similarly to other endocrine glands. Intrainsular PACAP plays a regulatory role in insulin and glucagon secretion and is implied in glucose homeostasis. Pancreatic PACAP has also been implicated in the regulation of beta cell proliferation (26).

Under pathological conditions, a few studies have dealt with changes in PACAP and receptor expression. Previous studies showed that pancreatic over-expression of PACAP increases in cerulein-induced inflammation leading to acute pancreatitis in a mouse model (27). PACAP, along with its receptors, has been

shown to be involved in cell proliferation and differentiation both under normal circumstances and in tumourous transformation (28-33). For some tumour cells, PACAP acts as a growth factor (30), while it inhibits growth of others (34). Whether it stimulates growth of pancreatic tumour cells, it is not known at present, however, a PACAP-response gene associated with proliferation and stress response has been described in pancreatic carcinoma (35). Stimulative role of tumour genesis of PACAP is proven by stimulation of c-Fos as well as c-Jun transcription, and PACAP strongly induces proliferation of the rat pancreatic carcinoma cell line AR4-2J via interaction with the G-protein coupled type 1 PACAP/VIP (PV1) receptor (36). PACAP and PAC1 receptor display specific alterations in several different tumour types, such as thyroid papillary carcinoma and testicular cancer (37,38). It is not known how expression of the peptide and its specific receptor changes in pancreatic cancer. Therefore, the aim of the present study was to investigate whether there is a change in the expression of PACAP and its PAC1 receptor in pancreas adenocarcinoma.

Materials and methods

Patients. A five-year-long period (September 2012-February 2017) was investigated. Preoperative and perioperative data of patients operated in our Department of Surgery because of pancreatic ductal carcinoma were collected. Operation type as well as histological findings, grading, and margin resection were investigated from the pathological tissue samples after diagnosis and treatments had been made (Ethical permission number: PTE/83069/2018).

Histology and immunhistology. After data collection new histological sections were made and prepared for further specific histological examination of PACAP and PAC1 receptor expression. Two-μm-thick paraffin sections fixed in 4% buffered formalin were processed for immunohistochemical staining. Sections were stained using standard immunohistochemistry with human anti-PACAP antibody (dilution of 1:200; Peninsula, CA, USA) and with human PAC1 receptor antibody raised in rabbit (dilution of 1:200; Sigma-Aldrich, Budapest, Hungary). Immunohistochemical staining was performed with EnVision FLEX Visualization Systems for Dako Omins (Dako, Denmark), similarly to our earlier descriptions (37). Liquid fast-red substrate kit (Abcam, UK) was used as a chromogen for the immunohistochemical staining. Pathological analysis was performed by an expert pathologist, using a semi-quantitative approach to evaluate the immunohistochemical staining intensity between no staining, weak, medium and strong staining. By omitting the primary antiserum, we performed a method control, which resulted in no staining. Well-identified structures, like insular cells, nerve elements of the myenteric plexus and intramural ganglia, served as positive control, as both PACAP and PAC1 receptor are known to be expressed in the insula and PACAP has been described in the nerve elements. Tumour cell staining intensity was compared to that of tumour-free tissue in the same pancreas tissue in a semi-quantitative way.

Results

Clinical data. Data of 19 patients (7 male, 12 female) were chosen to be investigated (mean age were 69.6 years; 54 to 74 years). Seven patients had Grade 2, 13 patients Grade 3 adenocarcinoma in the pancreas head, with icterus and significant weight loss. Five patients were operated by conventional Whipple operation, 14 patients underwent pylorus preserving pancreatoduodenectomy (PPPD). In every case operation was followed by a three-day-long Intensive Care Unit (ICU) observation. After ICU observation and further care in normal surgery unit all patients were emitted. The histological result of the resected pancreas tissue showed Grade 2 adenocarcinoma in 11 patients, Grade 3 adenocarcinoma in 7 cases, and mucinous adenocarcinoma in 1 patient. Tumour staging in all cases was pT3. Lymph node staging was N0 in 5 cases, the other specimens showed N1 stage. Resection margin was not affected (R0 resection) in 9 cases, samples from 7 patients showed narrow resection margin, 1 sample showed perineural invasion on ductus choledochus, another sample showed tumour cell infiltration on the wall of veins. In case of one patient, the tumour involved the common hepatic artery and portal vein (R2 resection).

Histology and immunhistology. The immunohistochemical staining showed that PAC1 was expressed in both the exocrine and endocrine parts of the pancreas, in accordance with earlier descriptions. We also confirmed the particularly strong staining of the pancreatic islets (Fig. 1A and B). In the adenocarcinoma, receptor staining was markedly weaker. In tissue samples, the border between tumourous and normal pancreas was also shown by the different staining intensity for the PAC1 receptor (Fig. 1C and D). Nerve elements did not show receptor positivity.

PACAP staining, on the other hand, was weaker in the exocrine part, and again very strong in the endocrine islets (Fig. 2A and B). Similarly to the PAC1 receptor staining, PACAP expression was also weaker in the adenocarcinoma parts of the tissue samples (Fig. 2C). Neither PACAP nor PAC1 receptor expression showed correlation with the tumour outcome. In contrast to the absence of PAC1 receptor, PACAP was also expressed in the intrapancreatic nerves (Fig. 2D) and ganglionic cells (Fig. 2E and H). Following Whipple operation, resected parts of the duodenum were also examined. We could confirm earlier descriptions regarding the presence of PACAP and its specific receptor in the duodenum. Myenteric and submucosal plexi were strongly stained for PACAP, including inter- and intramuscular as well as lamina propria nerve fibers and ganglionic cells in the myenteric plexus (Fig. 2F).

Discussion

In the present study we analysed normal and tumourous pancreas tissues within the same samples for PACAP and PAC1 receptor immunostaining. We observed a diminished expression for both the peptide and its specific receptor in the adenocarcinoma compared to the normal tissue, independent from tumour grade.

Several growth factors play an important role in pancreatic organogenesis and are also involved later in tumourgenesis. Among others, fibroblast growth factor (FGF) has been shown to be involved in the regulation of tumourous cell growth and differentiation in the pancreas (39). FGF receptor IIIb and IIIc play critical roles in the epithelio-mesenchymal transition in

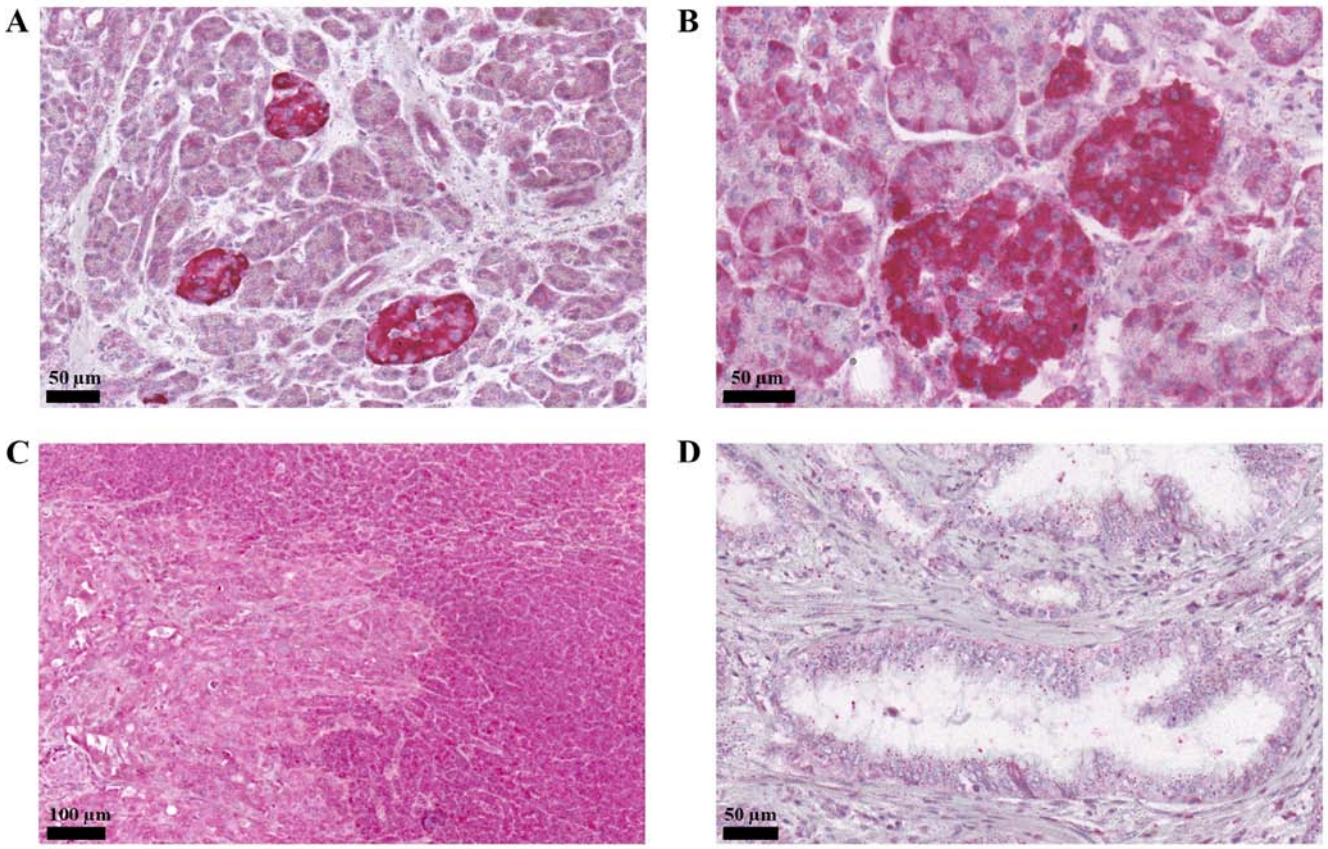

Figure 1. PAC1 receptor immunostaining in normal pancreas and ductal adenocarcinomas. (A and B) Strong staining of the pancreatic islets was observed. In the adenocarcinoma, receptor staining was markedly weaker (left side of C, and D). (C) The border between tumourous and normal pancreas was also shown by the different staining intensity for the PAC1 receptor.

spite of no expression in the ductal cells but showing very high expression levels in the islets (40,41). It has been demonstrated that increased nerve growth factor (NGF) expression correlates with poorer prognosis, increased inflammation and pain (42). The involvement of transforming growth factor beta (TGF beta) is unquestionable in tumour growth, including that of the pancreas (43). Overexpression of epidermal growth factor (EGF) occurs in the majority of ductal adenocarcinomas of the pancreas and is associated with poorer prognosis (44-46). The increased insulin-like growth factor expression has been found to be correlated with increased risk of pancreatic cancer (47). There is a continuous, urgent need for novel diagnostic markers for pancreatic cancer (48). The currently available markers have low sensitivity and specificity. Personalized treatment approaches call for more prognostic and treatment-predictive biomarkers (48-50).

PACAP, as a growth factor, plays an important role in the development of the nervous system and several peripheral organs (51-53). It is not surprising, therefore, that certain tumour types also express alterations in PACAP and/or receptor expression. Certain tumours show overexpression of the PACAP-ergic system, while others lack PACAP signalling. *In vitro* studies have demonstrated that PACAP is able to stimulate or inhibit tumour growth, depending on various factors, such as tumour type, differentiation stage, origin or environmental circumstances (54). For example, PACAP inhibits cell survival in retinoblastoma cells (34), reduces invasiveness in glioblastoma cells (55) and inhibits tumour growth in cervical carcinoma (56). On the other hand, it stimulates cell proliferation in an osteosarcoma cell line (57) and increases the number of viable cells in a colon tumour cell line (58). Even within the same cell line, different effects can be observed depending on exposure time, concentration and other circumstances. This dual effect has been described in a prostate cancer cell line, where short exposure to PACAP induces cell proliferation, while long-term exposure induces proliferation arrest (59). In a human retinoblastoma cell line, nanomolar concentrations of PACAP do not affect cell viability, while higher concentrations decrease cell survival (34).

PACAP/VIP receptors are known to play a leading role in cancer genesis and the VIP/PACAP receptors are expressed in the most frequently occurring human tumours (breast, prostate, ductal carcinoma of the pancreas, lung, colon, stomach, liver, and urinary bladder, lymphomas and meningioma). In these cases the receptors are predominantly VPAC1 type. On the other hand leiomyomas predominantly express VPAC2 receptors, whereas paraganglioma, pheochromocytoma, and endometrial carcinomas preferentially express PAC1 receptors (60). Recent studies have shown that VIP/PACAP-receptor expression can be found in only 65% of pancreatic ductal carcinomas (30). Both VPAC1 and 2 receptors have been identified in pancreatic tumour samples (30). Overexpression of these receptors (61) explains the attempts for the clinical use of radiolabelled VIP-analogues in various cancer types, including pancreas adenocarcinoma (30,62,63). However, contradictory data

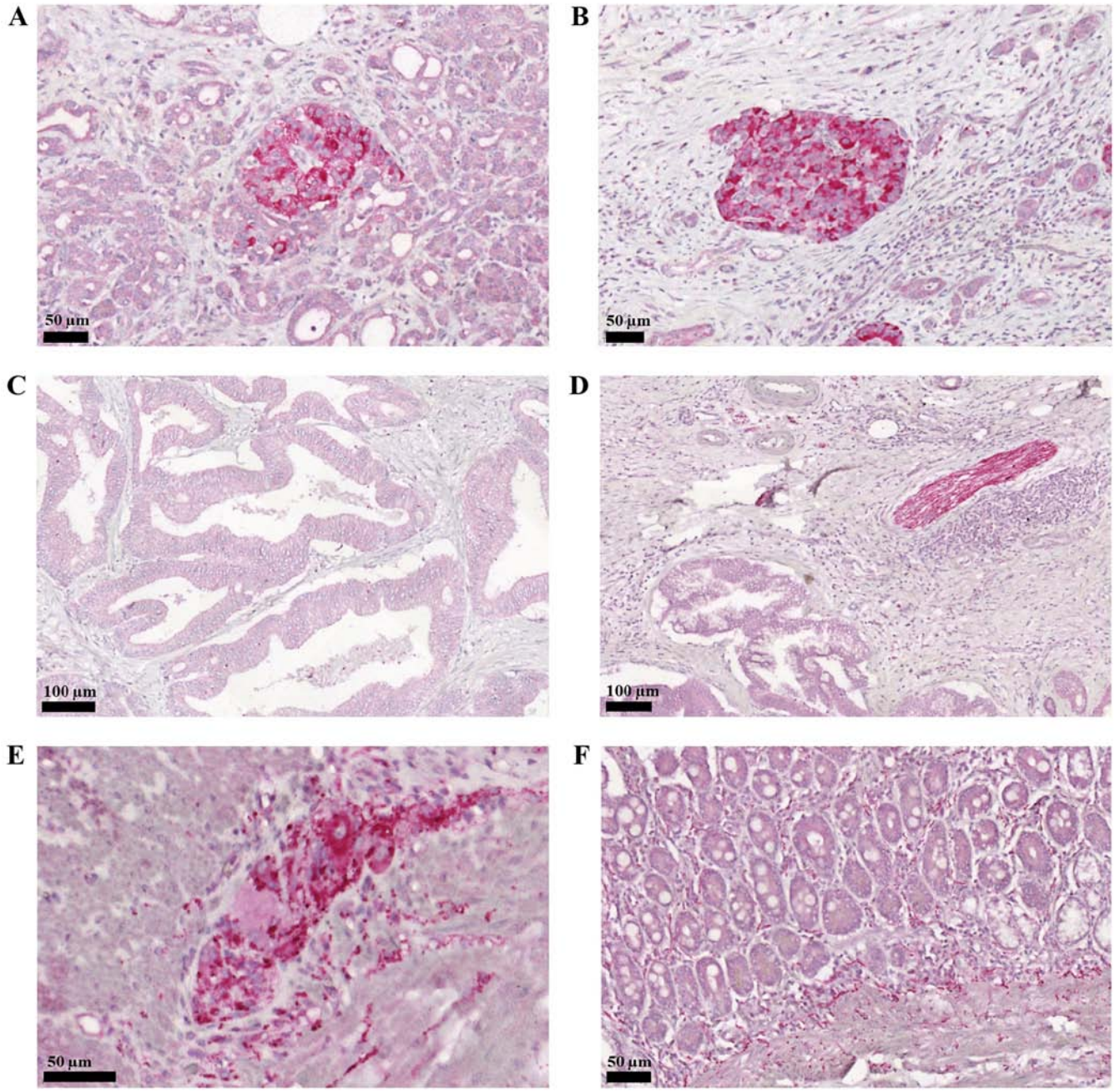

Figure 2. PACAP immunostaining in normal pancreas and ductal adenocarcinomas. (A and B) Strong staining was observed in the endocrine islets. (C) PACAP expression was weaker in the adenocarcinoma parts of the tissue samples. PACAP was also expressed in the (D) intrapancreatic nerves and (E) ganglionic cells. (F) Duodenal lamina propria nerve fibers also stained for PACAP.

have also been published, as according to the observations of Hessenius and coworkers (62), no imaging was seen with radiolabeled-VIP-analogues in pancreatic cancer patients, and *in vitro* binding studies in these tumours did not confirm overexpression of VPAC1.We found PAC1 receptor expression in the exocrine pancreas in nearly all cases, but very weak expression in the tumourous parts. Changes in PACAP expression have been shown in a few tumours by radioimmunoassay and immunohistochemistry (64). In earlier studies, we described lower PACAP tissue levels in lung, kidney and colon cancer, but higher levels in prostate cancer (64,65). A changed staining pattern has been described in different human testicular cancers (38) and in human thyroid papillary carcinoma (37). In the present study we observed that PACAP expression was weak in normal tissues in the exocrine pancreas, and nearly absent in the adenocarcinoma parts of the tissue samples. The limitation of our study is that we cannot draw final conclusion at the moment whether the reduction of PACAP and PAC1 receptor expression is a consequence of the adenocarcinoma development or the reduced PACAP signaling plays a role in pancreatic carcinogenesis. This should be further explored in future studies.

In summary, we found that both PACAP and PAC1 receptor expression is markedly decreased in human pancreatic ductal adenocarcinoma tissue samples, while staining remained strong in the endocrine islets. This suggests that decrease or lack of the PAC1

receptor/PACAP signalling may contribute to tumour growth and/or differentiation, details of which must be further explored.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The patients' data collection was performed by SF, ZV, VV, OK and DK. The histological sections were produced by BK, which were subsequently stained and examined by DR, OK, DT and AB. Figures were produced by DT. The manuscript was written by SF, DR, OK and DK.

Ethics approval and consent to participate

Data collection was permitted by Local Ethic Committee of University Pecs (use of patient data system of the Clinical Centre of University Pecs) (Permission number PTE/83069/2018).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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