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Expression of PACAP and PAC1 Receptor in Normal Human Thyroid Gland and in Thyroid Papillary Carcinoma

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Abstract Pituitary adenylate cyclase activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide-secretin-glucagon peptide family, isolated first from ovine hypothalamus. The diverse physiological effects of PACAP are known mainly from animal experiments, including several actions in endocrine glands. Alteration of PACAP expression has been shown in several tumors, but changes in expression of PACAP and its specific PAC1 receptor in human thyroid gland pathologies have not yet been investigated. Therefore, the aim of the present study was to investigate expression of PACAP and its PAC1 receptor in human thyroid papillary carcinoma, the most common endocrine malignant tumor. PACAP and PAC1 receptor expressions were investigated from thyroid gland samples of patients with papillary carcinomas. The staining intensity of follicular epithelial cells and thyroid colloid of tumor tissue was compared to that of tumor-free tissue in the same thyroid glands in a semi-quantitative way. Our results reveal that both PACAP(-like) and PAC1 receptor(-like) immunoreactivities are altered in papillary carcinoma. Stronger PACAP immunoreactivity was observed in active follicles. Colloidal PACAP immunostaining was either lacking or very weak, and more tumorous cells displayed strong apical immunoreactivity. Regarding PAC1 receptor, cells of the normal thyroid tissue showed strong granular expression, which was lacking in the tumor cells. The cytoplasm of tumor cells displayed weak, minimal staining, while in a few tumor cells we observed strong PAC1 receptor expression. This pattern was similar to that observed in the PACAP expression, but fewer in number. In summary, we showed alteration of PACAP and PAC1 receptor expression in human thyroid papillary carcinoma, indicating that PACAP regulation is disturbed in tumorous tissue of the thyroid gland. The exact role of PACAP in thyroid tumor growth should be further explored.

Keywords Papillary carcinoma · Tumor · Thyroid gland · PACAP · PAC1 receptor

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) with its two forms (PACAP38 and PACAP27) was originally isolated from the hypothalamus as a hypophyseotrop neuropeptide (Miyata et al. 1990). The widespread occurrence in the entire body was later demonstrated, with the highest levels of PACAP expression in the nervous system and endocrine organs (Clason et al. 2016; Girard et al. 2016; Matsumoto et al. 2016; Sandor et al. 2016; Vaudry et al. 2009). PACAP exerts its diverse biological actions on three receptors: the specific PAC1 receptor, binding only PACAP, and the VPAC1/2 receptors, which also bind VIP.

Several endocrine effects of PACAP have been revealed in subsequent studies (Kanasaki et al. 2015a; Koves 2016). PACAP influences the hormone secretion of various endocrine glands and neuroendocrine cells, affects the hypothalamo-hypophyseal system, pineal and adrenal glands, and acts on the gonadal steroid hormone secretion as well as the activity of the pancreatic islets (Bik et al. 2007; Faluhelyi

et al. 2006; Ges et al. 2013; Halvorson 2014; Kanasaki et al. 2015b; Park et al. 2010; Reglodi et al. 2012; Sanlioglu et al. 2012). The occurrence and effects in the thyroid gland are less well known. The distribution of PACAP and its receptors have been studied in the thyroid of various species, as well as the interaction with the hormone secretion and thyroid-mediated actions (Kanno et al. 2005). PACAP stimulates cAMP production in thyroid cells and interacts with the TSH receptors (Chen et al. 1993). VPAC2 receptors have been described in mouse thyroid follicular cells (Harmar et al. 2004), while VPAC1 receptors have been found in human thyroid gland (Reubi 2000). Subsequent studies have confirmed PAC1 and VPAC1 receptor mRNA in follicular cells and blood vessels, whereas low expression of VPAC2 receptor mRNA has also been found (Fahrenkrug and Hannibal 2011). A detailed immunohistochemical mapping of PACAP showed PACAP immunoreactive nerve fibers associated with blood vessels, thyroid follicles and parafollicular (C) cells (Fahrenkrug and Hannibal 2011).

PACAP has been shown to affect the hypothalamo-pituitary-thyroid axis. Contradictory data exist about its effect on TSH secretion. While early studies found no stimulatory effect of PACAP on TSH release from cultured pituitary cells (Culler and Paschall 1991; Hart et al. 1992), later studies have described that PACAP does influence the function of thyreotrophic cells in the pituitary of various species (Gracia-Navarro et al. 1992; Rawlings and Hezareh 1996; Okada et al. 2007). A recent study has shown that intravenous PACAP38 infusion in migraine patients induces TSH (Guo et al. 2016), while no change was found in normal men after infusion (Chiodera et al. 1996). In rats, a reduction was found 60 and 120 min after intracerebroventricular administration (Casperini et al. 2012). PACAP has been found to potentiate the effects of TRH (Kanasaki et al. 2015a, b). We have recently described that PACAP is an endogenous regulator of the hypothalamo-pituitary-thyroid axis by affecting T3-mediated negative feedback via cAMP-induced D2 expression of tanycytes (Egri et al. 2016). In this study, we have found that intracerebroventricular PACAP suppresses TRH and decreases TSH secretion, and knockout mice exhibit hypothyroid phenotype (Egri et al. 2016). In addition, an axis independent thyroxin-stimulating effect has also been described in a rat model of septic shock (Baranowska-Bik et al. 2006). Taken together, most data confirm that PACAP is involved in the regulation of thyroid hormone secretion, but the exact mechanism still needs to be elucidated. Furthermore, it is not known, how expression of PACAP and its receptors changes under pathological conditions of the thyroid gland.

PACAP and its receptors have been implicated in cell proliferation and differentiation both under normal circumstances and in tumorous transformation (Jung et al. 2011; Moody et al. 2003, 2016; Moody and Jensen 2016; Schulz et al. 2004, 2015). Alteration of PACAP expression has been shown in a few tumors by radioimmunoassay and immunohistochemistry (Tamas et al. 2016). Among others, we have found lower PACAP tissue levels in colon, kidney and lung cancer while higher levels were detected in prostate cancer (Szanto et al. 2012; Tamas et al. 2016). A changed staining pattern has been described in different human testicular cancers (Nakamura et al. 2014). Alterations in expression of PACAP and its specific PAC1 receptor in human thyroid gland pathologies has not yet been investigated. The most common endocrine tumor is the thyroid papillary carcinoma, the prevalence of which has doubled in the last 30 years due to increased radioactive exposure (Tronko et al. 2012). Many other factors can be accounted responsible for the continuous global increase of the papillary thyroid carcinoma (Shang et al. 2016). The aim of the present study was to investigate whether there is a change in expression of PACAP and its PAC1 receptor in thyroid papillary carcinoma.

Materials and Methods

PACAP and PAC1 receptor expression was investigated from thyroid gland samples of patients with papillary carcinomas from six patients (one male, five female) with a mean age of 55.33 years (from 41 to 73). The tumor size was between 3 and 36 mm and the clinical stage according to pTNM classification was between pT1a and pT3.

Thyroid tissue of the patients with total or sub-total thyroidectomy because of papillary thyroid carcinoma was processed for histological analysis using 2-μm-thick paraffin embedded sections fixed in 4 % buffered formalin. Sections were stained using standard immunohistochemistry with human anti-PACAP antibody raised in rabbit (Peninsula, CA, USA) in a dilution of 1:200 and with human PAC1 receptor antibody raised in rabbit (Sigma-Aldrich, Budapest, Hungary) in a dilution of 1:200. Immunohistochemical staining was performed with EnVision FLEX Visualization Systems for Dako Omins (Dako, Denmark). Pathological analysis was performed by an expert pathologist. A method control was performed by omitting the primary antiserum, which revealed no staining. The staining intensity of follicular epithelial cells and thyroid colloid of tumor tissue was compared to that of tumor-free tissue in the same thyroid glands in a semi-quantitative way (0: negative, +: weak, ++: medium, +++: strong staining).

Results

Our analysis of thyroid cancer patients shows staining heterogeneity in both tumorous and non-tumorous thyroid tissues. Colloid was stained in all normal parenchyma (with intensity: ++, +++) (Figs. 1, 2, 3, and 4). Inactive follicular cells (flat cells) showed markedly weaker intensity (with intensity 0 − +)

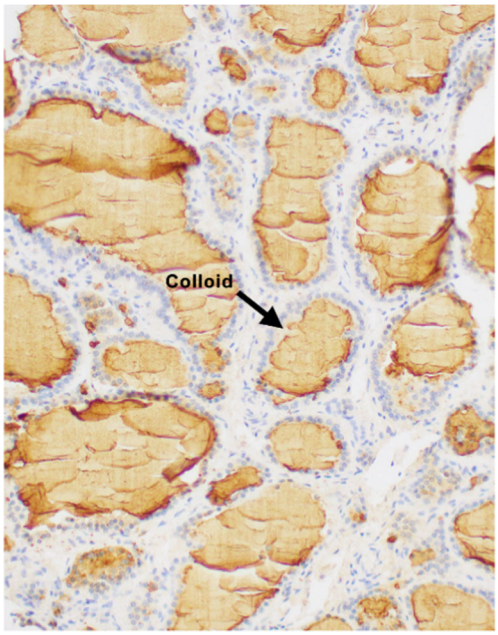

Fig. 1 Normal parenchyma without epithelial staining (*arrow*). Magnification: ×130

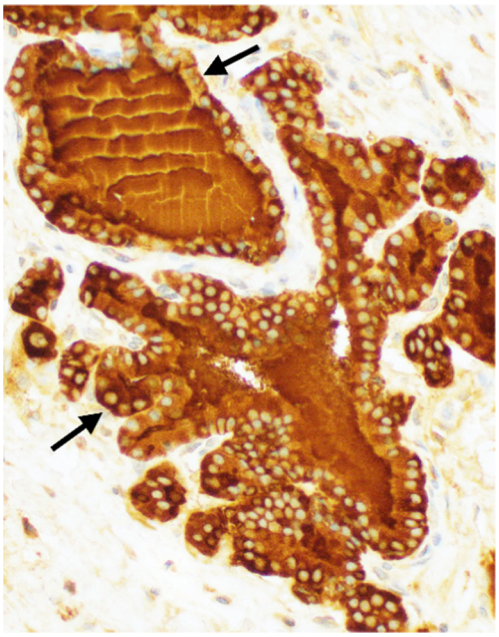

Fig. 3 Normal parenchyma with stronger staining of secreting cells (*arrows*). Magnification: ×270

(Figs. 1 and 2), while medium and strong intracytoplasmic staining intensity (++, +++) was observed in cuboidal and to columnar cells of active follicles (Figs. 3 and 4). In two cases, an intracytoplasmic granular staining pattern could be observed (Fig. 4).

In tumors, the colloid was less well stained, or staining was missing. When positive, staining was observed on the apical surface of tumorous cells, as a thin strong immunopositive layer (Fig. 5). The number of positive cells with stronger expression of PACAP was generally higher in the tumorous tissue compared to the normal thyroid gland (Figs. 6 and 7). In many cells, the intracytoplasmic staining showed apically stronger intensity (Fig. 8). The number of interpositioned cells with strong staining was higher in tumorous tissue (Figs. 9 and 10). We found no correlation between PACAP expression pattern and tumor stages, size or the age of the patient.

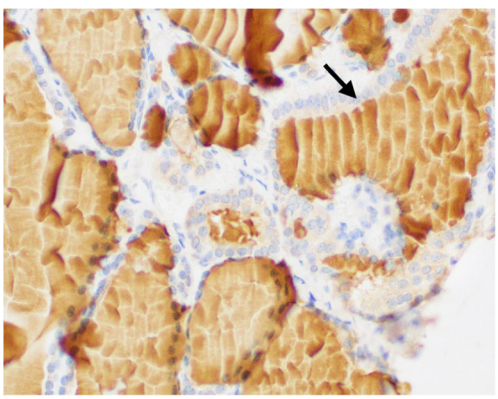

Fig. 2 Normal parenchyma with colloid staining (*arrow*). Magnification: ×300

PAC1 receptor expression also showed alteration in papillary cancer compared to normal tissue. Cells of the normal thyroid gland showed strong granular expression, which was lacking in the tumor cells (Fig. 11). The cytoplasm of tumor cells displayed weak, minimal staining (Figs. 11, 12, and 13). In a few tumor cells, we observed strong PAC1 receptor expression. This pattern was similar to that observed in the PACAP expression, but fewer in number (Figs. 11 and 12).

Discussion

In the present study, we analyzed normal and tumorous thyroid tissues within the same samples. We observed an altered expression intensity and pattern of both PACAP and its

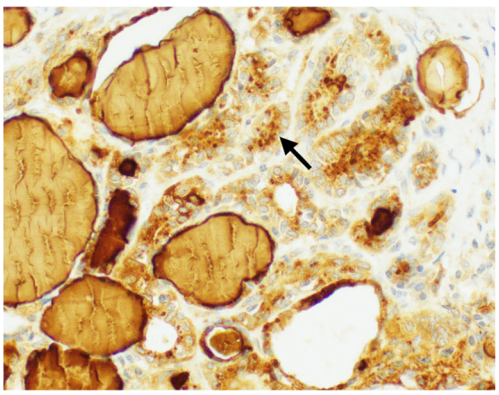

Fig. 4 Normal parenchyma with granular staining (*arrow*). Magnification: ×270

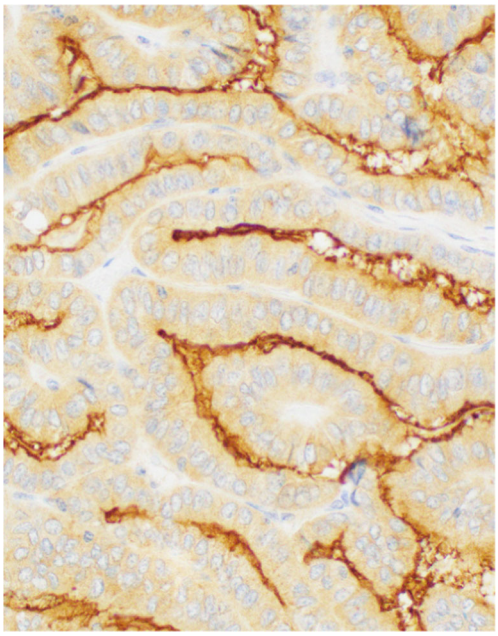

Fig. 5 Thin immunopositive layer on the apical surface of the tumor cells. Magnification: ×300

specific PAC1 receptor immunoreactivity, suggesting a functional significance of PACAP in thyroid tumor growth.

Papillary thyroid carcinoma accounts for the vast majority (85 %) of all malignant thyroid neoplasms. It is defined as an epithelial tumor showing evidence of papillary differentiation and it is characterized by distinctive nuclear features (Lloyd et al. 2011). Alteration in several growth factors and/or their receptors have already been reported in thyroid papillary carcinomas. For instance, it has been shown that papillary thyroid carcinoma expresses the GLP-1 (glucagon-like peptide-1) receptor and it is negatively correlated with tumor multifocality (Jung and Kwon 2014). The presence of IGF-I (insulin-like growth factor-1) receptors was also demonstrated in normal and neoplastic tissues of human thyroid gland, where the specific binding of radioactive IGF-1 in thyroid cancer tissues was significantly higher than in surrounding normal tissues. This

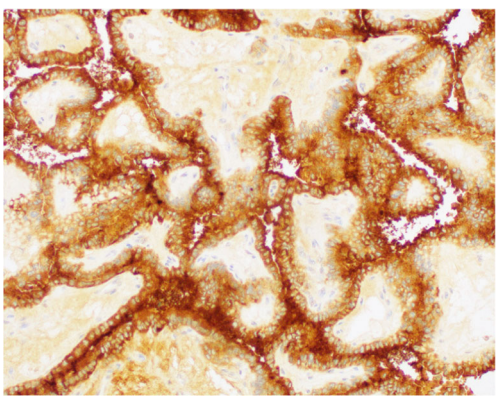

Fig. 6 Pronounced expression of PACAP in the tumor cells. Magnification: ×270

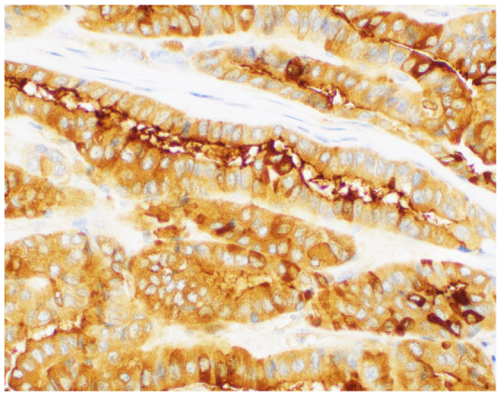

Fig. 7 Strong PACAP expression in the tumor cells. Magnification: ×330

could indicate a possible role of IGF-I and its receptors in the growth of thyroid cancer (Yashiro et al. 1989). It is also well documented that EGF (epidermal growth factor) receptors play an important role in the growth and differentiation of papillary carcinoma of the thyroid gland. EGF stimulates the DNA synthesis and proliferation of the thyroid cells and therefore has eventually an important role in the regulatory mechanism of normal and neoplastic thyroid cell growth. Previously, it has been shown that the binding of EGF is higher in thyroid neoplasms than in normal thyroid tissue. The binding characteristics also shows a positive correlation with a poorer tumor prognosis (Duh et al. 1985). Furthermore, the amount of EGF receptors which are found in the tumorous tissue is significantly higher than in the adjacent normal tissue (Mäkinen et al. 1988). Later, it has additionally been documented that neoplastic thyroid tissues not only have higher EGF binding than other

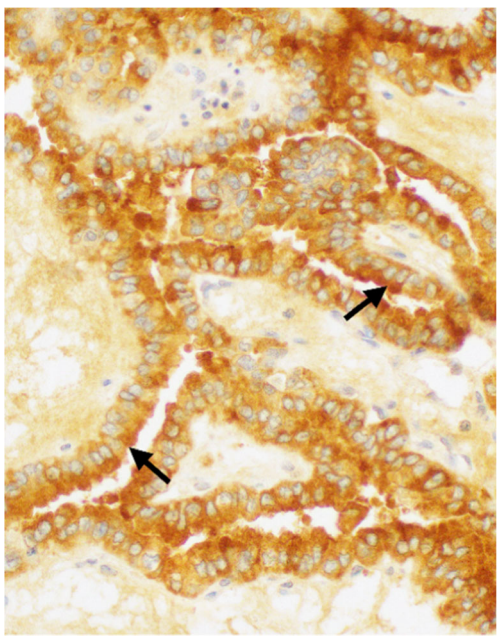

Fig. 8 Strong apical intracytoplasmic staining in the tumor cells (*arrows*). Magnification: ×270

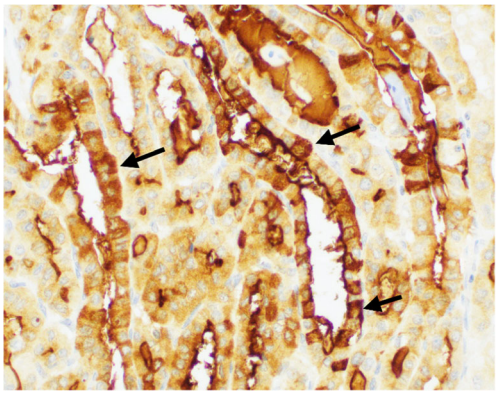

Fig. 9 Wedged tumor cells with strong staining (*arrows*). Magnification: ×300

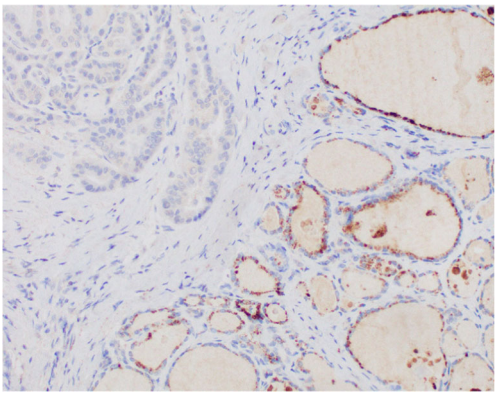

Fig. 11 PAC1-receptor staining in the tumor cells (*top left*) and in normal tissue (*right side*). Magnification: ×230

thyroid tissues, but there is also a significant correlation between EGF and TSH binding, and between EGF and TSH-induced adenylate cyclase activity (Duh et al. 1990). All these growth factors and several others have been proposed to play a role in thyroid tumor growth. Similar to EGF, PACAP also stimulates adenylate cyclase activity, although its role in thyroid tumor growth is not known. Elevation of cyclic AMP has been shown to be associated with suppressed papillary tumor growth in thyroid cell cultures (Matsumoto et al. 2008), but desensitization of the adenylate cyclase-induced activation and no effect of cyclic AMP stimulators or inhibitors on thyroid tumor growth have also been reported (Hölting et al. 1995; Tezelman et al. 1996). Earlier studies have shown that VIP, the peptide with the closest structural homology to PACAP, plays a physiologically important role in the regulation of the secretion and growth of normal and neoplastic thyroid tissues (Siperstein et al. 1988).

Recent human studies revealed that dysregulation of neuropeptides may play an important role in pathological processes, including age-related conditions, cognitive decline, inflammatory processes, and tumor growth (Moody et al. 2015; Ogren et al. 2010; Padua et al. 2016). PACAP is a very potent cytoprotective and trophic peptide influencing differentiation and growth of various tissues (Vaudry et al. 2009). In tumor cells, the peptide can exert very different actions depending on the cells of origin. In many types of tumor cells, PACAP has protective effects, and thus, it promotes survival and proliferation of tumors similarly to most normal tissue types. Such effects have been described in lung and prostate cancer cells (Gutierrez-Canas et al. 2003; Zia et al. 1995) as well as in pituitary adenomas (Oka et al. 1999). However, opposite effects have also been found in some types of malignant tumor cell lines. For example, PACAP exerts cytotoxic effects in Y79 retinoblastoma cells (Wojcieszak and Zawilska 2014) and leukemic myeloid cells (Hayez et al. 2004). Even in the same tumor type, contradictory results have been reported PACAP stimulates proliferation, but inhibits migration of glioblastoma cells (Cochaud et al. 2010; Dufes et al. 2003; Maugeri et al. 2016), while antiproliferative effects have also

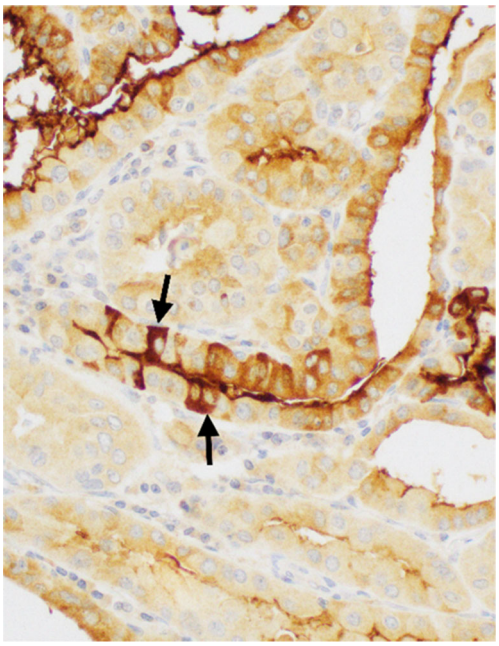

Fig. 10 Wedged tumor cells with strong staining (*arrows*). Magnification: ×300

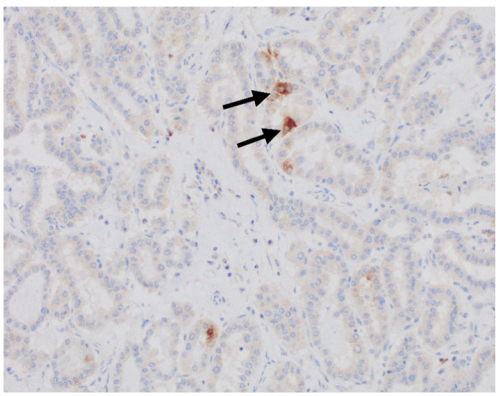

Fig. 12 Some tumor cells with strong PAC1-receptor staining (*arrows*). Magnification: ×230

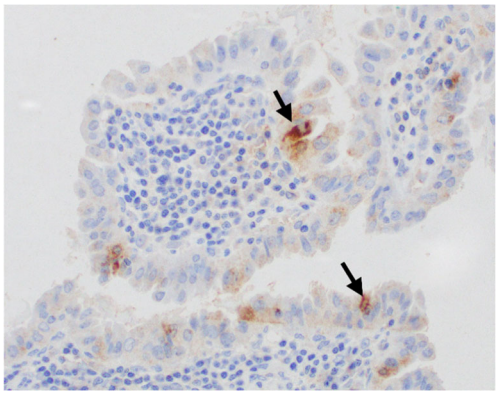

Fig. 13 Some tumor cells with strong PAC1-receptor staining (*arrows*). Other cells show minimal "dust-like" reaction. Magnification: ×300

been found (Sharma et al. 2001; Vertongen et al. 1996). Furthermore, PACAP deficient mice develop colon cancer after induced colitis in contrast to wild type mice (Nemetz et al. 2008). These effects have recently been reviewed by Moody and coworkers (Moody et al. 2016). The overexpression of the VIP and PACAP receptors have been reported in several tumor types (Moody et al. 2016; Schulz et al. 2004). The altered expression pattern of the peptide and the changes in receptor expression in different tumors may show correlation with tumor malignancy, such as it has recently been demonstrated in testicular cancer, where PACAP immunoreactivity displayed a different pattern in seminomas and embryonic cancer (Nakamura et al. 2014). The use of the diagnostic value of PACAP is hindered by the lack of commercially available highly selective diagnostic methods for determining PACAP expression and/or tissue concentration (Reglodi et al. 2016). However, recent studies have highlighted the possibility of using PACAP as a biomarker in several conditions, where the levels of PACAP-like immunoreactivity show correlation with the clinical stages and/or prognosis of the disease (Bukovics et al. 2014; Han et al. 2015; Ressler et al. 2011; Tajti et al. 2015). Therefore, it is important to map alteration of PACAP(-like) expression and that of its receptors in different pathological conditions.

Based on the present results, we can conclude that expression and distribution of both PACAP and its specific PAC1 receptor is altered in thyroid papillary cancer, indicating that PACAP regulation is disturbed in tumorous tissue of the thyroid gland. Whether PACAP plays a direct role in tumor growth (stimulation or suppression) or the altered expression pattern is a secondary consequence of the disturbed regulation of tissue growth and differentiation, is not known at the moment. However, based on the data indicating the physiological effects and alterations under pathological conditions it is suggested that the exact role and functional significance of PACAP in thyroid tumor growth should be further explored.

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