Fine-needle aspiration biopsy-RT-PCR expression analysis of prothymosin α and parathymosin in thyroid: novel proliferation markers?

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Fine-needle aspiration biopsy-based cytology has become an established and reliable diagnostic preoperative test in the evaluation of thyroid nodules. Despite the high specificity and sensitivity of the method, results might be doubtful in a significant number of cases. Genetic analysis of the aspirates by RT-PCR may contribute, in parallel to the cytology report, to a more precise diagnosis. Prothymosin α and parathymosin are two homologous chromatin remodeling proteins essential for cell cycle progression and proliferation of either normal or malignant cells. A semi-quantitative RT-PCR assay was developed to determine prothymosin α and parathymosin mRNA expression patterns in thyroid follicular cells obtained from the fine-needle aspiration biopsy specimens of patients diagnosed with simple nodular goitre, follicular adenoma, papillary and follicular well-differentiated carcinomas. Prothymosin α and parathymosin mRNA levels were found significantly elevated in well-differentiated carcinomas in relation to adenomas ($p<0.05$) and goitres ($p<0.05$), an event possibly linked to the proliferation activity of thyroid follicular cells. Further studies are required to establish prothymosin α and parathymosin as diagnostic proliferation markers in thyroid cancer, especially in cases of undetermined cellular morphology of follicular origin which reflect the most common cytohistopathological discrepancies.

Key words: prothymosin α; parathymosin; proliferation marker; cancer; thyroid.

Prothymosin α (ProTα), a nuclear oncoprotein-transcription factor, exhibits an essential intracellular role strongly related to cell cycle progression and proliferation of either normal or malignant cells [1]. ProTα mRNA expression is induced at the end of S and G2/M phases of cell cycle, in parallel with cyclin B levels [2]. Inhibition of ProTα synthesis by antisense oligonucleotides led to cell division arrest in myeloma cells [3]. ProTα transcripts are induced by growth stimulation of resting lymphocytes, NIH3T3 fibroblasts, thymocytes, and hepatocytes during liver regeneration [4]. Overexpression of ProTα has been shown to accelerate proliferation and retard differentiation in HL-60 cells [5]. The protein has been suggested as a cellular oncoprotein that induces transformation in rodent fibroblasts [6]. Similarly to other oncoproteins-transcription factors, ProTα is overexpressed in a variety of cancer tissues and cell lines [1]. Parathymosin (ParaT), a partially homologous protein [7], has also been implicated in cell proliferation processes. The protein has been suggested to accelerate proliferation by inhibiting glucocorticoid action [8]. ParaT levels have been found elevated in cancer tissues indicating a possible role in tumorigenesis [9,10]. The distribution of ProTα and ParaT in the nucleus has been related to transcription and replication sites, respectively [11], while both proteins have been found to interact with histone H1, indicating a role in chromatin remodeling [12,13].

ProTα and ParaT genes expression analysis have never been studied before in human thyroid tissue. In the present study, we decided to undertake the investigation of ProTα and ParaT as potential cellular proliferation markers in benign and malignant thyroid lesions. To this extend, a semi-quantitative RT-PCR assay was developed to determine ProTα and ParaT mRNA levels in thyroid follicular cells obtained from the remaining material within the needle used for the fine-needle aspiration biopsy (FNAB) of patients diagnosed with simple nodular goitre (SNG), follicular adenoma (FA), papillary

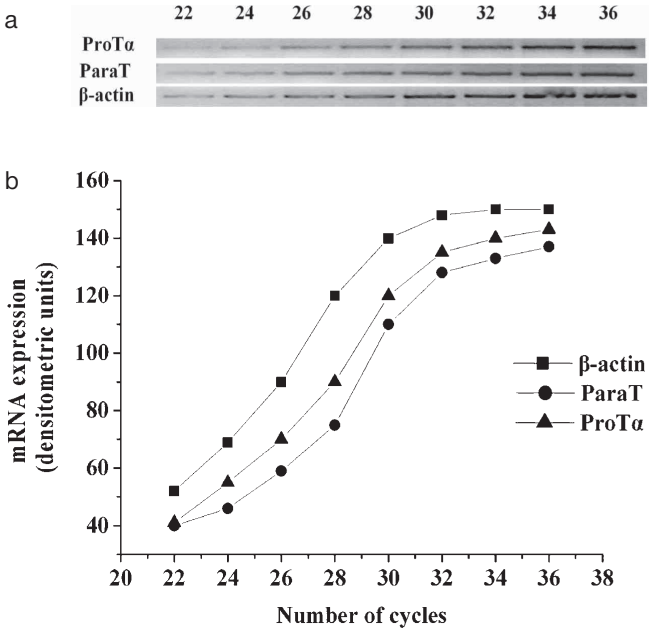

Figure 1. Cycle titration of ProTα, ParaT, and β-actin mRNAs expression. a. RT-PCRs for ProTα, ParaT, and β-actin were carried out using constant amounts of control RNA at different number of cycles (22, 24, 26, 28, 30, 32, 34, 36). Amplified products were visualized on an ethidium bromide-stained agarose gel 1.4%, and were quantified by densitometric scanning; b. Kinetic analysis of ProTα, ParaT, and β-actin amplification at different number of cycles. The signal intensity values (densitometric units) were plotted against the number of cycles. RT-PCR products for all genes showed an exponential amplification during 22-32 cycles and a plateau phase after 34 cycles.

(PTC) and follicular (FTC) well-differentiated carcinomas (WDC).

Patients and Methods

Patients

Thyroid follicular cells were obtained from 37 euthyroid patients (25 females and 12 males, aged 24-78 years) who underwent FNAB for diagnostic purposes. Patients included in the study had not receive any medication before the FNAB procedure. The initial diagnosis was based on physical examination, ultrasound and isotopic scanning of the thyroid. Patients with positive anti-thyroid peroxidase and anti-thyroglobulin antibodies were excluded from the study. The palpable nodule was aspirated using a 22-gauge needle with a biopsy pistol and 4-6 aspirates were taken. Aspirates from each biopsy were prepared for cytological examination and diagnosis, while the remaining material within the needle was washed out with 600 μl of the lysis buffer used for RNA isolation (Qiagen). The samples was then directly stored at -80°C. According to the cytological report the patients were sub-classified into those with SNG (n=14), FA (n=10), PTC (n=8) and FTC (n=5). The experiments were performed according to the regulations of the local ethics committee and all patients gave informed consent.

Semi-quantitative RT-PCR assay

Total RNA isolation from the fine needle aspirates was achieved using the RNeasy-kit (Qiagen) according to the recommendations of the manufacturer. Samples were diluted with RNase-free water in a final volume of 60 μl, treated with RNase-free DNAase I (Promega) and stored at -80°C until further use. RNA concentration was determined by measuring the optical density (OD) of the samples at 260 nm, using the cofactor of 1 OD = 40 μg/ml.

One step RT-PCR was performed on total RNA samples (100 pg) using a commercially available kit (Titan One Tube RT-PCR Kit, Roche). An appropriately designed set of primers, both spanning an intron, was used to amplify a specific part of human ProTα cDNA (255 bp). The sequences of the primers were: sense, 5'-CCACCAAGGACTTAAGGAG -3' (spanning intron 3); antisense, 5'-TCGTCATCCTCATCATCTTC-3' (spanning intron 4). A 310 bp fragment of ParaT cDNA was amplified using the following set of primers: sense, 5'-CCGGAATTCATGTCGGAGAAGCGTC-3'; antisense, 5'-TCCGCTCGAGTCACGCCGATGCCCCATT-3'. The exact positions of introns and exons of ParaT gene have not been determined in human genome. Oligonucleotide primers for ProTα and ParaT cDNA amplification were designed from the GenBank sequences (www.ncbi.nlm.nih.gov/Genbank). Semi-quantitation of ProTα mRNA levels was achieved by including human β-actin as internal control. A 575 bp part of β-actin cDNA was amplified using the following pair of primers (Roche): sense, 5'-CCAAGGCCAACCGCGAGAAGATGA-3'; antisense: 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'.

To increase the validity of the method, preliminary experiments were performed to determine the RT-PCR cycles corresponding to the exponential phase of ProTα, ParaT, and β-actin cDNA amplification, in order the RT-PCR product yield to quantitatively reflect the amount of the initial template RNA. For this purpose, RT-PCRs were carried out separately for each gene using a known amount (20pg) of control RNA (K562, Roche) at different number of cycles (22, 24, 26, 28, 30, 32, 34, 36). As shown in Figure 1, 30 cycles of amplification were found to lie within the exponential phase of ProTα, ParaT, and β-actin cDNA amplification.

The reaction components were set up in a final volume of 25 μl. The RT-PCRs were carried out in a GeneAmp PCR System 9600 Thermal Cycler (Perkin-Elmer). Reverse transcription was performed at 60°C for 30 min. cDNA amplification thermocycling conditions included a preliminary denaturation step at 94°C for 2 min, preceding 10 cycles of denaturation at 94°C for 30 s, annealing at 55°C (ProTα and ParaT) and 66°C (β-actin) for 30 s and extension at 68°C for 45 s, followed by 20 cycles with an additional 5 s elongation time per cycle, and a final extension step at 68°C for 3 min.

RT-PCR products (10 μl) were then visualized on an ethidium bromide-stained agarose gel 1.4% (Sigma).

A standard DNA molecular weight ladder (1 kb ladder, GIBCO-BRL) was used to provide appropriate size markers. The densitometric analysis of signal intensities was performed using the Scion-Image software program (www.scioncorp.com) under UV illumination. ProTα and ParaT mRNA expression profiles were normalized to the expression of the β-actin gene.

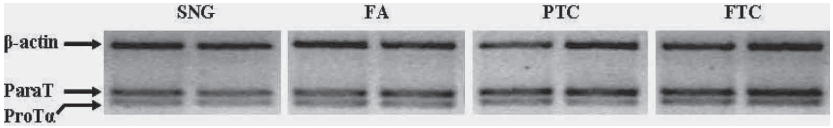

Figure 2. Semi-quantitative analysis of ProTα and ParaT mRNAs expression in FNAB samples. ProTα, ParaT, and β-actin RT-PCR products were analyzed on an ethidium bromide-stained agarose gel 1.4%. ProTα and ParaT mRNAs expression was normalized to the expression of β-actin gene.

Statistical analysis

The SPSS program (Version 11.0) was used for statistical analysis of the results. Values are presented as mean ± standard deviation. Differences between study groups were analyzed using the one-way ANOVA test. Correlations were performed using the Pearson's chi-squared test. A value of $p < 0.05$ was considered to be statistically significant.

Results

ProTα and ParaT mRNAs expression pattern was investigated in all FNAB spcecimens using a semi-quantitative RT-PCR assay (Figure 2). The mean percentage ProTα/β-actin and ParaT/β-actin optical densities ratios were calculated for each sample. ProTα mRNA levels were determined as follows: 41.05 ± 7.42 in SNG; 43.46 ± 9.78 in FA; 58.34 ± 13.98 in PTC; 70.26 ± 15.27 in FTC. The mean percentage ParaT mRNA levels were 60.45 ± 11.62 in SNG; 65.67 ± 12.83 FA; 81.84 ± 17.15 in PTC; 87.46 ± 19.77 in FTC. ProTα and ParaT mRNA levels were found significantly elevated in well-differentiated carcinoma group (FTC, PTC) in relation to SNG and FA ($p<0.05$) (Table 1). Furthermore, ParaT transcripts were elevated compared to ProTα in all histological types studied ($p<0.05$). No significant correlations were observed between protein's expression and age or gender of the patients.

Discussion

FNAB-based cytology of the thyroid gland has become an established and reliable diagnostic preoperative test that is routinely used as the first step in the evaluation of nodular thyroid disease. Although FNAB-based cytology has high specificity and sensitivity, results might be doubtful in a significant number of cases, mainly because of inadequacy of the samples or the presence of undetermined cellular morphology usually described as follicular neoplasia [15]. Previous studies have demonstrated that genetic analysis of the FNABs by RT-PCR may contribute, in parallel to the cytology to a more precise diagnosis. The leftover follicular cells within the needle can be easily used for RNA extraction, and then for RT-PCR amplification of specific mRNAs that are expressed in benign or malignant thyroid nodules. Semiquantitative RT-PCR is widely used to determine the levels of specific mRNAs, especially when the starting material is not adequate, like in FNAB, to perform direct measurements by Northern blot analysis. WINZER et al. reported the detection of thyroid-relevant mRNAs in a small number of follicular cells, such as can be obtained by FNAB [16]. RT-PCR detection of RET, calcitonin, and carcinoembryonic antigen transcripts in FNABs has been suggested as an efficient molecular tool in diagnosis of medullary thyroid carcinomas [17]. Using the same technique, human telomerase reverse transcriptase (hTERT) and FRA-1 genes have been found to be expressed only in neoplastic thyroid lesions [18].

ProTα as well ParaT are overexpressed in a variety of cancer tissues and cell lines indicating a possible role in cancer development. ProTα is overexpressed in colon, hepatocellular, breast, lung, and ovarian cancer, as well as in human neuroblastoma suggesting an essential role in carcinogenesis [1]. The protein has been suggested as a proliferation marker in hepatocellular and breast cancer [19,20]. Downregulation of ProTα gene with coincidental overexpression of connective tissue growth factor attenuates cell growth in human oral squamous cell carcinoma [21]. The inhibition of progression of prostatic intra-epithelial neoplasia to carcinoma by isoflavones has been attributed among the others to

Table 1. Prothymosin α (ProTα) and parathymosin (ParaT) expression profiles in simple nodular goitre (SNG), follicular adenoma (FA), papillary (PTC) and follicular (FTC) well-differentiated carcinomas as assessed by semi-quantitative RT-PCR assay. Differences between study groups were analyzed using the one-way ANOVA test. NS: statistically non-significant.

	ProTζ		ParaT	
	Mean ± SD (%)	p value	Mean ± SD (%)	p value
SNG vs. FA	41.05 ± 7.42 vs. 43.46 ± 9.78	*NS*	60.45 ± 11.62 vs. 65.67 ± 12.83	*NS*
SNG vs. PTC	41.05 ± 7.42 vs. 58.34 ± 13.98	0.035	60.45 ± 11.62 vs. 81.84 ± 17.15	0.030
SNG vs. FTC	41.05 ± 7.42 vs. 70.26 ± 15.27	0.016	60.45 ± 11.62 vs. 87.46 ± 19.77	0.020
FA vs. PTC	43.46 ± 9.78 vs. 58.34 ± 13.98	0.043	65.67 ± 12.83 vs. 81.84 ± 17.15	0.040
FA vs. FTC	43.46 ± 9.78 vs. 70.26 ± 15.27	0.017	65.67 ± 12.83 vs. 87.46 ± 19.77	0.022
PTC vs. FTC	58.34 ± 13.98 vs. 70.26 ± 15.27	*NS*	81.84 ± 17.15vs. 87.46 ± 19.77	*NS*

downregulation of ProTα expression [22]. Inhibition of nuclear ProTα expression in breast cancer cells using antisense methodology resulted in the inhibition of estradiol E2-induced breast cancer cell proliferation [23]. ProTα has been proposed as a prognostic tumor marker in breast, lung and bladder cancer [24-26]. High levels of the protein have been associated with poor prognosis and a worse outcome. Moreover, ProTα has been recently suggested as an anti-apoptotic factor that promotes cell survival [27]. ParaT has been studied to a lesser extend. High levels of the protein have been detected in colon and breast cancer when related to the neighboring normal tissues [9,10].

In the present study, using a semiquantitative RT-PCR assay, ProTα and ParaT gene expression patterns were investigated in hyperplastic and neoplastic thyroid follicular cells obtained from the remaining material within the needle used for diagnostic FNAB. We found that after cytological preparation of the aspirates, the remaining material within the needle was adequate for RNA isolation and RT-PCR amplification of both cDNAs. ProTα and ParaT transcripts were found significantly elevated in WDC (PTC, FTC) in relation to FA and SNG, and thus, their levels may reflect the proliferation activity of thyroid follicular cells. We showed that ParaT is overexpressed in relation to ProTα in all histological types studied. It has been previously reported that both polypeptides share a reciprocal tissue distribution; ProTα is predominantly expressed in lymphoid tissues such as spleen and thymus, while ParaT is mainly expressed in non-lymphoid tissues such as liver and kidney [28]. A single-stand conformation polymorphism analysis (SSCP) performed on ProTα cDNA fragments failed to detect any polymorphisms (unpublished data). In accordance with the aforementioned results, we have recently showed that ProTα immunoexpression is significantly elevated in well-differentiated thyroid carcinomas in relation to adenomas, goitres and normal thyroid tissue. Moreover, ProTα expression was directly correlated with the proliferation index as assessed by Ki-67 immunoreactivity [29].

In conclusion, ProTα and ParaT mRNA expression profiles were determined for the first time in human thyroid tissue using the remaining material within the needle used for FNAB diagnostic. We showed that FNAB-RT-PCR is a convenient methodology for studying the expression profiles of candidate genes that may participate in the pathogenesis of thyroid neoplasias. Furthermore, in combination with molecular scanning techniques such as SSCP or direct sequencing, mutations can be evaluated. ProTα and ParaT mRNA transcripts were found significantly elevated in carcinomas when related to adenomas and goitres, and thus, may provide an objective measure of thyroid follicular cells proliferation activity. ProTα and ParaT holds a promise as diagnostic proliferation markers in identifying specific histological types of thyroid lesions, and thus to contribute, in parallel to the cytology report, to a more precise diagnosis, especially in cases of undetermined cellular morphology of follicular origin which reflect the most common cytohistopathological discrepancies. The prognostic value of both proteins in thyroid carcinomas has to be evaluated.

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