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PGP9.5 mRNA could contribute to the molecular-based diagnosis of medullary thyroid carcinoma

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Abstract

The protein gene product 9.5 (PGP9.5) is a ubiquitin hydrolase that is widely expressed in neuronal tissues at all stages of neuronal differentiation and is a known neuroendocrine marker. Medullary thyroid carcinoma (MTC) arises from parafollicular cells and is reported to overexpress several mRNAs such as *RET*, calcitonin, and *CEA*. These markers are thought to be useful in determining a molecular-based diagnosis of MTC. We examined the expression levels of PGP9.5 mRNA in 80 thyroid tissues using real-time quantitative reverse transcription (RT-PCR) and found that PGP9.5 mRNA was overexpressed in all 11 MTCs examined, both hereditary and sporadic, but not in other histological tumour types. Furthermore, by RT-PCR, PGP9.5 mRNA was detected only in aspirates from three medullary carcinomas, and not in aspirates from other tumour types. These results demonstrate that, in addition to the expression of *RET*, calcitonin and *CEA*, PGP9.5 mRNA expression may contribute to the molecular-based diagnosis of MTCs.

Keywords: Medullary thyroid carcinoma; PGP9.5; Fine-needle aspiration biopsy

1. Introduction

Thyroid tumours are often diagnosed by fine-needle aspiration biopsy (FNAB) [1]. Cytological examination of FNAB by a skillful pathologist who is an expert in thyroid tumours provides the most reliable information for the diagnosis of thyroid neoplasms. However, in some clinical situations, slide samples are not adequate for cytological examinations due to poor fixation, and a well-trained expert pathologist is not always available to make the diagnosis. In such cases, a more objective method is required for an accurate diagnosis. We previously introduced a method of preoperative molecular-based diagnosis of thyroid carcinomas, which we named aspiration biopsy RNA diagnosis (ABRD) [2–4]. ABRD allows cytological and molecular-based diagnoses to be performed simultaneously by extracting RNA from the leftover cells in the needle used for the FNAB. ABRD thus provides both RNA information and a cytological diagnosis without subjecting the patient to any further invasive procedures.

Medullary thyroid carcinoma (MTC) is a relatively rare tumour and represents approximately 5% of all thyroid malignancies [5]. As APUDomas, various neuropeptides were reported to have originated from MTCs. In a previous study, we diagnosed MTCs by detecting *RET*, calcitonin, and *CEA* mRNAs in FNABs and confirmed that these mRNAs are useful markers in making molecular-based diagnoses [6].

PGP9.5, a ubiquitin hydrolase, is widely expressed in neuronal tissues and is thought to be a neuroendocrine marker [7,8]. The ubiquitin-proteasome pathway degrades cytosolic and nuclear proteins via an adenosine triphosphate (ATP)- and ubiquitin-dependent mechanism which involves the regulation of cell cycle genes [9]. Hanna and colleagues examined the expression of PGP9.5 protein in MTCs by immunohistochemistry and found it was expressed in 12 of 17 (71%) MTCs [10].

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In our present study, we measured the relative expression levels of PGP9.5 mRNA to beta-actin mRNA in normal thyroid tissues and various thyroid tumours, including 11 MTCs and thyroid-derived fibroblast cultures. We used a real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique in order to estimate to what extent the overexpression of PGP9.5 mRNA in MTCs is a useful marker in molecular-based diagnoses such as ABRD. In addition, we examined, by RT-PCR, the expression of PGP9.5 in aspirates from MTCs.

2. Materials and methods

2.1. Extraction of RNA from thyroid tissues

Tissue samples from thyroid tumours or normal thyroid tissues from the opposite lobe of the carcinomas were obtained by surgery after the patients' informed consent. All tissues were frozen in liquid nitrogen immediately after resection. Total RNA was extracted according to the method of Chomczynski and Sacchi described in Ref. [11].

2.2. RNA extraction from thyroid-derived fibroblasts in culture

RNA from thyroid-derived fibroblasts was extracted from seven normal thyroid tissues as previously described [12]. In brief, approximately 500 mg of each tissue was cut into small fragments and the fragments were digested at 37 °C in Ham's F-12 medium containing 10% v/v fetal calf serum (Gibco, Gaithersburg, MD, USA) and 2 mg/ml collagenase (CLS2, Funakoshi, Tokyo, Japan) for 3 h. Cells were collected by centrifugation at 500*g* for 10 min and there were sparsely seeded in three 10-cm culture dishes. Cells were cultured at 37 °C in Ham's F-12 medium containing 10%v/v fetal calf serum and the medium was replaced every 3 days. After 10 days, when most of the surface of each dish was covered by proliferating fibroblasts, cells were scraped and collected by centrifugation at 500*g* for 10 min. Total RNA was extracted as described above.

2.3. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was performed essentially as previously described in Ref. [13]. In brief, RNAs from 10 normal thyroid tissues, 10 adenomatous goitres, 15 follicular adenomas, 15 follicular carcinomas, 15 papillary carcinomas, four anaplastic carcinomas and 11 MTCs, and RNAs from seven cultures of fibroblasts were subjected to real-time quantitative RT-PCR analysis (Table 1). Reverse transcription was performed using 1 μg of total RNA in a RT mixture containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates (dNTPs), 200 U M-MLV reverse transcriptase (Gibco), 2 U/μl RNase inhibitor (Takara, Shiga, Japan), and 2.5 μM oligo dT (Gibco) in a total volume of 20 μl at 37 °C for 60 min. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System and a TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's protocol. One microlitre of the first-strand cDNA was used in the following assay. The two primers and one TaqMan probe used for the quantification of PGP9.5 (Gene Bank NM004181) and beta-actin (Gene Bank X00351) mRNAs were: (PGPF (0.5 μM): 5′-GCCACCTCTATGAACTTGATG-3′ (bases 583–603)), (PGPR (0.5 μM): 5′-GGTGAATTCTCTGCAGACCTT-3′ (bases 669–689)), and (PGP-TM (10 pmol):5′-FAM-TGTCCTCTGAACTGGCGCCATGGTTC-TAMRA-3′(bases 623–648)); and (ACF (0.5 μM): 5′-TGGACATCCGCAAAGACCTG-3′ (bases 901–920)), (ACR (0.5 μM): 5′-CCGATCCACACGGAGTACTT-3′ (bases 1047–1066)), and (AC-TM (10 pmol): 5′-FAM-CACCACCATGTACCCTGGCATTGCC-TAMRA-3′ (bases 947–971)), respectively.

The conditions for the TaqMan PCR were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A recombinant pGEM T-vector (Promega, Tokyo, Japan) containing either PGP9.5 or beta-actin cDNA was constructed by PCR-cloning with the same set of primers used in TaqMan PCR and were used as standard samples. The amplification plots of the PCR reaction were used to determine the threshold cycle (C_T). The C_T value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value (optimal ΔRn) was first detected. The initial copy number of the target mRNA was calculated from a plot of the C_T against the input target

Table 1
Tissues of MTC used in this study

No.	Gender	Age (years)	Sporadic/hereditary
1	Male	49	Hereditary (MEN2A)
2	Male	46	Hereditary (MEN2A)
3	Female	63	Sporadic
4	Female	20	Hereditary (MEN2A)
5	Female	57	Sporadic
6	Male	68	Sporadic
7	Male	17	Hereditary (MEN2B, metastatic lymph node)
8	Female	23	Hereditary (MEN2B)
9	Female	38	Sporadic
10	Female	70	Sporadic
11	Female	10	Hereditary (MEN2A)

MTC, medullary thyroid carcinoma; MEN, multiple endocrine neoplasia.

Table 2
Expression levels of PGP9.5 mRNA in thyroid tumours

Tissue	Number	PGP9.5 mRNA/beta-actin mRNA (mean±S.D.)	Minimum	Maximum
Normal thyroid	10	0.93±0.59	0.26	1.93
Adenomatous goitre	10	3.16±2.17	0.22	6.53
Follicular adenoma	15	2.61±3.35	0.10	9.19
Follicular carcinoma	15	3.30±5.30	0.10	16.45
Papillary carcinoma	15	1.94±2.59	0.20	8.60
Anaplastic carcinoma	4	4.45±6.35	0.79	13.94
Fibroblast culture	7	6.93±4.01	3.37	14.72
Medullary carcinoma	11	352.1±334.9	100.60	1,153.56

S.D., standard deviation.

quantity. As negative controls, RNAs from thyroid tumours were amplified without reverse-transcription. They showed no amplification signal in the real-time PCR analysis.

2.4. ABRD

ABRD was performed as previously described. In brief, RNA samples were obtained preoperatively from 3 patients suspected of having MTC. Ten preoperative aspirates from other types of tumours (three follicular adenomas and seven papillary carcinomas) were used as negative controls. Reverse transcription was performed as described above using whole RNA samples. Primers used for the PCR amplification of PGP9.5 cDNA and beta-actin cDNA as an internal control were: (PGPF2: 5′-GGCTGGAAGAGGAGTCTCT-3′ (bases 172–190)), (PGPR: 5′-GGTGAATTCTCTGCAGACCTT-3′ (bases 669–689)), and (ACF: 5′-TGGACATCCGC AAAGA CCTG-3′ (bases 901–920)), (ACR: 5′-CCGATCCACA CGGAGTACTT-3′ (bases 1047–1066)), respectively.

Each reaction mixture consisted of 1 μl of cDNA, 0.5 μM each primer, 2 μl of 10×Ex Taq buffer, 1.6 μl of 2 mM dNTP mix, 0.5 U of Ex Taq polymerase (Takara), and nuclease-free water to a final volume of 20 μl. The reaction mixture was subjected to 24 and 30 cycles of denaturation (94 °C; 30 s), annealing (55 °C; 30 s), and extension (72 °C; 30 s) for PGP9.5 and beta-actin cDNAs, respectively. After PCR amplification, 5 μl of reaction mixture was run on an 1.5% w/v agarose gel. The gel was stained with SYBR Green I (Takara), then analysed with a Fluor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

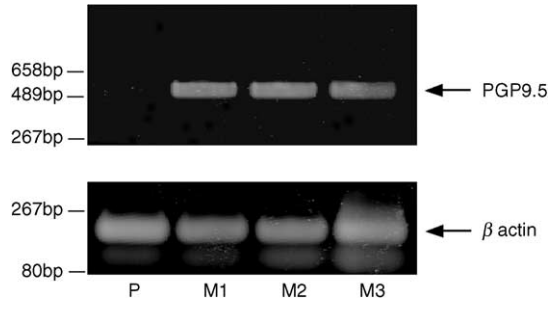

Fig. 1. Expression of PGP9.5 mRNA in preoperative aspirates from MTCs. P, an aspirate from a papillary carcinoma as a negative control; M1, an aspirate from a MTC; M2 and M3, aspirates from recurrent lymph nodes of MTCs; bp, base pairs.

2.5. Statistical analysis

Statistical analysis of differences between the groups was done using the Mann–Whitney *U* test. *P* values of <0.05 were considered significant.

3. Results

By real-time quantitative RT-PCR, expression of PGP9.5 mRNA was observed in all the tissues examined, including normal thyroid tissues, fibroblast cultures. Extremely high expression levels of PGP9.5 mRNA were observed in all of the MTC samples (Table 2). A significant increase in the expression levels of PGP9.5 mRNA compared with normal thyroid tissues and thyroid tumours (except for anaplastic carcinomas and MTCs), was observed in the thyroid-derived fibroblast cultures (Table 3). Aspirates were

Table 3
Comparison of PGP9.5 mRNA levels between various histological classifications by *P* values

	N	AG	FA	FC	PC	AC	FB	MC
N		0.0140	NS[a]	NS	NS	NS	<0.01	<0.01
	AG		NS	NS	NS	NS	0.0358	<0.01
		FA		NS	NS	NS	0.0288	<0.01
			FC		NS	NS	0.0136	<0.01
				PC		NS	<0.01	<0.01
					AC		NS	<0.01
						FB		<0.01

N, AG, FA, FC, PC, AC, FB and MC represent normal thyroids, adenomatous goitres, follicular adenomas, follicular carcinomas, papillary carcinomas, anaplastic carcinomas, thyroid-derived fibroblast cultures and medullary carcinomas, respectively.

[a] Not significant statistically.

obtained from a MTC (case 1: a 72-year-old female), and from two metastatic lymph nodes of MTC (case 2: a 17-year-old male, multiple endocrine neoplasia (MEN) 2B; and case 3: a 45-year-old female) after patients' informed consent. By RT-PCR, PGP9.5 mRNA was detected in the aspirates from three medullary carcinomas, but not in the 10 aspirates from the other tumour types. Representative data are shown in Fig. 1.

4. Discussion

PGP9.5 was first isolated as a specific cytoplasmic marker for neuroendocrine cells. Ubiquitination of cellular proteins and targeting them for subsequent degradation via ubiquitin-mediated proteolysis is a potentially important mechanism regulating the cell cycle. In tumours, increased deubiquitination of cell cycle-related proteins, such as cyclins, by PGP9.5 contributes to the increased or uncontrolled growth of tumour cells [14]. In fact, there are some studies reporting a relationship between the expression of PGP9.5 and the grade of malignancy. PGP9.5 was highly expressed in non-small cell lung cancer and the expression of PGP9.5 was closely associated with the advanced stages of lung cancer [15]. PGP9.5-negative pancreatic cancer patients showed significantly better survival rates compared with those who were PGP9.5-positive, and PGP9.5 may be a novel marker indicating prognosis [16]. PGP expression is related to tumour progression and may be useful as a marker for invasive colorectal cancer [17]. However, for thyroid tumours of follicular origins it is not clear if the expression levels of PGP9.5 mRNA relate to the patient's prognosis, because there was no significant difference in expression levels between adenomas and carcinomas and between well-differentiated carcinomas and anaplastic carcinomas.

Expression of the PGP9.5 protein has been observed in fibroblasts in mature wounds [18]. We found an increased expression of PGP9.5 mRNA in thyroid-derived fibroblast cultures. It is likely that the increased expression levels of PGP9.5 mRNA in some thyroid tumours is caused not only by an increased proliferation rate of the tumour cells, but also by contamination with connective tissues containing fibroblasts.

In a previous study, we reported that RT-PCR detection of *RET*, calcitonin, and *CEA* mRNAs in FNABs was an efficient molecular adjunct in the diagnosis of MTC, for these mRNAs are detectable in all MTCs, but not in other tumour types [6]. Hanna and colleagues also previously reported that the expression of PGP9.5 protein was detectable in 12 of 17 (71%) MTCs by immunohistochemistry [10]. In our present study, we found PGP9.5 mRNA was overexpressed in all MTCs that we examined. This discrepancy may be due to the sensitivity of the assay used, because in Hanna's study the carcinoembryonic antigen (CEA) protein was detected in only 88.9% of MTCs, while we detected *CEA* mRNA in all MTCs examined [6]. PGP9.5 mRNA was expressed approximately 10^2-fold more in the MTC samples than in the other thyroid tumour samples. Furthermore, by RT-PCR, PGP mRNA was detectable only in aspirates from MTCs. These results suggest that like *RET*, calcitonin and *CEA* mRNAs, PGP9.5 mRNA could help in the molecular-based diagnosis of MTCs.

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