Tokai J Exp Clin Med., Vol. 24, No. 1, pp.13-20, 1999

Immunohistochemical Expressions of Prohormone Convertase (PC) 1/3 and PC2 in Carcinoids of Various Organs

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(Received November 24, 1998; Accepted December 10, 1998)

In order to clarify the expression of prohormone convertase (PC) 1/3 and PC2 in various carcinoids and non-carcinoid endocrine tumors, we performed indirect immunoperoxidase staining on total of 19 cases of carcinoids (9 cases of bronchial carcinoids, 4 cases of rectal carcinoids, 4 cases of gastric carcinoids and 2 cases of bile duct carcinoids). Our study also included 7 non-carcinoid endocrine tumors. Seventy-nine% and 26% of carcinoids highly or strongly expressed positive staining for PC1/3 and PC2, respectively. High and strong expressions (3+ or 4+) of both PC1/3 and PC2 were noted in only bronchial carcinoids. Strong expressions for only PC1/3 were noted in rectal carcinoids. Bile duct carcinoids also demonstrated higher expressions of PC1/3 than those of PC2. These results suggested that high expressions of both PC1/3 and PC2 in bronchial carcinoids might reflect their diverse and frequent peptide production. The expressions of PC1/3 mRNA and PC2 mRNA detected by in situ hybridization in the bronchial carcinoids and rectal carcinoids were correlated with immunoexpressions of both of the antigens. The granular immunoexpression pattern of PC1/3 and PC2 visualized by confocal laser scanning microscopy would suggest the site of post-translational processing in the secretory granules. Non-carcinoid endocrine tumors showed low expressions (+ or 2+) of PC1/3 and PC2, except for thyroid medullary carcinoma showing high immunoexpression of PC1/3. Other non-carcinoid endocrine tumors (parathyroid adenomas and adrenal pheochromocytomas) revealed low immunoexpressions for both PC1/3 and PC2.

Key words : Prohormone Convertase (PC), Carcinoid, Endocrine tumor

INTRODUCTION

It has been well known that peptide hormones are generally produced as larger prohormone molecules which undergo intracellular proteolytic digestion designated as post-translational processing. The proteolytic enzymes are represented by PC1/3, PC2, PC4, PC5, PC6 and PC7. Among them, PC1/3 and PC2 are mainly involved in the processing of peptide prohormones such as proopiomelanocortin (See Fig. 1). PC1/3 and PC2 belong to the KEX2 family which usually digest proteins at the pair of dibasic amino acids, i.e. Arg-Arg or Lys-Arg.

Immunohistochemical studies for PC1/3 and PC2 have been done by several investigators (1-3) on various endocrine tumors including carcinoids, pancreatic islet cell tumors, and pituitary adenomas. Lloyd et al. (3) especially emphasized the roles of PC1/3

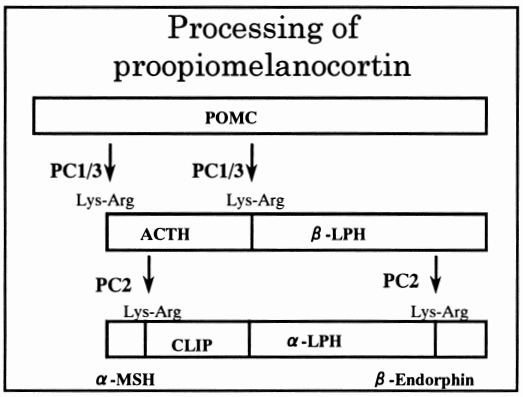

Fig. 1 Proopiomelanocortin is processed by PC1/3 and PC2

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and PC2 as neuroendocrine markers including chromogranin A and synaptophysin. Among various neuroendocrine tumors, carcinoids are known as the tumors occurring in the diffuse neuroendocrine system (4) and produce various neuropeptides. The diversity of peptide production in carcinoids depends roughly on the histogenetic tissues. i.e. foregut, midgut and hindgut. Bronchial carcinoids of foregut origin have been reported to produce the diverse peptides (5). Our immunohistochemical study was aimed at elucidating the pattern of PC expressions according to histogenetic background and types of carcinoid and further speculating the role and clinical complication of PC1/3 and PC2 in the functioning expression of carcinoids. For comparison, this study also included non-carcinoid endocrine tumors which were composed of 2 pheochromocytomas, a medullary carcinoma of thyroid and 4 parathyroid adenomas.

Table 1 Presented cases of various carcinoids and and non-carcinoid endocrine tumors

Case No	Diagnosis	age (years)	sex
1	BRC	49	M
2	BRC	53	M
3	BRC	43	M
4	BRC	68	F
5	BRC	22	F
6	BRC	72	M
7	BRC	28	F
8	BRC	72	F
9	BRC	46	M
10	RC	37	M
11	RC	50	M
12	RC	59	M
13	RC	60	M
14	GC	49	F
15	GC	63	M
16	GC	50	F
17	GC	42	F
18	BDC	63	M
19	BDC	56	M
20	PAA	26	M
21	PAA	17	M
22	PAA	38	F
23	PAA	73	M
24	PHA	71	F
25	PHA	51	M
26	MCT	66	F

BRC : Bronchial carcinoid, RC : Rectal carcinoid,
GC : Gastric carcinoid, BDC : Bile duct carcinoid,
PAA : Parathyroid adenoma, PHA : Pheochromocytoma,
MCT : Medullary carcinoma of thyroid

MATERIAL AND METHODS

Tumor samples

All cases studied are presented in Table 1. Total 19 cases of carcinoids (bronchial, 9 cases; gastric, 4 cases; bile duct, 2 cases; and rectal, 4 cases) and 9 cases of non-carcinoid endocrine tumors (2 adrenal pheochromocytomas, a medullary carcinoma of thyroid: MCT and 4 parathyroid adenomas) were subjected to the following immunohistochemical studies. ISH was also performed for the rectal carcinoids and bronchial carcinoids.

Immunohistochemistry

The tissue fragments were fixed in 10% formalin and embedded in paraffin. Deparaffinized sections were stained by indirect immunoperoxidase method. Rabbit polyclonal antibodies to PC1/3 (ST-28) and PC2 (ST-29), which were kindly supplied by Dr. S. Tanaka, were used at a 1: 1000 dilution. The specificities of anti-PC1/3 antibody and anti-PC2 antibody have been reported previously in our studies on the pancreas (2). The sites of antigen-antibody reaction were visualized by DAB (3, 3'- diaminobenzidine tetrahydrochloride). For negative control study, sections were incubated in the absence of the primary antibodies.

Non-radioisotopic in situ hybridization (ISH), technique

ISH was performed according to the method reported previously (6). In brief, paraffin sections were attached to 3'- aminopropyl triethoxysilane-coated glass slides. The sequences of the oligonucleotide probes (30 mers) for PC1/3 and PC2 are shown in Table 2 (after the method of Dr. RV Lloyd) (3). The oligonucleotides were synthesized and then biotinylated by the 3'- end-labeling method using the terminal labeling kit (Enzo Biochem., New York, USA).

Paraffin-embedded specimens were sectioned at 4 μm in thick, deparaffinized, rinsed in 2x sodium citrate (SSC), and treated with 0.2N HC1 at room temperature (RT) for 20 min. They were then treated with 10 μg/mL proteinase K at 37℃ for 30 min, followed by treatment with 0.25% acetic anhydride in 0.1mol/L triethanolamine at

Table 2 Oligonucleotide probes used for the detection of PC1/3 mRNA and PC2 mRNA

probe	Sequence
PC1/3	AGA GGG GTG GAG AAG ATG GTG GAT CCA GGG
PC2	AGA GTG CCC CGT ACA TCG ACC AGG TGG TGC

(after Dr. Lloyd)

RT. The slides were then rinsed in 2x SSC and prehybridized at 37℃ for 30 min. The hybridization solution was comprised of 10% dextran sulfate, 3x SSC, 1x Denhardt's solution (0.02% Ficoll. and 0.02% bovine serum albumin), and 0.02% polyvinylpyrrolidone), 100 μg/mL salmon sperm DNA, 125 μg/mL yeast transfer RNA, 10 μg/mL polyadenylic-cytidylic acid, 1mg/mL sodium pyrophosphate, pH 7.4, and 50% formamide. The hybridization was carried out at 37℃ overnight with the biotinylated probes diluted in the above solution. The final probe concentrations were 1ng/μL. After the hybridization, slides were washed with 2x SSC, and the hybridization signals were then detected with nitroblue terazolium and 5-bromo-4-chloro-3-indopylophosphate substrate using Dako's ISH detection kit. The control experiments were carried out as follows; (i) ISH with sense probes; (ii) ISH without probes.

Confocal laser scanning microscopy (CLSM)

The CLSM (LSM-410, carl Zeiss, Jena, Germany) was employed on immunostaining sections to elucidate the localization of PC1/3 and PC2 by reflectance confocal mode, equipped with 488 nm Argon laser and/or 543 nm Helium Neon Laser (for DAB) for the bronchial carcinoid. Optical reflectance confocal signals of PC1/3 and PC2 labeled were observed using a dichroic beam splitter (NT 80/20/543, Carl Zeiss. Jena, Germany).

RESULTS

Immunohistochemistry

Immunohistochemical positivity was graded as follows according to the proportion of positively stained tumor cells. i.e.-: no stained cells. +: less than 25%. 2+: 25-50%. 3+: 50-75% and 4+: more than 75%. The results of immunohistochemistry for PC1/3 and PC2 are summarized in Table 3.

Totally, 95% (18/19) of carcinoids were positive (+ to 4+) for PC1/3. Eight cases of 9 bronchial carcinoids and 4 rectal carcinoids, 2 cases of 4 gastric carcinoids and one of 2 bile duct carcinoids showed 3+ or 4+ of PC1/3. In contrast, 58% (11/19) of carcinoids were positive for PC2. Only five cases of bronchial carcinoids showed strong positivity (3+ or 4+) for the PC2. As for non-car-

Table 3 Expression of PCs for carcinoids and non-carcinoid endocrine tumors

Case No	Diagnosis	PC 1/3	PC 2
1	BRC	4+	4+
2	BRC	3+	4+
3	BRC	3+	3+
4	BRC	4+	4+
5	BRC	4+	2+
6	BRC	3+	−
7	BRC	4+	2+
8	BRC	+	−
9	BRC	3+	3+
10	RC	4+	−
11	RC	4+	−
12	RC	4+	2+
13	RC	4+	+
14	GC	3+	−
15	GC	3+	+
16	GC	+	2+
17	GC	−	−
18	BDC	+	−
19	BDC	3+	−
20	PAA	2+	−
21	PAA	+	2+
22	PAA	+	+
23	PAA	+	2+
24	PHA	+	+
25	PHA	+	+
26	MCT	3+	+

−	+	2+	3+	4+
negative staining	−25%	−50%	−75%	−100%

BRC : Bronchial carcinoid, RC : Rectal carcinoid, GC : Gastric carcinoid, BDC : Bile duct carcinoid, PAA : Parathyroid adenoma, PHA : Pheochromocytoma, MCT : Medullary carcinoma of thyroid

cinoid endocrine tumors, only MCT showed strong positivity for PC1/3. Figs. 2, 3, and 4 demonstrate representative stainings in various carcinoids. Most cases of rectal carcinoids were strongly positive for PC1/3 but were less frequently positive or negative for PC2. The gastric and bile duct carcinoids were less frequently positive for PC2 comparing to the other carcinoids. The staining for both PC1/3 and PC2 was characteristic of granular fashion in the cytoplasm of the tumor cells. The staining of PC1/3 and PC2 showed low expressions (+ or 2+) in non-carcinoid endocrine tumors (pheochromocytomas and parathyroid adenomas), but MCT showed strong positivity for PC1/3.

In situ hybridization

PC1/3 mRNA was strongly positive, while PC2 mRNA was weakly or negatively expressed for rectal carcinoids (Fig. 5). Both mRNAs were detected for bronchial carcinoids (Fig. 6). The localization of mRNA signal was found in the cytoplasm of the carci-

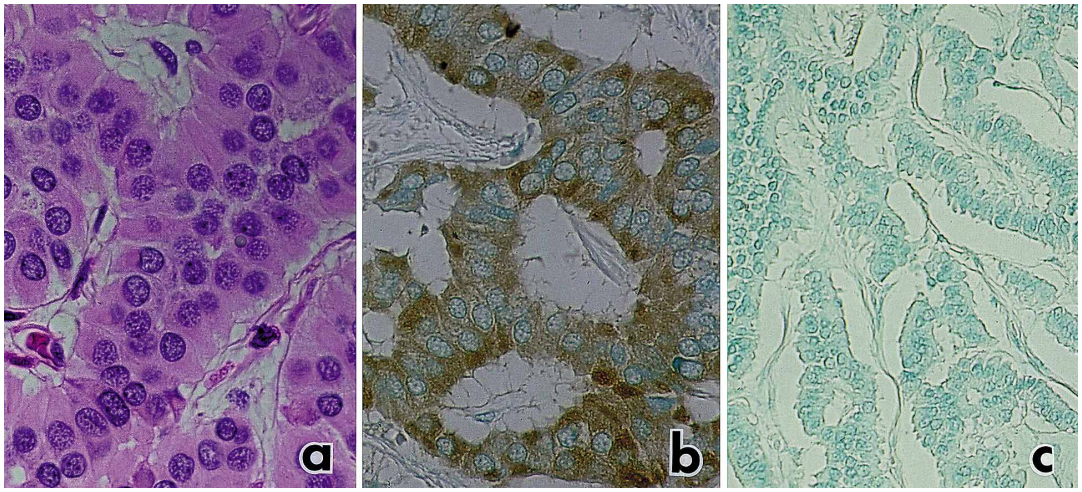

Fig. 2 Immunohistochemical expressions for PC1/3 and PC2 for rectal carcinoid (Case 10)
a. HE stain, b. PC1/3, C. PC2
Rectal carcinoid shows only expression of PC1/3.

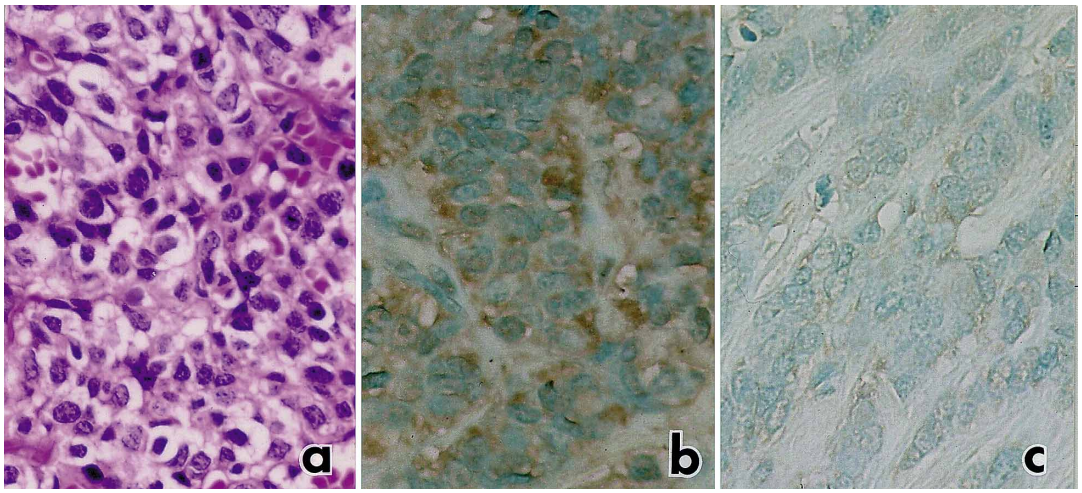

Fig. 3 Immunohistochemical expression for PC1/3 and PC2 for bronchial carcinoid (Case l)
a. HE stain, b. PC1/3, C. PC2
Bronchial carcinoid reveals expressions of both PC1/3 and PC2.

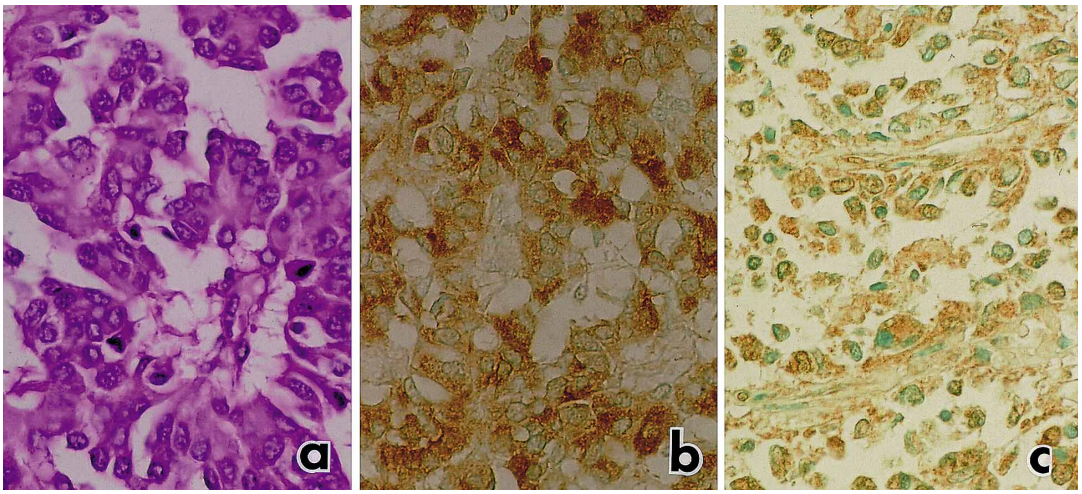

Fig. 4 Immunohistochemical expression of PC1/3 and PC2 for the bile duct carcinoid (Case 19)
a. HE stain, b. PC1/3, C. PC2
Bile duct carcinoid demonstrates only PC1/3 expression.

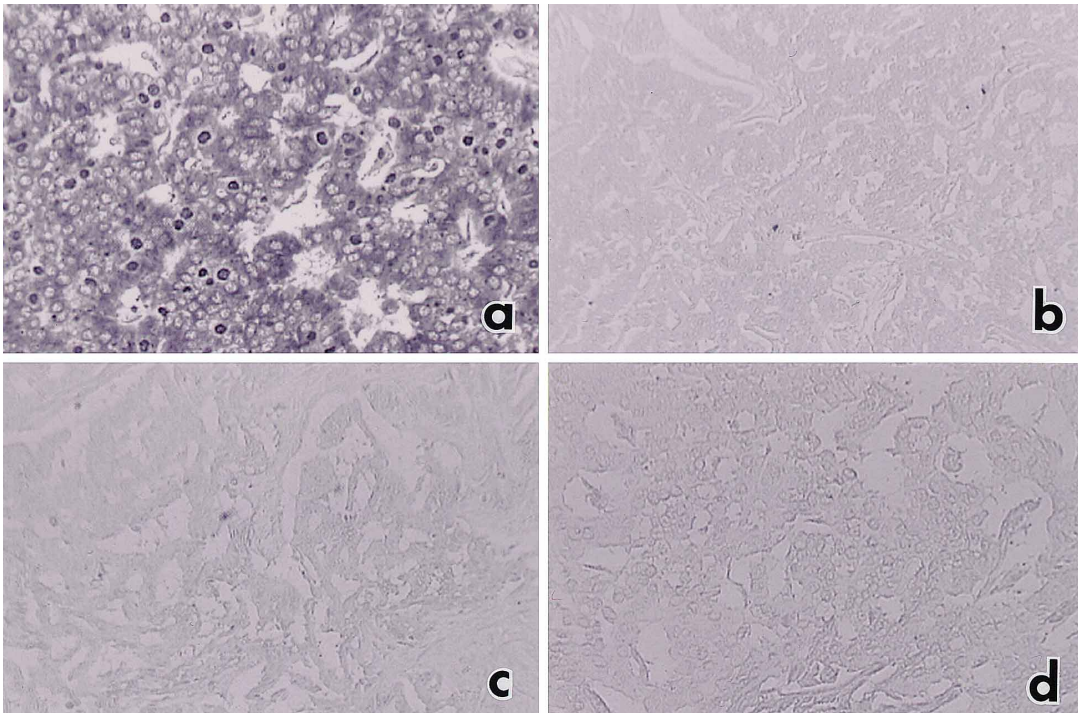

Fig. 5 Expression of mRNA for PC1/3 and PC2 in the rectal carcinoid (Case 10) by in situ hybridization treated with antisense or sense oligonucleotide probe.
a. antisense oligonucleotide probe for PC1/3
b. sense oligonucleotide probe for PC1/3
c. antisense oligonucleotide probe for PC2
d. sense oligonucleotide probe for PC2
Rectal carcinoid expresses only PC1/3 mRNA.

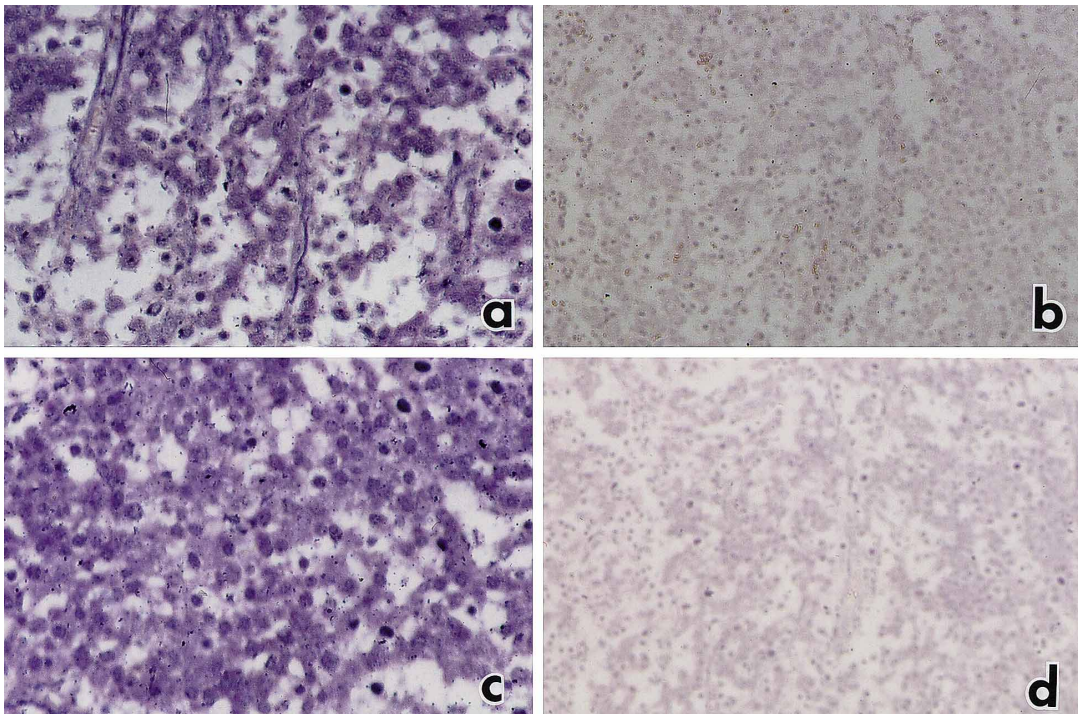

Fig. 6 Expression of mRNA for PC1/3 and PC2 in the bronchial carcinoid (Case 1) by in situ hybridization treated with antisense or sense oligonucleotide probe.
a. antisense oligonucleotide probe for PC1/3
b. sense oligonucleotide probe for PC1/3
c. antisense oligonucleotide probe for PC2
d. sense oligonucleotide probe for PC2
Bronchial carcinoid expresses both PC1/3 and PC2 mRNA.

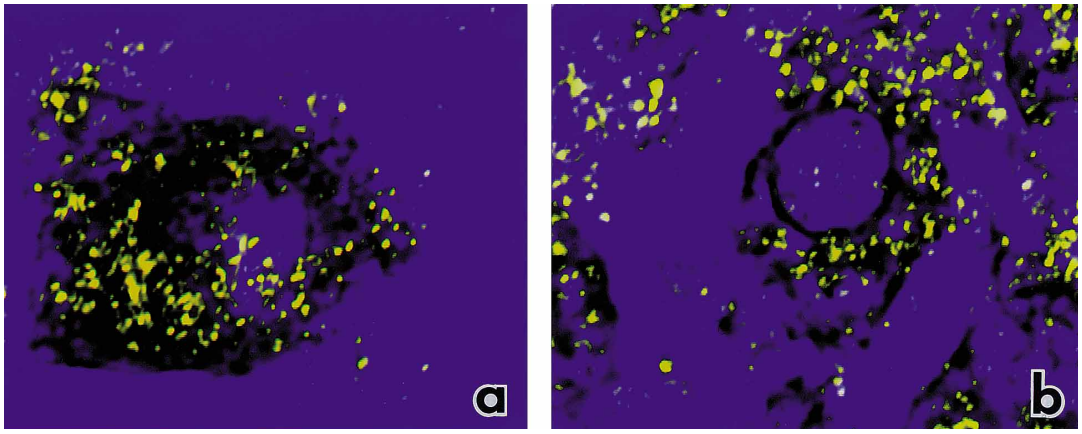

Fig. 7 Combined reflectance and transmittance image of PC1/3 and PC2 signals (yellow color) in the bronchial carcinoid showing granular immunoexpression pattern.
a. PC1/3 signals, b. PC2 signals
PC1/3 and PC2 signals demonstrates in the diffuse-granular pattern.

noid tumor cells.

Confocal laser scanning microscopy

Immunolocalization of PC1/3 and PC2 revealed diffuse-granular pattern in the cytoplasm on the bronchial carcinoid.

DISCUSSION

Our immunohistochemical studies on various carcinoids showed high and strong positivity (3+ or 4+) in 79% for PC1/3 and in 26% for PC2. It has been clarified that PC1/3 is the major processing enzyme in carcinoids . No cases of pheochromocytoma and parathyroid adenomas showed strong staining. Ninety-five% of carcinoids were positive (+ to 4+) for PC1/3. while 58% were positive for PC2. These results suggest that PC1/3 could serve more significantly as a neuroendocrine marker for various neuroendocrine tumors represented by carcinoids. Lloyd et al. (3) demonstrated higher expression of PC1/3 mRNA and PC2 mRNA in the neuroendocrine tumors comparing non-neuroendocrine tumors and indicated their role as neuroendocrine markers . Scopsi et al. (4) also demonstrated immunohistochemical diversity of PC1/3 and PC2 in neuroendocrine cells according to the organs where neuroendocrine cells lie. They also pointed out high frequency of PC immunoexpressions in various neuroendocrine tumors including carcinoids. Our results emphasized prominent immunohistochemical expressions of PC1/3 and PC2 particularly in bronchial carcinoids comparing to other types of carcinoids. It has been well known that diversity of peptide production is closely associated with the location where peptide hormone producing tumors occur, as follows. The bronchial carcinoids are most diverse in produced peptides (7-9) ; calcitonin has been reported to be produced in 30% of bronchial carcinoids; gastrin-releasing peptide in 25%; ACTH in 5%. Thirteen% of gastric carcinoids produce gastrin (10), and 50% of rectal carcinoids produce glicentin and pancreatic polypeptide (6). Regarding the relationship between peptide production and the subcellular expression pattern of PCs, Itoh et al. reported the expressions of PC1/3 and PC2 in insulinomas and that of PC2 in glucagonomas and indicated the correlative expressions of PCs and peptide production(2) . Beinfeld MC et al. also reported that functional relationship between PC1/3, PC2 and peptide hormones (11, 12). From these study, it could be speculated that high PC1/3 and PC2 expressions in the bronchial carcinoids may be well correlated with their diverse peptide production.

In the carcinoids, PC1/3 and PC2 were localized in the cytoplasm as granular fashion. On the CLSM, PC1/3 and PC2 signals were identified as diffuse-granular manner in the cytoplasm. These features strongly suggested that PC1/3 and PC2 were presented in the secretory granules. Itoh et al. demonstrated the colocalization of PC1/3 and insulin as well as PC2 and glucagon in the same secretory granules by immunoelectron microscopy, which suggested that the site of post-translational processing of prohormones lied in the secretory granules.

Our studies indicated higher expressions of PC1/3 than PC2 in the carcinoids, suggesting that PC1/3 would serve as a more significant neuroendocrine marker. The high and strong expressions for both PC1/3 and PC2 in bronchial carcinoids may correspond to their diverse peptide production. The granular localization of the PC1/3 and PC2 may reflect the site of post-translational processing in the secretory granules (13). In the rectal and bronchial carcinoids, the expressions of PC1/3 and PC2 detected by immunohistochemistry was closely correlated with those of mRNA by ISH. Rectal carcinoids and pancreatic glucagonomas often demonstrate the immunoexpression of preproglucagon. However, in the functioning expression, the former are clinically silent because of abscence or low incidence of PC2 and the latter commonly reveal clinical symptoms. In considering these differences, PC2 would be a key enzyme in the expression of functions. We conclude that PC1/3 and PC2 are of particular significance in analyzing the clinical functional expressions and peptide hormone diversity in some carcinoid tumors.

ACKNOWLEDGEMENT

The authors thank Dr. S .Tanaka for providing antibodies against PC1/3 and PC2.

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