



Quantitative measurement of telomerase reverse transcriptase, thyroglobulin and thyroid transcription factor 1 mRNAs in anaplastic thyroid carcinoma tissues and cell lines

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Abstract. Anaplastic thyroid carcinomas (ATC) are undifferentiated tumors that show rapid progression and dissemination. The recent increase in knowledge about cancer stem cells has attracted marked interest in ATC, since these lesions are suggested to be closely related to thyroid stem cells (TSCs). Due to the rarity of ATCs, however, the gene expression patterns that characterize these lesions have not been fully clarified. Using real-time quantitative reverse transcription-polymerase chain reaction, we measured the mRNA expression levels of three representative genes, telomerase reverse transcriptase (hTERT), thyroglobulin and thyroid transcription factor 1 (TTF-1) in twelve frozen tissue samples of ATC and six cell lines derived from ATCs. All twelve ATC specimens and six ATC cell lines showed an increased expression of hTERT mRNA but the expression levels of hTERT mRNA did not show a clear difference between ATCs and other thyroid tumors. The mean expression level of thyroglobulin mRNA in the ATCs and ATC cell lines was $<10^2$ times higher than that in the differentiated thyroid carcinomas. All twelve ATCs showed a loss of TTF-1 mRNA expression, but two cell lines, TCO-1 and 8505C, expressed TTF-1 mRNA. In conclusion, ATC tissues and cell lines were characterized by the expression of hTERT and a loss of thyroglobulin expression, concordant with the general recognition. However, the loss of TTF-1 expression can not characterize ATC cells, as some ATC cell lines expressed TTF-1 mRNA abundantly. This information could contribute to clarifying the nature of ATCs and could be useful in detecting TSCs, which have not yet been identified.

Introduction

Thyroid tumors are relatively common, especially in women of middle age. In differentiated thyroid carcinomas, including papillary and follicular carcinomas, distant metastases and local recurrence occur frequently, but fatal cases are rare, as these carcinomas usually show slow growth. Conversely, anaplastic thyroid carcinomas (ATC) are rare tumors and have a relentless and deadly clinical course with rapid progression and dissemination (1).

Since the discovery of cancer stem cells in various kinds of human tumors, many thyroid researchers have focused their study on ATCs, as certain studies have hypothesized that thyroid cancer cells are derived from remnants of fetal thyroid cells instead of normal follicular cells and that ATCs are derived from thyroid stem cells (TSCs), which have not yet been identified (2-5).

Stem cells or cancer stem cells are usually identified by using antibodies against their surface antigens or the specific expression pattern of their mRNAs. In either case, genes with an expression level that differs markedly when compared with that in other types of cells, need to be identified (2,5). Thus, the gene expression pattern that differentiates ATC cells from other cells is of great interest since this pattern can provide a clue for identifying TSCs.

However, information that can be used to further examine TSCs is far from satisfactory for the following two reasons: i) As ATCs are rare tumors, there are only a limited number of reports on specific gene expression in ATCs and very few ATC tissues have been examined in each study. ii) Most of the studies, especially those using immunohistochemistry, were not performed quantitatively (1,6). Thus, although an extreme difference in gene expression is usually necessary when using a cell separation apparatus such as a cell sorter, the extent of the differences in gene expression is not clear.

In previous studies, the increased expression of telomerase reverse transcriptase (hTERT) mRNA and protein and the loss of expression of some thyroid specific genes, such as thyroglobulin and thyroid transcription factor 1 (TTF-1), were observed in ATCs (7-9). It is generally recognized that these genes are useful markers for identifying ATC cells. Considering the above facts, we measured the copy numbers

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Table I. Characteristics of ATCs used in this study.

Case no.	Age	Gender	Histological subtype
1	77	F	Giant cell
2	77	F	Spindle cell
3	63	F	Pleomorphic
4	48	M	Pleomorphic
5	82	F	Spindle cell
6	80	F	Squamoid cell
7	35	F	Giant cell
8	61	F	Giant cell
9	63	F	Giant cell
10	73	F	Giant cell
11	80	F	Pleomorphic
12	54	F	Spindle cell

ATCs, anaplastic thyroid carcinomas.

of hTERT, thyroglobulin and TTF-1 mRNA in various kinds of cells and tissues derived from the thyroid including twelve ATCs and six cell lines, in order to evaluate the usefulness of these genes in distinguishing ATC cells from other cells in the thyroid.

Materials and methods

Extraction of RNA from thyroid tissues and cells. Tissue samples from ten follicular adenomas, six follicular carcinomas, eight papillary carcinomas, twelve ATCs and ten normal thyroid tissues from the lobe contralateral to the carcinoma, were collected at surgery after obtaining informed consent from each patient. Tissue samples of six medullary carcinomas were also collected to be used as an analogue of C cells. The clinical and pathological characteristics of ATCs are shown in Table I. All tissues were frozen in liquid nitrogen immediately after resection. The study protocol was approved by the institutional ethics committee.

Six cell lines derived from ATCs, SW579, KMH2, 8305C, 8505C, ASH-3 and TCO-1, were cultured in RPMI-1640 with 10% fetal bovine serum (Invitrogen, Tokyo, Japan). SW579 was purchased from the American Type Culture Collection (Manassas, VA, USA) and other cell lines were provided by the Human Science Research Resource Bank (Osaka, Japan). Total RNAs of these tissues and cell lines were extracted as described previously (6). RNAs from six thyroid-derived fibroblast cultures were also extracted as previously described (10).

Reverse transcription. Reverse transcription was performed using 1 μ g total RNA in an RT mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 0.5 mM deoxynucleotide triphosphates (dNTPs) (Takara, Shiga, Japan), 200 U M-MLV reverse transcriptase (Invitrogen), 2 U/ μ l RNase inhibitor (Takara), and 2.5 μ M

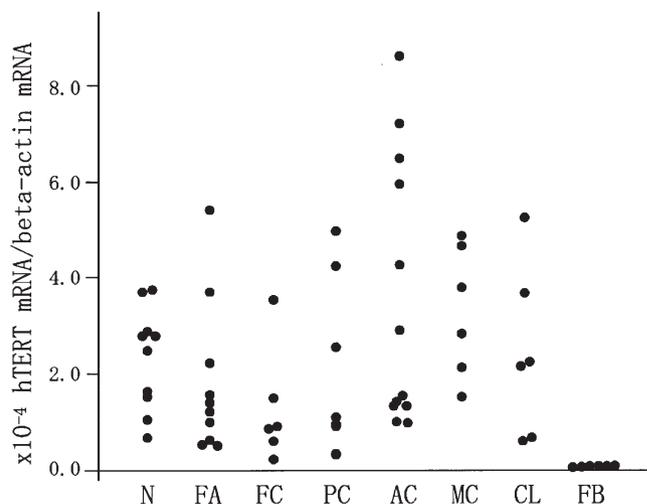


Figure 1. Expression levels of telomerase reverse transcriptase mRNA relative to beta-actin mRNA. The results are shown as the means from duplicate determinations. N, normal thyroid tissues; FA, follicular adenomas; FC, follicular carcinomas; PC, papillary carcinomas; AC, anaplastic thyroid carcinomas; MC, medullary carcinomas; CL, anaplastic thyroid carcinoma cell lines; FB, thyroid-derived fibroblasts.

random hexamer (Invitrogen) in a total volume of 20 μ l at 42°C for 60 min.

Real-time quantitative polymerase chain reaction. Real-time quantitative polymerase chain reaction (TaqMan PCR) using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was performed as described previously (6). One microliter of the first strand cDNA was used in the following assay. The two primers and one TaqMan probe used for the quantification of hTERT (*TERT*, GenBank NM003219), thyroglobulin (*TG*, GenBank X05615), TTF-1 (*TTF1*, GenBank BC006221) and beta-actin (*ACTB*, GenBank X00351) mRNAs were: [TERTF (0.5 μ M): 5'-CGGAAGAGTGTCTGGAGCAA-3' (base 1784-1803)], [TERTR (0.5 μ M): 5'-GGATGAAGCGGAGTCTGGA-3' (base 1928-1910)], and [TERT-TM (140 nM): 5'-FAM-CTGCACCCTCTTCAAGTGCTGTCTGATTCC-TAMRA-3' (1846-1817)]; [TGF (0.5 μ M): 5'-CTGCTGGCTCCACC TTGTTT-3' (base 2071-2090)], [TGR (0.5 μ M): 5'-CAGG GCGTGGGCGATTCTT-3' (base 2230-2211)], and [TG-TM (140 nM): 5'-FAM-CACTTCGAGTTCAGGAATGGCCT GACCCT-TAMRA-3' (2196-2167)]; [TTF1F (0.5 μ M): 5'-TCCAGAACCACCGCTACA-3' (base 746-763)], [TTF1R (0.5 μ M): 5'-ACGGTTTGCCGCTTTTAC-3' (base 928-910)], and [TTF1-TM (140 nM): 5'-FAM-ACTGCTG CTGAGCCTGTTGCTGC-TAMRA-3' (879-858)]; and [ACF (0.5 μ M): 5'-TGGACATCCGCAAAGACCTG-3' (base 901-920)], [ACR (0.5 μ M): 5'-CCGATCCACA CGGAGTACTT-3' (base 1066-1047)], and [AC-TM (140 nM): 5'-FAM-CACCACCATGTACCCTGGCATTGCC-TAMRA-3' (base 947-971)], respectively. All primers and probes were purchased from Operon Biotechnologies (Tokyo, Japan). The conditions for TaqMan PCR were as follows: 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. hTERT, thyroglobulin, TTF-1 or beta-actin cDNA, held in the recombinant pGEM Easy T-Vector (Promega, Madison, WI,

SPANDIDOS PUBLICATIONS Expression levels of hTERT mRNA in fibroblasts, ATC cell lines.

Tissue or cell	No. of cases	X10 ⁻⁴ hTERT mRNA/ beta-actin mRNA (mean ± SD)
Fibroblasts	6	0.0298±0.00113
ATCs and ATC cell lines	18	3.13±2.49

hTERT, telomerase reverse transcriptase; ATC, anaplastic thyroid carcinoma.

Table III. Expression levels of thyroglobulin mRNA in differentiated carcinomas, ATCs and ATC cell lines.

Tissue or cell	No. of cases	Thyroglobulin mRNA/ beta-actin mRNA (mean ± SD)
Differentiated carcinoma	14	0.670±0.502
ATCs and ATC cell lines	18	0.00410±0.0107

ATC, anaplastic thyroid carcinoma.

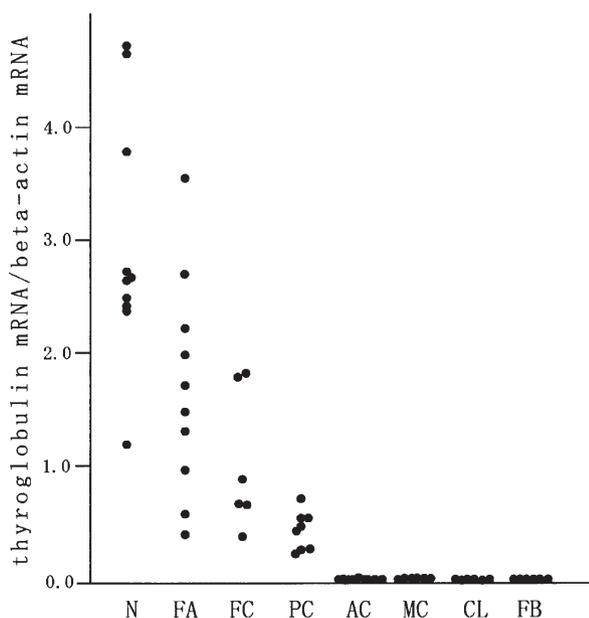


Figure 2. Expression levels of thyroglobulin mRNA relative to beta-actin mRNA. The results are shown as the means from duplicate determinations. N, normal thyroid tissues; FA, follicular adenomas; FC, follicular carcinomas; PC, papillary carcinomas; AC, anaplastic thyroid carcinomas; MC, medullary carcinomas; CL, anaplastic thyroid carcinoma cell lines; FB, thyroid-derived fibroblasts.

USA), were constructed by PCR-cloning with the same set of primers used in TaqMan PCR and these products were then 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 (ΔR_n) above the line of the optimal value (optimal ΔR_n) was first detected. The initial copy number of the target mRNA was calculated by a plot of the C_T against the input target quantity.

Results

High levels of hTERT mRNA expression were observed in all ATCs and cell lines derived from an ATC. Unexpectedly, a considerable number of normal thyroid tissues and differ-

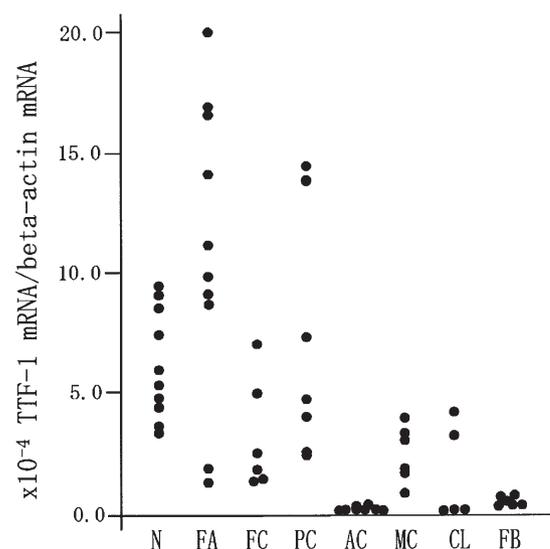


Figure 3. Expression levels of thyroid transcription factor 1 mRNA relative to beta-actin mRNA. The results are shown as the means from duplicate determinations. N, normal thyroid tissues; FA, follicular adenomas; FC, follicular carcinomas; PC, papillary carcinomas; AC, anaplastic thyroid carcinomas; MC, medullary carcinomas; CL, anaplastic thyroid carcinoma cell lines; FB, thyroid-derived fibroblasts.

entiated thyroid tumors including follicular adenomas, follicular carcinomas and papillary carcinomas also expressed a high level of hTERT mRNA (Fig. 1). Thus, there were no clear differences in the expression levels between differentiated tumors and ATCs. The mean expression level of hTERT in the ATCs and ATC cell lines was $<10^2$ times higher than that in the fibroblasts (Table II).

The expression of thyroglobulin mRNA was restricted to the normal thyroid tissues and differentiated tumors (Fig. 2). The mean expression level of thyroglobulin mRNA in the differentiated carcinomas (papillary and follicular carcinomas) was $<10^2$ times higher than that in the ATCs and ATC cell lines (Table III).

TTF-1 mRNA was expressed in the normal thyroid tissues and differentiated tumors. It was also expressed in the medullary carcinomas and two ATC cell lines, 8505C and TCO-1 (Fig. 3). The mean expression level of TTF-1 mRNA

Table IV. Expression levels of TTF-1 mRNA in differentiated carcinomas and ATCs.

Tissue or cell	No. of cases	X10 ⁻⁴ TTF-1 mRNA/ beta-actin mRNA (mean ± SD)
Differentiated carcinoma	14	5.69±4.79
ATCs	12	0.0838±0.0975

TTF-1, thyroid transcription factor 1; ATCs, anaplastic thyroid carcinomas.

in the differentiated carcinomas was <50x higher than that in the ATCs (Table IV).

Fig. 4 shows a comparison of the expression levels of thyroglobulin and hTERT mRNAs. All ATCs and ATC cell lines showed high expression levels of hTERT mRNA and a loss of expression of thyroglobulin mRNA. Fig. 5 shows a comparison of the expression levels of TTF-1 and hTERT mRNAs. All ATCs showed high expression levels of hTERT mRNA and a loss of TTF-1 mRNA expression. Normal thyroid, differentiated tumors, and medullary carcinomas could be distinguished from ATCs by the high expression level of TTF-1 mRNA. However, two of the six ATC cell lines, TCO-1 and 8505C, showed high expression levels of both hTERT and TTF-1 mRNAs. The classification of tissues and

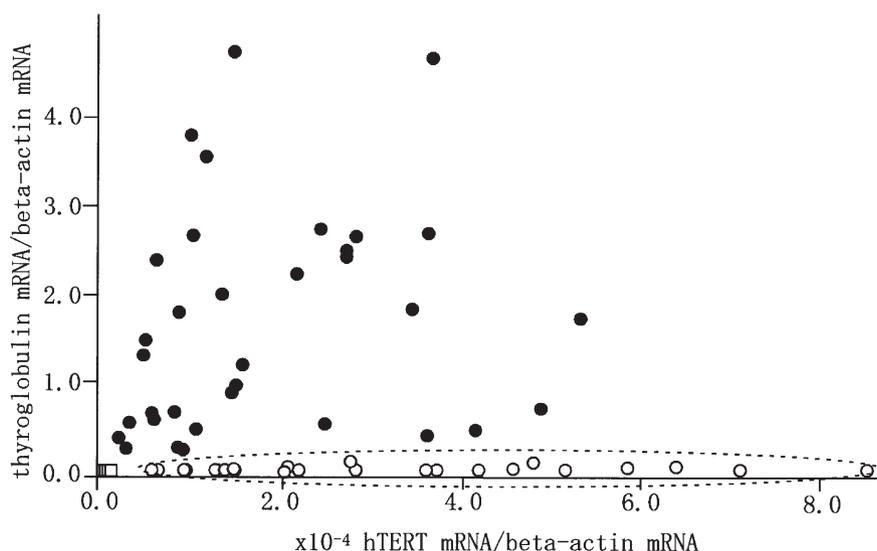


Figure 4. Comparison of the expression levels of telomerase reverse transcriptase and thyroglobulin mRNAs. The results are shown as the means from duplicate determinations. Closed circles, normal thyroid tissues and differentiated thyroid tumors; open circles, anaplastic thyroid carcinomas (ATCs) and ATC cell lines; grey circles, medullary carcinomas; open squares, thyroid-derived fibroblasts. The circle with the broken line shows the area where ATCs and ATC cell lines cluster.

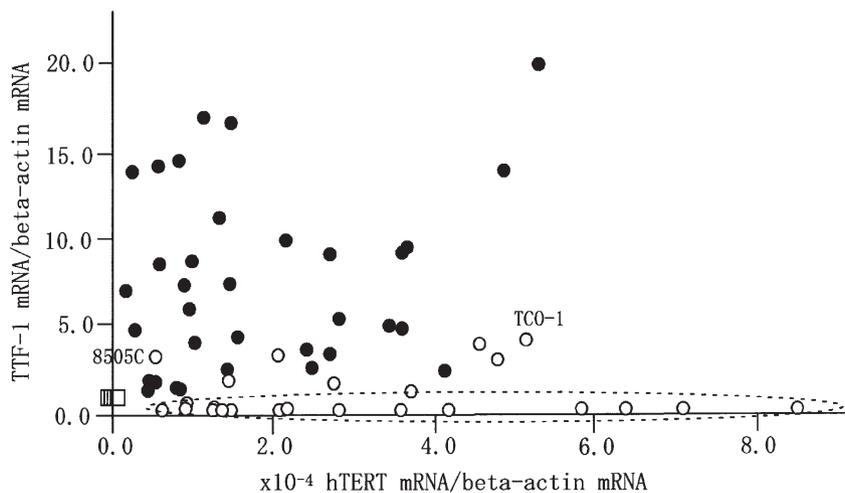


Figure 5. Comparison of the expression levels of telomerase reverse transcriptase and thyroid transcription factor 1 mRNAs. The results are shown as the means from duplicate determinations. Closed circles, normal thyroid tissues and differentiated thyroid tumors; open circles, anaplastic thyroid carcinomas (ATCs) and ATC cell lines; grey circles, medullary carcinomas; open squares, thyroid-derived fibroblasts. The circle with the broken line shows the area where ATCs cluster.

SPANDIDOS PUBLICATIONS Classification of thyroid-derived tissues and cells based on the expression of three genes.

hTERT	Thyro-globulin	TTF-1	Tissues and cells
+	+	+	Normal thyroid, follicular tumor, papillary carcinoma
+	-	+	Medullary carcinoma ATC cell line
+	-	-	ATC, ATC cell line
-	-	-	Fibroblast

hTERT, telomerase reverse transcriptase; TTF-1, thyroid transcription factor 1; ATC, anaplastic thyroid carcinoma.

cells in the thyroid based on the expression levels of these three genes is summarized in Table V.

Discussion

As ATC is a rare tumor, there are only a limited number of reports describing intensive analyses of the gene expression profile in ATCs. In previous studies, certain genes such as α 1 tubulin, Ubch10, and thymosin β 10 were overexpressed in ATCs (11-13). However, the reason for the overexpression of these genes in ATCs is closely related to the high proliferation rate of these cells. Thus, such genes are not likely to be useful in identifying resting ATC cells or related TSCs in thyroid tissues.

In the thyroid, thyroid follicular cells, peripheral blood cells, fibroblasts, and C cells, which are the origins of medullary carcinoma, are the major components besides tumor cells. Among these cells, fibroblasts show a gene expression pattern similar to that of poorly differentiated thyroid tumor cells (10). In this study, as shown in Fig. 1 and Table II, we demonstrated that fibroblasts can be clearly distinguished from ATC cells by the expression of hTERT mRNA.

In previous studies using immunohistochemistry, an overexpression of the hTERT protein was observed in ATCs but not in the differentiated tumors or normal thyroid (14). However, in our quantitative measurement study, some normal thyroid tissues and differentiated tumors also showed an increased expression of hTERT mRNA. Thus, the differentiation of ATCs from other thyroid tumors by the expression level of hTERT mRNA was quite difficult. This discrepancy could be caused by lymphocytes infiltrating normal thyroid tissues or differentiated tumors, as infiltrating lymphocytes in the thyroid showed a high expression level of hTERT mRNA (15). However, this was not likely to happen in the majority of the tested cases as they did not show lymphocyte infiltration by pathological examination (data not shown). Thus, the possibility that normal thyrocytes or differentiated tumor cells themselves express high levels of hTERT mRNA remains.

ATC is characterized by the increased expression of hTERT mRNA and the loss of thyroglobulin expression. However, medullary carcinomas, used in this study as analogues of C cells, were also classified into the same category. These two can be distinguished by the expression of TTF-1, as is shown in Figs. 3 and 5; medullary carcinomas but not ATCs expressed TTF-1 mRNA.

Discordant with the general recognition that ATCs or cells derived from ATCs are characterized by the loss of TTF-1 mRNA expression, two of the six ATC cell lines analyzed in this study showed a high expression of TTF-1 mRNA, presenting a gene expression profile similar to that of medullary carcinoma.

This fact leads to the following two speculations. The common origins of ATCs and ATC cell lines, probably TSCs, do not express TTF-1 mRNA and the two cell lines came to express TTF-1 mRNA by further differentiation. Alternatively, the possible TSCs do express TTF-1 mRNA but ATCs and some ATC cell lines lost the expression during the repeated proliferation and de-differentiation process. In this case, tumor cells that do not lose TTF-1 mRNA expression could become differentiated thyroid tumor cells that also express thyroglobulin. A recent study analyzing gene expression during fetal thyroid development supports the latter idea, since it proved the existence of cells that express TTF-1 but not thyroglobulin mRNAs at the very beginning of thyroid development (16).

The present study of the quantitative measurement of three representative genes in ATC cells provided an insight into the nature of these cells. These data provide a clue for clarifying the precise mechanism of ATC carcinogenesis and the biological characteristics of TSC, which remain mostly unknown at present.

Acknowledgements

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References

1. Ain KB: Anaplastic thyroid carcinoma: behavior, biology, and therapeutic approaches. *Thyroid* 8: 715-726, 1998.
2. Reya T, Morrison SJ, Clarke MF and Weissman IL: Stem cells, cancer, and cancer stem cells. *Nature* 414: 105-111, 2001.
3. Takano T: Fetal cell carcinogenesis of the thyroid: a hypothesis for better understanding of gene expression profile and genomic alternation in thyroid carcinoma. *Endocr J* 51: 509-515, 2004.
4. Takano T and Amino N: Fetal cell carcinogenesis: a new hypothesis for better understanding of thyroid carcinoma. *Thyroid* 15: 432-438, 2005.
5. Takano T: Fetal cell carcinogenesis of the thyroid: Theory and practice. *Semin Cancer Biol* 17: 233-240, 2007.
6. Takano T, Miyauchi A, Yokozawa T, *et al*: Preoperative diagnosis of thyroid papillary and anaplastic carcinomas by real-time quantitative reverse transcription-polymerase chain reaction of oncofetal fibronectin messenger RNA. *Cancer Res* 59: 4542-4545, 1999.
7. Hoang-Vu C, Boltze C, Gimm O, *et al*: Expression of telomerase genes in thyroid carcinoma. *Int J Oncol* 21: 265-272, 2002.
8. Giuffrida D and Gharib H: Anaplastic thyroid carcinoma: current diagnosis and treatment. *Ann Oncol* 11: 1083-1089, 2000.

9. Fabbro D, Di Loreto C, Beltrami CA, Belfiore A, Di Lauro R and Damante G: Expression of thyroid-specific transcription factors TTF-1 and PAX-8 in human thyroid neoplasms. *Cancer Res* 54: 4744-4749, 1994.
10. Takano T, Miyauchi A, Matsuzuka F, Kuma K and Amino N: Expression of oncofetal fibronectin messenger ribonucleic acid in fibroblasts in the thyroid: a possible cause of false positive results in molecular-based diagnosis of thyroid carcinomas. *J Clin Endocrinol Metab* 85: 765-768, 2000.
11. Takano T, Hasegawa Y, Miyauchi A, *et al*: Overexpression of α 1 tubulin mRNA in thyroid anaplastic carcinoma. *Cancer Lett* 168: 51-55, 2001.
12. Pallante P, Berlingieri MT, Troncione G, *et al*: UbcH10 overexpression may represent a marker of anaplastic thyroid carcinomas. *Br J Cancer* 93: 464-471, 2005.
13. Takano T, Hasegawa Y, Miyauchi A, *et al*: Quantitative analysis of thymosin beta-10 messenger RNA in thyroid carcinomas. *Jpn J Clin Oncol* 32: 229-232, 2002.
14. Ito Y, Yoshida H, Tomoda C, *et al*: Telomerase activity in thyroid neoplasms evaluated by the expression of human telomerase reverse transcriptase (hTERT). *Anticancer Res* 25: 509-514, 2005.
15. Saji M, Xydas S, Westra WH, *et al*: Human telomerase reverse transcriptase (hTERT) gene expression in thyroid neoplasms. *Clin Cancer Res* 5: 1483-1489, 1999.
16. Trueba SS, Auge J, Mattei G, *et al*: PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis-associated malformations. *J Clin Endocrinol Metab* 90: 455-462, 2005.