RAPID COMMUNICATION

Tensin3 is a novel thyroid-specific gene

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Abstract

Thyroid-specific genes, such as thyroid peroxidase, thyroglobulin, Na⁺/I⁻ symporter and thyroid-stimulating hormone receptor, play fundamental roles in thyroid function and relate to many pathological conditions. Using sequence specific-differential display, we detected three genes that showed higher expression levels in normal thyroid tissues than in thyroid tumor tissues. After subcloning and sequencing analysis, one of the genes was revealed to be tensin3. The expression level of tensin3 was examined with real-time quantitative PCR analysis. Its expression levels were more depressed in thyroid tumor tissues than in normal thyroid tissues. The decrease was even more evident in two anaplastic carcinomas. High and moderate levels of tensin3 mRNA expression were observed in the thyroid and placenta respectively. Tensin3 mRNA was expressed only in low levels in other tissues, such as the brain, heart, lung, liver, pancreas, kidney, skeletal muscle, white blood cells and prostate. These results show that tensin3 is a novel thyroid-specific gene and further investigations may reveal its relation to thyroid function or thyroid disease.

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Introduction

Thyroid-specific genes, such as thyroid peroxidase (TPO), thyroglobulin (TG), Na⁺/I⁻ symporter (NIS) and thyroid-stimulating hormone receptor (TSHR) play fundamental roles in the functions of thyroid follicular cells, especially in the synthesis and secretion of thyroid hormone (Haddad & Sidbury 1959, Baas *et al.* 1986, Smanik *et al.* 1996). These genes relate to many thyroid diseases and are used in their clinical diagnoses. The genes are usually underexpressed in thyroid tumor tissue. For example, Lazar *et al.* (1999) have reported the decreased expression of TPO, TG, NIS and TSHR in thyroid carcinomas.

In a previous study, we developed a modified method of differential display, sequence specific-differential display (SS-DD), which allows the rapid screening of specific changes in mRNAs in tumor tissues (Takano *et al.* 1997). In SS-DD, a 16- to 18-base degenerate primer instead of a 10-mer primer is used to prevent false positive results by performing the polymerase chain reaction (PCR) with a high annealing temperature. In addition, Ex-Taq polymerase instead of Taq polymerase is used to produce longer PCR products, which helps to avoid amplification of the 3' non-coding region.

Using SS-DD, we have detected three genes in which the expression levels were higher in normal thyroid tissues than in thyroid tumor tissues. One of these genes was identified as acid ceramidase, but the other two genes showed unknown sequences (Maeda *et al.* 1999). We cloned one of these unknown genes and found that it is identical to the genes named tensin3 (Cui *et al.* 2004) or TEM6 (Carson-Walter *et al.* 2001) in other studies. Interestingly, in human tissues, this gene is expressed in the thyroid in a restricted manner, which suggests it is a novel thyroid-specific gene.

Materials and methods

Extraction of RNA

Tissue samples were obtained at surgery, and were quickly frozen in liquid nitrogen. Total RNA was extracted according to a method described previously (Chomczynski & Sacchi 1987). All tissues were histologically classified according to WHO guidelines (Hedinger *et al.* 1989). The study protocol was approved by the institutional ethical committee. Informed consent was obtained from all patients.

Sequence specific-differential display

SS-DD was performed essentially as previously described (Takano *et al.* 1997). In brief, RNAs from nine normal thyroid tissue samples, five papillary carcinomas, two follicular adenomas and two follicular carcinomas were used for the screening using SS-DD. All primers were purchased from Funakoshi (Tokyo, Japan). DNase digestion of 10 µg total RNA was performed with DNase I (Takara, Sigma, Kyoto, Japan), at 37 °C for 30 min. The RNA pellet was phenol/chloroform/isoamyl alcohol-extracted, and was precipitated with ethanol, followed by reconstitution with distilled water. Two micrograms total RNA were converted to cDNA in an reverse transcriptase (RT) mixture containing 8 µl 5 × RT buffer, 8 µl 2.5 mM deoxynucleosidetriphosphate (dNTP) (Takara), 0.4 µl 100 mM dithiothreitol, 2 µl 200 U/µl reverse transcriptase (Gibco, Gaithersburg, MD, USA), 0.4 µl 140 µU/µl RNase inhibitor (Takara), and 200 µM oligo dT (Funakoshi) in a total volume of 40 µl (adjusted adding nuclease-free water) at 37 °C for 60 min. Two degenerate primers were used in the following PCR. One, SH2 (2.0 µM): 5'-TTCCTGGTG(A/C)G(A/G)(G/C)A(G/C)(A/T/U)(G/C)-3', was based on the consensus sequence for the Src homology 2 (SH2) domain (Matuoka *et al.* 1992), and the other, DD3'-2 (2.0 µM): 5'-GTTTTTTTTTTT TTTTTTTT(G/A/C)-3', was designed to anneal to the poly A tail. Each reaction mixture consisted of 1 µl cDNA, 200 µM of each primer, 4 µl 10 × Ex Taq buffer, 4 µl 2.5 mM dNTP mix, 2 U Ex Taq polymerase, and nuclease-free water to a final volume of 40 µl; 10 × Ex Taq buffer, 2.5 mM dNTP mix, and Ex Taq polymerase were obtained from Takara. The PCR was performed at 94 °C for 2 min, then 2 cycles of denaturation (94 °C, 1 min), annealing (40 °C, 3 min) and extension (72 °C, 5 min), followed by 40 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min with 5 s of auto-segment extension for each cycle). After PCR amplification, 4.0 µl reaction mixture were run on a 3.5% acrylamide gel in a Tris-HCl/boric acid/EDTA (TBE) buffer (pH 8.0) using 360-mm glass sequencing plates (Takara). After electrophoresis, one of the glass plates was removed, and the gel was stained with SYBR Green I (Wako, Osaka, Japan) and analyzed with a Fluor Imager (Molecular Dynamics, Sunnyvale, CA, USA). The bands of interest were cut with a razor, and PCR products were extracted by boiling at 100 °C for 5 min. The PCR products were amplified by PCR with the following two primers: SH2-R (2.0 µM): 5'-ATGCGAATTCTTTCCTGGTG(A/C)G(A/G)(G/C)A-3' and DD3'-R2 (0.5 µM): 5'-ATGCGAATTCGTTTTTTTTTTTTTTTTTTT-3' in a reaction mixture containing 5 µl 10 × Ex Taq buffer, 4 µl 2.5 mM dNTP mix, 2.5 U Ex Taq polymerase and nuclease-free water to a final volume of 50 µl. The PCR

was carried out for 30 cycles at 94 °C, 60 °C and 72 °C for 1 min. Purified PCR products were cloned into a pMOSBlue vector using a pMOSBlue T-vector kit (Amersham, Bucks, UK) according to the manufacturer's instructions. DNA sequencing was performed using a Taq Dye Primer Cycle Sequencing Kit and a 373 A DNA sequencer (Perkin-Elmer, Foster City, CA, USA).

Cloning of an unknown gene

Cloning of an unknown gene was carried out using a human thyroid cDNA library (Takara) with hybridization analysis. A cDNA fragment for hybridization was amplified from a human thyroid cDNA with RT-PCR using the following two primers: 5SN1SC (0.5 µM): 5'-TTCCTGTCCCACAACTTCTCACG-3' (base 3202–3225 of tensin3 cDNA), and 3SN1SC (0.5 µM): 5'-ATATCCGCCTTGTACCAGAACTTG-3' (base 3516–3539 of tensin3 cDNA). The amplified probe was labeled with an AlkPhos labeling kit and the CDP-Star detection reagent (Amersham Pharmacia Biotech, UK) was used for signal detection. Hybridization and detection were performed according to the manufacturer's protocol.

Reverse transcription-PCR

Reverse transcription-PCR (RT-PCR) was performed as previously described (Takano *et al.* 1997). cDNAs from four thyroid tissue samples and human cDNAs (Clontech, Palo Alto, CA, USA) from brain, heart, lung, liver, pancreas, kidney, placenta, skeletal muscle, white blood cells (WBC) and prostate were analyzed. The following primers were used for the PCR: one was 5SN1SC (0.5 µM): 5'-TTCCTGTCCCACAACTTCTCACG-3' (base 3202–3225 of tensin3 cDNA (GenBank AF417489)), and the other was 3SN1SC (0.5 µM): 5'-ATATCCGCCTTGTACCAGAACTTG-3' (base 3516–3539 of tensin3 cDNA). The PCR was performed at 95 °C for 10 min, and 35 cycles of 95 °C, 60 °C and 72 °C for 30 s. Four microliters of each PCR product were electrophoresed on a 1.5% SeaKem GTG agarose gel (Takara) in a Tris-HCl/acetate/EDTA (pH 8.0) buffer. The gel was stained with ethidium bromide, then analyzed with a Fluor Imager.

Real-time quantitative RT-PCR of tensin3 mRNA

Real-time quantitative RT-PCR (TaqMan RT-PCR) using the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) was performed according to the manufacturer's protocol. The amplified sequence was different from that amplified in the RT-PCR analysis. The two

primers and one TaqMan probe used for the quantification of tensin3 mRNA were: 5 TMSN1 (0.5 µM): 5'-CATATTTCTGGGAGCCTGACG-3' (base 3773–3792), 3 TMSN1 (0.5 µM): 5'-TCAAGTACCACACATTGCAG-3' (base 3940–3958), and SN1-TM (10 pmol): 5'-FAM-CAGAGAGAGATCCATTGGAGGAA-TAMRA-3' (base 3851–3873). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (GenBank BC013310) was used as an internal control since the expression levels of GAPDH mRNA in normal thyroid tissues, follicular adenomas, papillary carcinomas and follicular carcinomas were almost equal (Takano *et al.* 2000). The two primers and one TaqMan probe used for the quantification of GAPDH were: TMGPD5 (0.5 µM): 5'-TCCATGACAACCTTTGGTATC-3' (base 551–570), TMGPD3 (0.5 µM): 5'-AAGGTCATCCCTGAGCTAGA-3' (base 715–734), and GPD-TM (10 pmol): 5'-FAM-AGAATCATCCCTGCCTCTACT-TAMRA-3' (base 671–693). The analyzed cDNAs were reverse-transcribed from RNAs extracted from 28 normal thyroid tissues, 13 follicular adenomas, 10 adenomatous goiters, 15 papillary carcinomas, 5 follicular carcinomas and 2 anaplastic carcinomas, as described above. Human cDNAs (Clontech) from brain, heart, lung, liver, pancreas, kidney, placenta, skeletal muscle, WBC and prostate were also analyzed. The conditions for the TaqMan RT-PCR were as follows: 95 °C for 10 min and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Recombinant pGEM T-vectors (Promega, Tokyo, Japan) containing either tensin3 or GAPDH cDNA were constructed by PCR-cloning with the same sets of primers used in the TaqMan RT-PCR, and were used as standard samples.

Statistical analysis

The Mann-Whitney test was used to determine differences between the tensin3 mRNA/GAPDH mRNA of normal thyroid tissues and that of thyroid tumors. *P* values of less than 0.05 were considered to be significant.

Analysis of the SAGE database

A tag counting analysis based on the SAGE libraries was performed with the public database (<http://www.ncbi.nlm.nih.gov/projects/SAGE/index.cgi?cmd=tagsearch>).

Results

Screening of the specific changes in mRNAs in thyroid tumors with SS-DD

In the results of electrophoresis, three bands, SN1, SN2 and SN3, which decrease in tumor tissues, were

observed (Fig. 1). A band of SN1 was cut out from the gel, and the extracted PCR products were then reamplified with PCR with the primers, SH2R and DD3'-R2. The reamplified PCR products were cloned into a pMOSBlue vector, and sequencing analysis was carried out. The sequence of SN2 corresponded to the 1482–2330 bp position of acid ceramidase mRNA (Maeda *et al.* 1999). The other two clones showed no homology to known sequences.

Sequence of an unknown gene

Using a human thyroid cDNA library, we screened about 10 000 clones, and found 3 positive clones. The detected clones were 5076 bp and had a huge 3' non-coding region. The predicted amino acid sequence showed the existence of an SH2 domain and a phosphotyrosine binding (PTB) domain. Thus, we named this gene the thyroid-specific PTB domain protein (TPP) (GenBank AB062750). This gene has also been cloned by two other groups. Carson-Walter *et al.* (2001) named it TEM6 (GenBank AF3787560) and Cui *et al.* (2004) named it tensin3 (GenBank AF417489). The comparison of these three reported sequences is summarized in Fig. 2. The coding region of tensin3 mRNA is the longest, 1445 amino acid residues, among the three genes.

Using cDNAs from human thyroid tissues, the expression of the 5' sequence of tensin3 mRNA, which is lacking in TPP mRNA, was confirmed with RT-PCR analyses using a couple of pairs of specific sense- and antisense-primers for 5' region tensin3 mRNA (data not shown).

Quantitative analysis of tensin3 mRNA

To confirm the decreased expression of tensin3 mRNA in thyroid tumors, TaqMan RT-PCR was carried out. The expression levels of tensin3 mRNA were decreased in papillary carcinomas, follicular carcinomas and follicular adenomas. Its expression was decreased markedly in the 2 anaplastic carcinomas (Fig. 3).

RT-PCR and quantitative RT-PCR analyses of tensin3 mRNA in human tissues

To confirm the specific restricted expression of tensin3 mRNA in the thyroid, semi-quantitative RT-PCR was carried out using cDNAs from the brain, heart, lung, liver, pancreas, kidney, placenta, skeletal muscle, WBC and prostate. As shown in Fig. 4, tensin3 mRNA was expressed at high levels in thyroid tissues. Moderate expression of tensin3 was observed in the placenta and it was only weakly expressed in other tissues.

Figure 1 Representative image of SS-DD using RNA from normal thyroid tissues and from thyroid papillary carcinomas. PCR products derived from normal thyroid tissues (N), papillary carcinomas (P), follicular adenomas (FA) and follicular carcinomas (FC) were separated on an acrylamide gel. The gel was stained with SYBR Green I, and the fluorescent image was detected with a Fluor Imager. A band, SN1 (indicated by a box) was cut out from the gel for extraction.

Figure 2 Comparison of the sequences of tensin3 mRNA, TEM6 mRNA and TPP mRNA. The sequence of the region base 2254–4388 of tensin3 mRNA (indicated by stippled bars) was common with TPP mRNA and TEM6 mRNA. The actin-binding domain (ABD) I is coded in the NH₂ terminus regions of tensin3 mRNA, and the Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains are coded in the COOH terminus regions of tensin3 mRNA, TEM6 mRNA and TPP mRNA respectively. The open reading frame of tensin3 mRNA is base 10–4347. A bold line with an asterisk shows the position of the probe used for the library screening.

Figure 3 Tensin3 mRNA/GAPDH mRNA in quantitative RT-PCR analysis. Each closed circle shows the mean of a duplicate assay. * $P<0.005$, ** $P<0.01$.

Analysis of the SAGE database

Based on the sequence of TPP mRNA, the following ten bases after the last CATG in the 3'-noncoding region are TTAAAAGTCA. There were only six libraries from normal human tissues with this tag (Table 1). The tag count was high in the thyroid and placenta, which is identical to the result obtained with RT-PCR.

Discussion

Cui *et al.* (2004) cloned tensin3 as a member of the tensin family, to which Cten (Lo & Lo 2002), tensin1 and tensin2 belong, and tensin3 localizes in focal adhesions of the plasma membrane (Chen *et al.* 2002). According to a recent study (Cui *et al.* 2004), the NH₂ terminus of

Figure 4 RT-PCR and quantitative RT-PCR analyses of tensin3 mRNA in human tissues. cDNAs from brain, heart, lung, liver, pancreas, kidney, placenta, skeletal muscle, white blood cells (hWBC), prostate and four thyroid tissues were analyzed. The upper panel shows the image of electrophoresis after RT-PCR, and the lower panel shows the results of quantitative measurement of tensin3 mRNA. GAPDH mRNA was used as an internal control.

Table 1 SAGE library data for tag TTAAGTCA

Library name	Tags per million	Tag counts	Total tags
Normal thyroid	94	11	115 938
Prostate	67	4	59 553
Placenta	59	7	118 083
Lung	44	4	89 143
Kidney	23	1	41 857
Liver	14	1	66 861
Heart	11	1	84 357

tensin3 contains an actin-binding domain (ABD) I, and the COOH terminus contains an SH2 domain and a PTB domain. Tyrosine phosphorylation of tensin3 is induced by epidermal growth factor (EGF) in MDA-MB-468 cells in a time- and dose-dependent manner, and tensin3 may be related to the cell migration promoted by EGF. Cui *et al.* (2004) reported that tensin3 was expressed as a 180 kDa protein in various cell types, although they did not examine the expression in the thyroid.

TEM6 was first reported by St Croix *et al.* in 2000. They identified 46 transcripts named TEMs (tumor endothelial markers), which were significantly up-regulated in tumors compared with normal endothelium. However, they did not undertake further study on the TEM6 gene.

In the present study, we confirmed the overexpression of tensin3 mRNA in the human thyroid. Tensin3 mRNA was also expressed in thyroid tumors, although its expression levels were much lower in tumor tissues and even lower in anaplastic carcinomas. The restricted expression of tensin3 in the thyroid was confirmed by both quantitative RT-PCR and the SAGE database analysis. In the SAGE database analysis, the SAGE tag was also detected in the prostate, placenta, lung, kidney, liver and heart. However, in three of these six libraries, the corresponding tag sequence appeared only once, which suggests the possibility that the expression levels of tensin3 in these tissues are much lower than those expected from the data of 'tags per million'.

The role of tensin3 in the thyroid cell is not clear. Chiang *et al.* (2005) generated mutant mice with a

disrupted *tensin3* gene and suggested that the *tensin3* gene might be related to a sub-population of Silver-Russell syndrome patients. Interestingly, although Chiang *et al.* did not examine the thyroids or thyroid function in these mice, the mice were distinct dwarves, which suggest the existence of inherited hypothyroidism.

Finally, the results of our presented study show that *tensin3* mRNA is expressed in a restricted manner in the human thyroid. This fact suggests that *tensin3* may play some fundamental role in thyroid function and may relate to some thyroid diseases, as do the other thyroid-specific genes such as TPO, TG, NIS and TSHR.

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