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Catestatin-A Novel Neuropeptide in Carcinoid Tumors of the Appendix

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Abstract. *Background: The aim of the study was to investigate the immunohistochemical distribution of the novel neuropeptide catestatin in carcinoid tumors. Catestatin, a novel 21 amino acid neuropeptide derived from chromogranin A, was determined immunohistochemically in 30 carcinoid tumors of the appendix and various carcinoid tumors of other localities. Materials and Methods: Paraffin-embedded tissues from 30 carcinoid tumors of the appendix and 16 other carcinoid tumors (5 bronchus-, 5 stomach-, 2 small bowel-, 4 large bowel carcinoid tumors) were incubated with antibodies specific for catestatin, chromogranin A and chromogranin B. Immunohistochemical staining of catestatin was compared to staining with chromogranin A and B. Western blot analysis was performed in one patient with ileal carcinoid. Results: Thirty patients (20 women, 10 men) with carcinoid tumors of the appendix and 16 patients with other localized carcinoid tumors were investigated. Twenty-six of the appendiceal tumors were localized in the apex of the appendix and 4 tumors in the midportion; none of the tumors was localized at the base of the appendix. Median tumor diameter was 10.7mm (range 4-18mm). Immunoreactivity to catesatatin was positive in 28 patients (negative in 2, 0-10% in 11 patients, 11-50% in 14 patients, 51-100% in 3 patients). In 16 patients with carcinoid tumors in various other localizations, catestatin was also expressed. Western blot analysis of ileal carcinoid showed abundant catestatin reactivity with accelerated processing of chromogranin A in the tumor tissue. Conclusion: Catestatin derived from chromogranin A, which is the most widely distributed marker of neuroendocrine tumors, is expressed in high frequency in carcinoid tumors of the appendix (93.3%). Catestatin also was expressed in other carcinoid tumors. This is the first investigation of catestatin in carcinoid tumors demonstrating its possible utilization as a tumor marker.*

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Chromogranin A (CgA), the major soluble protein in the core of catecholamine storage vesicles, is a ubiquitous component of secretory vesicles throughout the neuroendocrine system (1). The recently characterized neuropeptide catestatin cleaved from CgA (bovine CgA 344-364) was first described by Mahata *et al*. in 1997 (2). It consists of 21 amino acids and inhibits catecholamine release as a nicotinic cholinergic antagonist. Other functioning peptide sequences of CgA are pancreastatin (porcine CgA 240-288), which impairs glucose tolerance by inhibiting glucose-stimulated insulin release from pancreatic islet beta cells (3), vasostatin (human CgA 1-76), which acts as a vascular smooth muscle dilatating agent (4) and parastatin (porcine CgA 347-419), which inhibits hormone secretion by parathyroid chief cells (5). CgA is widely distributed in neuronal and neuroendocrine tissue and is, therefore, the most important marker for neuroendocrine tumors (6,7). The aim of the immunohistochemical study was to investigate whether this novel neuropeptide is expressed in carcinoid tumors of the appendix and carcinoid tumors in other localizations.

Carcinoid tumors of the appendix are the most frequent of the carcinoid tumors but fortunately the ones with the best prognosis. These tumors are seldom diagnosed preoperatively, because symptoms are usually not tumor-related. Appendectomy is the therapy of choice. Prognosis of appendiceal carcinoids is excellent in general and dependent linearily on the diameter of the tumor. The metastatic potential of carcinoid tumors of the appendix is low when the tumor diameter is smaller than 2 cm. Good prognosis of appendiceal carcinoids may also be caused by the circumstance that the whole organ bearing a small tumor is removed surgically. Tumor cells of appendiceal carcinoids are derived from subepithelial endocrine cells, which are different to tumor cells of other localized

carcinoid tumors which are derived from epithelial endocrine cells (8). Right hemicolectomy is only suggested for tumors with a diameter of more than two centimeters or in case of tumor infiltration of the base of the appendix.

Materials and Methods

Formalin-fixed paraffin-embedded tissues from 30 carcinoids of the appendix and 16 various carcinoid tumors were retrieved from the files of the Department of Pathology, University of Innsbruck, Austria. The appendiceal tumors were reclassified according to the criteria outlined by Burke *et al.* (9).

Immunohistochemistry. The routinely processed tumor samples were formalin-fixed (4%) and paraffin-embedded. Five-mm sections on poly-L-lysine-coated slides were used after drying for 10h at 56˚C. After dewaxing and peroxidase blocking, slides were washed in Tris buffer (pH: 6.5) for 10 min (20˚C). The immunoreactions were performed by incubation with biotinylated swine-anti-catestatin antibody at 20˚C, followed by incubation with biotinylated swine-anti-rabbit antibody (1:500, 30 min, 20˚C, DAKO, Denmark, E0353). Then slides were incubated for 30 min with streptavidine 1:800 at 20˚C (DAKO P0397). Finally the tissue slides were developed in DAB and counterstained with hemalum.

Antiserum to catestatin. Polyclonal rabbit antisera recognizing the catestatin region of bovine $CgA_{344-364}$(RSMRLSFRARGYGFR GPGGLQL) was developed by a modification of protocols previously described for other chromogranin peptides (10,11).

Antiserum to chromogranin A. An anti-chromogranin A antibody was purchased from BioGenex, San Ramon, CA, USA.

Antiserum to chromogranin B. Preparation of the rabbit polyclonal antibody against a synthetic peptide of chromogranin B was described previously (12).

Antiserum to CD31. A monoclonal anti-CD31 antibody was purchased from Dako, Copenhagen, Denmark.

Analysis of immunohistochemistry. The relative intensity of staining in comparison to the positive control was assessed. Staining intensity was classified into 3 groups: grade I: 1-10%, grade II: 11-50%, grade III: 51-100% of control.

Positive control was performed on sections of adrenal medulla, while negative control was performed by omitting the primary antiserum.

Western blot. An extract of tumor tissue was prepared by homogenizing the tissue in boiling lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4 and protease cocktail inhibitors) using a tissumizer. The extract was microwaved for 15 sec and centrifuged at 6100 x g for 10 min to settle the debris. The supernatant was transferred to a fresh tube. Samples were diluted 1:10 and the protein concentration determined using Bradford's reagent. Ten mg of each sample were subjected to electrophoresis. NuPAGE Novex Bis-Tris gels with MES SDS running buffer were used to electrophorese reduced samples according to the manufacturer‘s instructions.

Table I. *Catestatin staining of 16 carcinoid tumors in various other localities.*

Localization of carcinoid	no staining	grade 1	grade 2
Bronchus			
typical carcinoid	+		
typical carcinoid	+		
atypical carcinoid	+		
atypical carcinoid		+	
atypical carcinoid			+
Stomach			
Type I carcinoid	+		
Type I carcinoid		+	
Type I carcinoid			+
Type III carcinoid			+
Type III carcinoid			+
Small bowel			
Ileum			+
Ileum			+
Large bowel			
Rectal carcinoid	+		
Liver metatasis of rectal	+		
Rectal carcinoid		+	
Sigmoid carcinoid	+		

Samples were also analyzed by 2-dimensional gel electrophoresis. Ready-made immobiline pH gradient (IPG) dry strip gels (Amersham Pharmacia Biotech, Piscataway, NJ, USA) with linear pH gradients 3-10 were used to resolve proteins by isoelectric focusing (IEF). IPG strips were rehydrated for 18 h prior to IEF (500V, 1h; 1000V, 1h, 8000V, 3h). At the end of the run, the IPG strips were equilibrated with SDS buffer system in the presence of DTT and iodoacetamide. In the second dimension, samples were electrophoresed using the NuPAGE system described above. At the end of electrophoresis, gels were transferred to nitrocellulose membranes according to the manufacturer's instructions using Xcell II™ blot module (Invitrogen, Carlsbad, CA, USA). Western blot analysis was done using primary polyclonal antibodies raised against human catestatin and goat anti-rabbit IgG conjugated to horseradish peroxidase. Immunodetection was by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Results

Specimens from 30 patients with the diagnosis of appendiceal carcinoid tumor and specimens of 16 carcinoids of various localities were investigated. In appendiceal carcinoids there were 20 women and 10 men. For appendix carcinoids the specimens were reclassified by the pathologist (EC) according to the criteria outlined by Burke *et al.* (9). Twenty three patients had classical

carcinoid tumor of the appendix, 6 were of tubular type and 2 patients had goblet cell carcinoid. In 22 specimens, infiltration of the mesoappendix was present. Vascular invasion was suspected light microscopically in 25 patients, but was excluded after incubation with CD31 antibody in all patients. In 6 patients serosal involvement was detected by light microscopy. Twenty-six tumors were localized at the tip of the appendix, 4 in the midportion and none at the base of the appendix. Median tumor diameter was 10.7 mm (range: 4-18 mm). Twelve patients had concommittant severe appendicitis, but none of the patients presented with perforated appendicitis. Immunoreactivity to catestatin in appendix carcinoids was positive in a high proportion (28 of 30 patients), while only 2 patients showed absolutely no immunoreactivity to catestatin (Figure 1). Immunoreactivity to CgA was also positive in 28 patients and negative in 2. One of these chromogranin-negative patients was also negative for catestatin staining, but one of the chromogranin-negative patients was positive for catestatin. All patients with mesoappendiceal infiltration showed positivity of catestatin staining (7 grade I, 12 grade II, 3 grade III).

The 16 specimens of carcinoid tumors in other localities (5 bronchus-, 5 gastric-, 2 small bowel-, 4 large bowel carcinoids) also showed immunoreactivity to catestatin (Table I, Figure 2). In normal crypt cells there was very poor staining of catestatin (Figure 3). Western blot analysis of an ileal carcinoid tumor showed abundant immunoreactivity of catestatin in the tumor tissue compared to the control tissue of normal bowel (Figure 4).

Discussion

Chromogranin A (CgA) is a highly acidic secreteory protein which was first detected in catecholamine storage vesicles of the adrenal medulla (13,14). It belongs to the chromogranin/secretogranin family which also includes chromogranin B and secretogranin II (15). CgA consists of 431 amino acids and is encoded by eight exons. The structure of this protein has 8-10 conserved dibasic sites which are potential cleavage sites. Catestatin was identified by Mahata *et al.* by using CgA peptide sequences and by testing the inhibiting efficacy of catecholamine release from PC12 cells (2,16). $CgA_{344-364}$ was the active domain which inhibited catecholamine release in IC_{50}s in the range of 200-400nM (2,17).

Carcinoid tumors as neuroendocrine tumors produce many different neuropeptides like serotonin, chromogranins (chromogranin A, B, C and secretoneurin, 18), tachykinins (substance P, neurokinin *etc.*), bradykinin, kallikrein and ACTH, CRF, ADH, histamine, gastrin and somatostatin as parathyroid hormone-like peptide in foregut carcinoids and glucagon in hindgut carcinoids (20). Except flushing in substance P-secreting ileal carcinoids, there exist no correlations of special peptide patterns to certain clinical behaviors of carcinoid tumors. However, elevated chromogranin correlates with poorer outcome and/or the existence of liver metastases (21). In hormone-producing neuroendocrine tumors, the hormone itself and the endocrine symptoms produced by the hormone are markers for disease progression. In non-functioning neuroendocrine tumors, markers like CgA or pancreatic polypeptide are useful for immunohistochemical diagnosis of these tumors (22). Carcinoid syndrome with symptoms like flushing, diarrhoea, right heart failure and sometimes bronchial constriction occurs due to elevated serotonin plasma levels.

The prognosis of appendix carcinoids is generally excellent. Five-year survival for patients with disease limited to the appendix is 94% (23). The most important prognostic parameter is tumor size at diagnosis. More than 95% of carcinoid tumors are less than 2 cm in diameter (23, 24).Thirlby (19) presented 46 metastasizing carcinoid tumors of the appendix and only 5 of these tumors had a tumor diameter less than 2 cm. In 1974, as a special type the so-called goblet cell carcinoid was described, which shows a dual endocrine and glandular differentiation (9). Histologically these tumors show mucus producing cells. In our series there were two goblet cell carcinoids which are known to have a worse outcome as compared to classical carcinoids (22). Both goblet cell carcinoid tumors were immunoreactive for catestatin.

At the moment CgA is considered to be the best diagnostic and therapeutic marker for neuroendocrine tumors, especially if there is no hormone secretion. CgA is increased in 50-100% of patients with various endocrine tumors and correlates with tumor load (22). Even in patients with MEN I-syndrome, CgA was elevated in all patients with small, but radiologically visible tumors and in 60% with biochemically unequivocal but radiologically not visible tumors (25). Except for the high correlation of catestatin expression and mesoappendiceal tumor infiltration, we were not able to correlate a certain tumor feature with a specific neuropeptide pattern. Catestatin was also expressed in high frequency in various carcinoid tumors. Ileal carcinoids and gastric type III tumors showed very abundant and intense staining of catestatin, while only one hindgut carcinoid showed grade I staining. In Western blot analysis a high processing rate of chromogranin A in tumor tissue compared to control tissue was remarkable. The underlying immunohistochemical investigations demonstrate that catestatin, as a cleavage product of chromogranin A, is richly expressed in carcinoid tumors of the appendix, therefore catestatin can be used for imunnohistochemical confirmation of appendiceal carcinoid tumors.

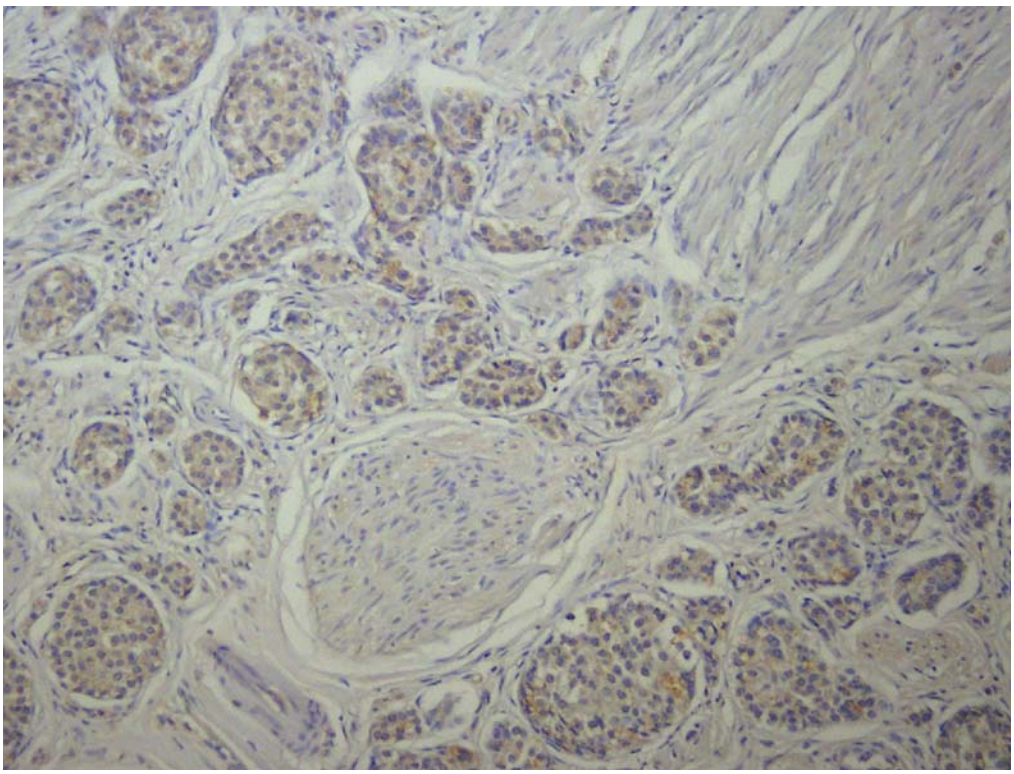

Figure 1. *Immunohistochemical staining with antiserum specific for catestatin of a classical carcinoid tumor of the appendix.*

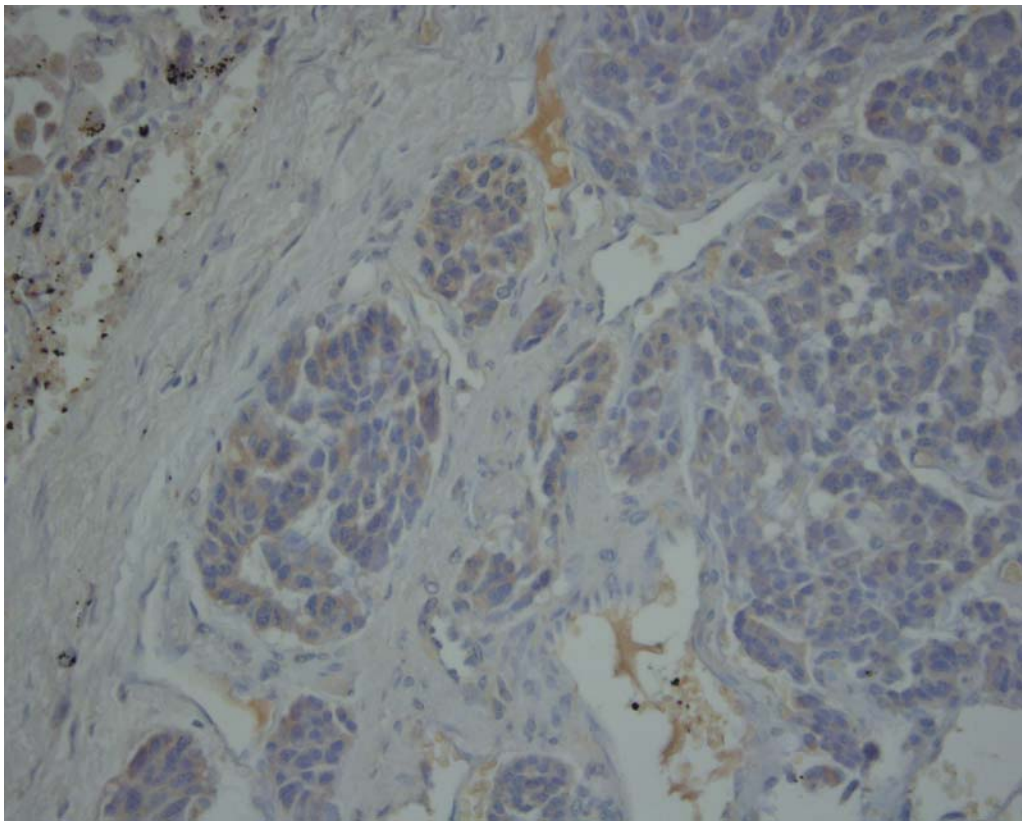

Figure 2. *Catestatin staining in atypical bronchial carcinoid tumor is shown.*

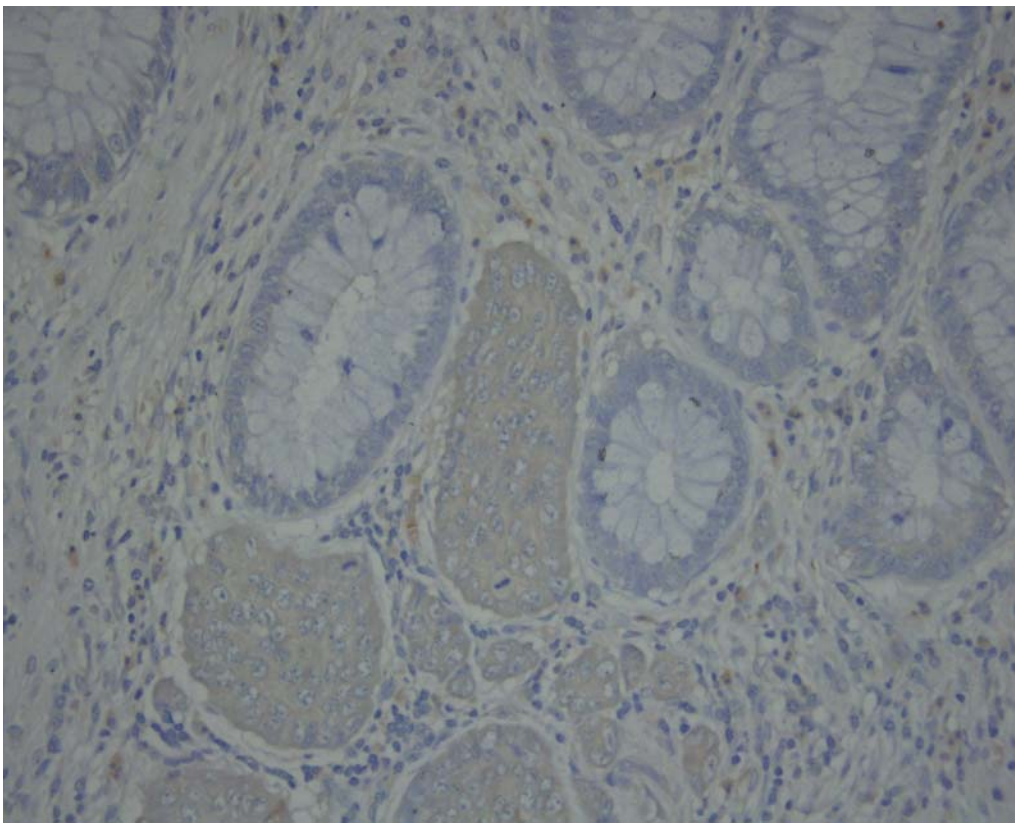

Figure 3. *Catestatin staining of ileum carcinoid with adjacent low staining of crypts of not affected mucosa.*

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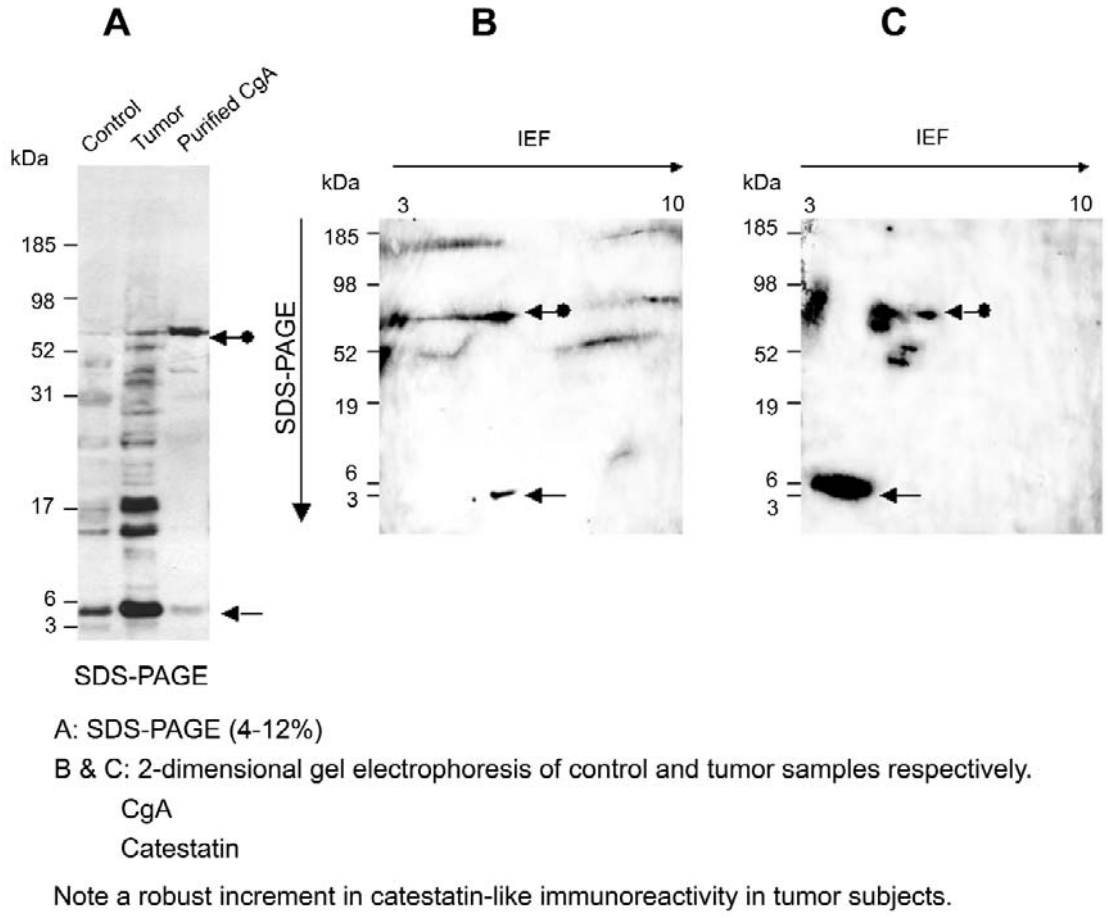

Figure 4. *Western blot analysis of ileal carcinoid tumor.*

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