Introduction

Fine needle aspiration (FNA) cytology is the best diagnostic approach for thyroid nodules selecting patients who need to be operated. Papillary thyroid carcinoma (PTC), the most frequent thyroid malignancy, can be diagnosed by FNA cytology when its characteristic nuclear features are seen. Recently, testing for *BRAF* mutation in thyroid FNA material is reported to provide additional diagnostic information for PTC (1). However, follicular thyroid carcinoma (FTC), the second common thyroid malignancy, cannot be differentiated from benign follicular adenoma (FA) with FNA cytology because diagnostic hallmarks of FTC (vascular and capsular invasion) are hardly detected by cytology. Moreover, thyroid nodules showing follicular growth pattern including nodular hyperplasia and follicular variant of PTC are often not easily discriminated from follicular neoplasms (FN) on FNA cytology (2). FNA has similar defect in diagnosing Hürthle cell neoplasm (HCN) including Hürthle cell adenoma (HCA) and Hürthle cell carcinoma (HCC), which are considered as variants of FN. About 15–30% of FNA specimens are reported as "follicular neoplasm" or "suspicious follicular neoplasm" (3). Current guidelines recommend initial surgery for these categories to get pathologic diagnosis (4), but only 20–30% of the cases turn out to be malignancy which need surgery indeed (5). Some of those who reveal a carcinoma are advised to undergo a second operation, a complete thyroidectomy (6). To obviate these unnecessary surgeries, there should be methods to differentiate FTC from FA in preoperative setting. Various genetic or protein markers such as

PAX8/PPARγ rearrangement, *Ras* point mutation, telomerase activity, galectin-3 and trefoil factor 3 expression have been suggested as indicators for differential diagnosis of FN (6-9). More recently, some researchers performed genome-wide screening of FN using microarray study and suggested sets of gene combination for differential diagnosis (10-12). They reported that FTC including minimally invasive ones could be differentiated from FA correctly with each different set of target genes of their own. However, it is generally accepted that diagnostic reproducibility is reliable only for the widely invasive FTC group and there are considerable interobserver and intraobserver discrepancies in the diagnosis of minimally invasive FTC or FA (13). Moreover, some of FA show cell atypism, increased mitotic activity and/or tumor necrosis without definite capsular or vascular invasion and they might be regarded as precursors of malignancy. Virtually, it is not uncommon that cases initially diagnosed as FA turn out to be FTC due to distant metastasis after the operation (14). In this study, we validated 14 candidate genes that have been suggested as indicators for differential diagnosis of FN in genome-wide expression profiling studies with quantifying mRNA expression of each gene with real time-PCR in our FN fresh frozen tissue specimens. We also compared levels of mRNA expression of these genes according to the pathologic diagnosis, histologic features such as extent of invasion or cell atypism and immunohistochemical staining results.

Materials and methods

Patient data and tissue samples

A total of 16 consecutive patients with a diagnosis of FN or suspicious FN based on the FNA cytology for thyroid nodule underwent lobectomy or total thyroidectomy at Chung-Ang University hospital during the period of 2011 – 2013. Primary tumor tissues were obtained at time of surgery and normal thyroid tissue was obtained from the ipsilateral or contralateral lobe of each patient. All tumor samples were obtained with permission of patients and in accordance with the guidelines of the respective Institutional Review Boards. The tumor tissue and normal tissue of thyroid were respectively dissected to 0.2 x 0.2 x 0.2 cm and obtained tissues were snap frozen in liquid nitrogen, and stored at – 80℃. Histopathological evaluation of the primary surgical specimens was done according to the criteria of World Health Orgainization, whereby 9 tumors were diagnosed as FA, 6 as FTC, 1 as papillary carcinoma. All 6 FTC were classified as minimally invasive FTC and no widely invasive FTC. The 6 tissues diagnosed as FA, the 6 tissues as FTC from final pathologic report, and 6 normal thyroid tissues were used for analysis of gene expression levels.

RNA extraction and cDNA synthesis

Frozen tissues were homogenized by homogenizer in Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was prepared according to the manufacturer s protocol (Bioneer K3090). The concentration of RNA was measured using a

spectrophotometer Nano-Drop, ND-100 (Nano-Drop Technologies). RNA was reverse transcribed into cDNA as described. 20 μl reverse transcription reaction mixture containing 1 μg of template RNA, 2 μl of 10 x RT buffer (Promega), 2 μl of 5 mM dNTP s, 2 μl of 10 μ M Oligo dT primer, 0.25 μl of 40 units/ μl RNAse inhibitor, 1 μl of Omniscrip reverse transcriptase, RNase free water were incubated at 37℃ for 60 min, heated to 95℃ for 5-10 min, and the quick-chilled on ice.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantification

The gene expression levels of the 14 test genes and 1 control gene were quantified using RT-PCR. The 14 test genes were selected based on previous studies, which had reported the difference of gene expression levels for FA and FTC. The *β-actin* was evaluated as endogenous control. The sequences of PCR primers were noted in Table 1. For the polymerase chain reaction, twenty microliters of PCR master mix was incubated with 1 μl of cDNA, 0.5 μl of 10 μ M forward primer, 0.5 μl of 10 μM reverse primer, 10 μl of 2X PCR premix and 8 μl of D.W. The amplification profile involved denaturation at 94℃ for 30 sec, primer annealing at 58℃ for 30 sec, and extension at 72℃ for 1 min. The mixture was brought to 4℃. After the amplification, 10 μl of the RT-PCR products were separated in 2% agarose gels, stained with ethidium bromide and analyzed by direct visualization under ultraviolet light.

Table 1. Sequences of primers used in RT-PCR

Number	Gene	Primer	Sequence of oligonucleotide	Product size (bp)	Cycles
1	*TERT*	Forward	GGCGACGACGTGCTGGTTCA	380	28
		Reverse	CAGAAACCACGGTCACTCGG		
2	*TFF3*	Forward	GCTGTCCTCCAGCTCTGCTG	190	30
		Reverse	GCATTCTGCTTCCTGCAGGG		
3	*DDIT3*	Forward	GACCTGCAAGAGGTCCTGTC	340	22
		Reverse	CTTCAGCTAGCTGTGCCACT		
4	*ARG2*	Forward	CAACCAGGAACTGGCTGAGG	410	22
		Reverse	CCATGACCTTCTGGATACCA		
5	*ITM1*	Forward	CACCATCGTCACGTACCACC	430	25
		Reverse	GGCTGCCATGTGCTCTGATG		
6	*C1orf24*	Forward	GGCTCAGCCTCCAGCCAGCT	420	25
		Reverse	GTCTGGGAAATGCCTGTCAG		
7	*PLAB*	Forward	CGAGGACCTGCTAACCAGGC	350	22
		Reverse	GTGCGGACGAAGATTCTGCC		
8	*CCND2*	Forward	GCAGAACCTGCTCACCATCG	380	25
		Reverse	CAGCTGCCAGGTTCCACTTC		
9	*PCSK2*	Forward	GCCATGGCCGATGGCGTGAA	390	30
		Reverse	GGAGGTGAGCACAGTCAGAT		
10	*c-Met*	Forward	GTGCTTGCACCTGGCATCCT	420	25
		Reverse	GACATGTCGCTGGCAGGTCC		
11	*EMMPRIN*	Forward	CGACCAGTGGGGAGAGTACT	430	22
		Reverse	CAGCACCAGCACCTCAGCCA		
12	*Adrenomedullin*	Forward	CCTGATGTACCTGGGTTCGC	340	25
		Reverse	CAGCTTCTGCACCGTGCACG		
13	*Autotaxin*	Forward	GAGCTCGTTCCAGTCGTGTC	480	28
		Reverse	GGGCGAACAAACCCTGCAGG		
14	*TGF β II*	Forward	GCACATCGTCCTGTGGACGC	500	28
		Reverse	CAACTCCCAGTGGTGGCAGG		
15	*β-actin*	Forward	GTACGTTGCTATCCAGGCTG	370	22
		Reverse	GAACCGCTCATTGCCAATGG		

TERT, telomerase reverse transcriptase; *TFF3,* trefoil factor 3 (intestine); *DDIT3,* DNA damage-inducible transcript 3; *ARG2*, arginase 2; *ITM1*, integral membrane protein 1; *C1orf24*, chromosome 1 open reading frame 24; *PLAB*, prostate differentiation factor; *CCND2*, cyclin D2; *PCSK2*, protein convertase 2; *EMMPRIN*, extracelluar matrix metalloproteinase inducer; TGF, transforming growth factor

Real time RT-PCR assay

Real time RT-PCR was performed to quantify expression levels of the 14 genes. Quantitative PCR was performed using CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) with SYBR Green master mix (Bio-Rad #172-5121) and primers (Table 2). The primers for candidate molecular markers were designed using Primer3-web interface, a primer design program (http://primer3.ut.ee). Triplicates were prepared with 4-fold prediluted cDNA. For quantitative PCR, ten microlitres of PCR master mix was incubated with 1 μl of cDNA, 1 μl of 4 μM forward primer, 1 μl of 4 μM reverse primer, 5 μl of 2X PCR premix and 2 μl of D.W. The thermal cycling condition comprised an initial denaturation at 95℃ (10 min), followed by 40 cycles of amplification at 95℃ (5 s) and 60℃ (10 s). Melt curves were generated to verify the purity of the amplicons. Data were obtained as average Ct values and normalized against the endogenous controls, *β-actin* as ΔCt. Expression of each target gene, normalized to an endogenous reference and relative to a calibration standard is measured as fold change using the comparative Ct method ($2^{-\Delta\Delta Ct}$ where $\Delta\Delta$Ct = ΔCt of adenoma and carcinoma − ΔCt of normal tissue) (15).

Table 2. Sequences of primers used in real time RT-PCR

Number	Gene	Primer	Sequence of oligonucleotide	Product size (bp)
1	*TERT*	Forward	CTA CTC CTC AGG CGA CAA GG	110
		Reverse	TGG AAC CCA GAA AGA TGG TC	
2	*TFF3*	Forward	CAT GTC ACC CCC AAG GAG	104
		Reverse	AAG GTG CAT TCT GCT TCC TG	
3	*DDIT3*	Forward	CAG AAC CAG CAG AGG TCA CA	100
		Reverse	CTC CCT TGG TCT TCC TCC TC	
4	*ARG2*	Forward	ACT CCA GTC CCC AAA GAT GA	98
		Reverse	GCT CTG CTA ACC ACC TCA GC	
5	*ITM1*	Forward	CTC CAC TCG TCT GTT TGC TG	92
		Reverse	CAG CCA GGA ACC TGG TAG TC	
6	*C1orf24*	Forward	GGA AAT GAT CAC TGG GGA TG	99
		Reverse	CCC TTC ATC TTA GGC AGC AG	
7	*PLAB*	Forward	CTA CAA TCC CAT GGT GCT CA	91
		Reverse	TAT GCA GTG GCA GTC TTT GG	
8	*CCND2*	Forward	CAC TTG TGA TGC CCT GAC TG	98
		Reverse	ACG GTA CTG CTG CAG GCT AT	
9	*PCSK2*	Forward	CTG CAC TCT GAG GCA TTC TG	93
		Reverse	CAG GTC AGA CCC AGG TTA GC	
10	*c-Met*	Forward	CAT GGA AAT GCC TCT GGA GT	96
		Reverse	CTG ACA TAC GCA GCC TGA AG	
11	*EMMPRIN*	Forward	GAC GAC CAG TGG GGA GAG TA	100
		Reverse	ACT TCA CGG CCT TCA CTC TG	
12	*Adrenomedullin*	Forward	CTA CCG CCA GAG CAT GAA C	92
		Reverse	AGA TCT GGT GTG CCA GCT TC	
13	*Autotaxin*	Forward	TGC AAT AGC TCA GAG GAC GA	100
		Reverse	AGA AGT CCA GGC TGG TGA GA	
14	*TGF β II*	Forward	CTG TAT GCA GAG GCT GAC CA	93
		Reverse	CTT CTC TAG CCA GGC AGC AC	
15	*β-actin*	Forward	CGT CAT ACT CCT GCT TGC TG	94
		Reverse	CCA GAT CAT TGC TCC TCC TGA	

TERT, telomerase reverse transcriptase; *TFF3,* trefoil factor 3 (intestine); *DDIT3,* DNA damage-inducible transcript 3; *ARG2*, arginase 2; *ITM1*, integral membrane protein 1; *C1orf24*, chromosome 1 open reading frame 24; *PLAB*, prostate differentiation factor; *CCND2*, cyclin D2; *PCSK2*, protein convertase 2; *EMMPRIN*, extracelluar matrix metalloproteinase inducer; TGF, transforming growth factor

Pathology and immunohistochemical study

All follicular neoplasm cases were microscopically re-examined by a pathologist with a special interest in thyroid (professor Kim Hee Sung) and pathologic features including degree of cell atypia, presence of oncocytic change, number of capsular and vascular invasion sites were described. Immunohistochemical study was performed on 4 mm thick sections using the labeled streptavidin-biotin peroxidase complex system (LSAB2) in a Ventana autostainer (Ventana Medical Systems, Tucson, AZ, USA). Heat-induced antigen retrieval was carried out for Galectin-3, Ki67 and HBME-1 and sections were incubated with primary antibodies (Table 3) for 30′ -60′ at room temperature. After primary antibodies, all sections were blocked for endogenous avidin and biotin by incubating with avidin solution for 20′ followed by biotin solution for 20′ (Dako's avidin/biotin blocking system, X0590). Positive controls were small bowel for Galectin-3, an epithelioid mesothelioma for HBME1 and spleen for Ki67. Appropriate negative controls by substituting primary antibody with isotype-matched mouse or rabbit IgG were also included (Table 3). The protein expression was initially assessed along a scale of 0-100% of tumor cells showing immunoreactivity. After evaluating normal and hyperplastic thyroid tissues, positive (increased) expression was defined as presence of ≥10% immunoreactive thyrocytes.

Table 3. Antibodies used in immunochemical study

Antibody	Clone	Dilution	Source
Galectin-3	Monoclonal	1:80	Thermo Fisher Scientific, CA, USA
Ki-67	Monoclonal	1:200	Thermo Fisher Scientific, CA, USA
HBME1	Monoclonal	1:50	Thermo Fisher Scientific, CA, USA

Statistical analysis

A Kruskal-Wallis test was used to determine whether there was a significant difference in the mean ratios of gene expression level between the three groups of normal thyroid tissues, FA and FTC. Mann-Whitney test was used to compare the mean mRNA ratios between each two groups of normal thyroid tissues, FA and FTC. Spearman correlation analysis was performed to identify the relation between fold changes of gene expression and clinicopathologic factors. All statistical determination were performed on computer (SPSS for Window, ver. 18; SPSS Inc., Chicago, IL). A $P < 0.05$ was considered statistically significant.

Results

RT-PCR

For assessment of the levels the various mRNAs in FN, the band density on agarose gel was evaluated with the aid of Labwork V8.0 software (Ultra-violet Product, Cambridge, UK). The ratio of PCR products level of test genes to beta actin as a housekeeping gene were compared among normal tissue, FA and FTC.

We could identify ethidium bromid-stained bands on agarose gels in all RT-PCR products of 14 genes. We did not evaluate the density of the bands of RT-PCR products to quantify the expression level of each gene. Instead, we performed real time RT-PCR for a better quantification of expression levels of 14 test genes.

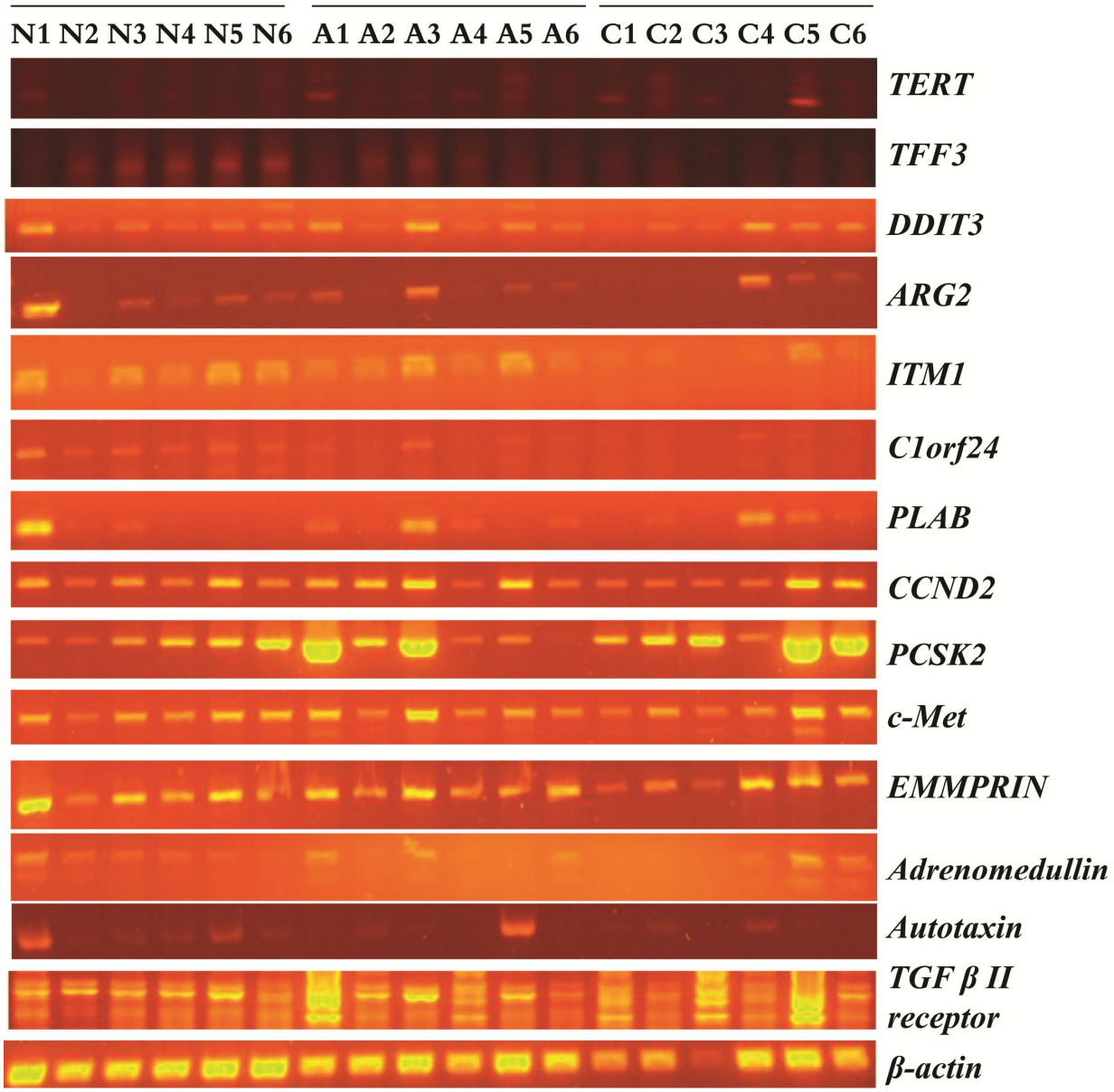


Figure 1. Results of RT-PCR of 14 candidate genes. N, normal tissue; A, adenoma; C, carcinoma

Real time RT-PCR

The fold changes of expression of 14 test genes between the three groups were evaluated by non-parametric Kruskal-Wallis test. Among 14 test genes, 5 genes (*TERT*, *TFF3*, *DDIT3*, *ITM1* and *C1orf24*) were differently expressed with statistical significance in the three groups (Fig 2). *TERT* gene expression showed high fold changes in FA and FTC than in normal tissue. However it did not show statistically significant difference between FA and FTC. The expression of *TFF3* were lower in FA and FTC rather than in normal tissue. There was no difference of fold changes of *TFF3* gene expression between FA and FTC. The fold changes of *DDIT3* gene expression were more increased in FTC than normal tissue, but statistical difference was found between FA and FTC. Though expression of *ITM1* was increased in FA rather than in normal tissue, there was no difference between FA and FTC. *C1orf24* was highly expressed in FTC compared to normal tissue or FA. *C1orf24* was the only gene whose expression was significantly different between FA and FTA (Fig 2).

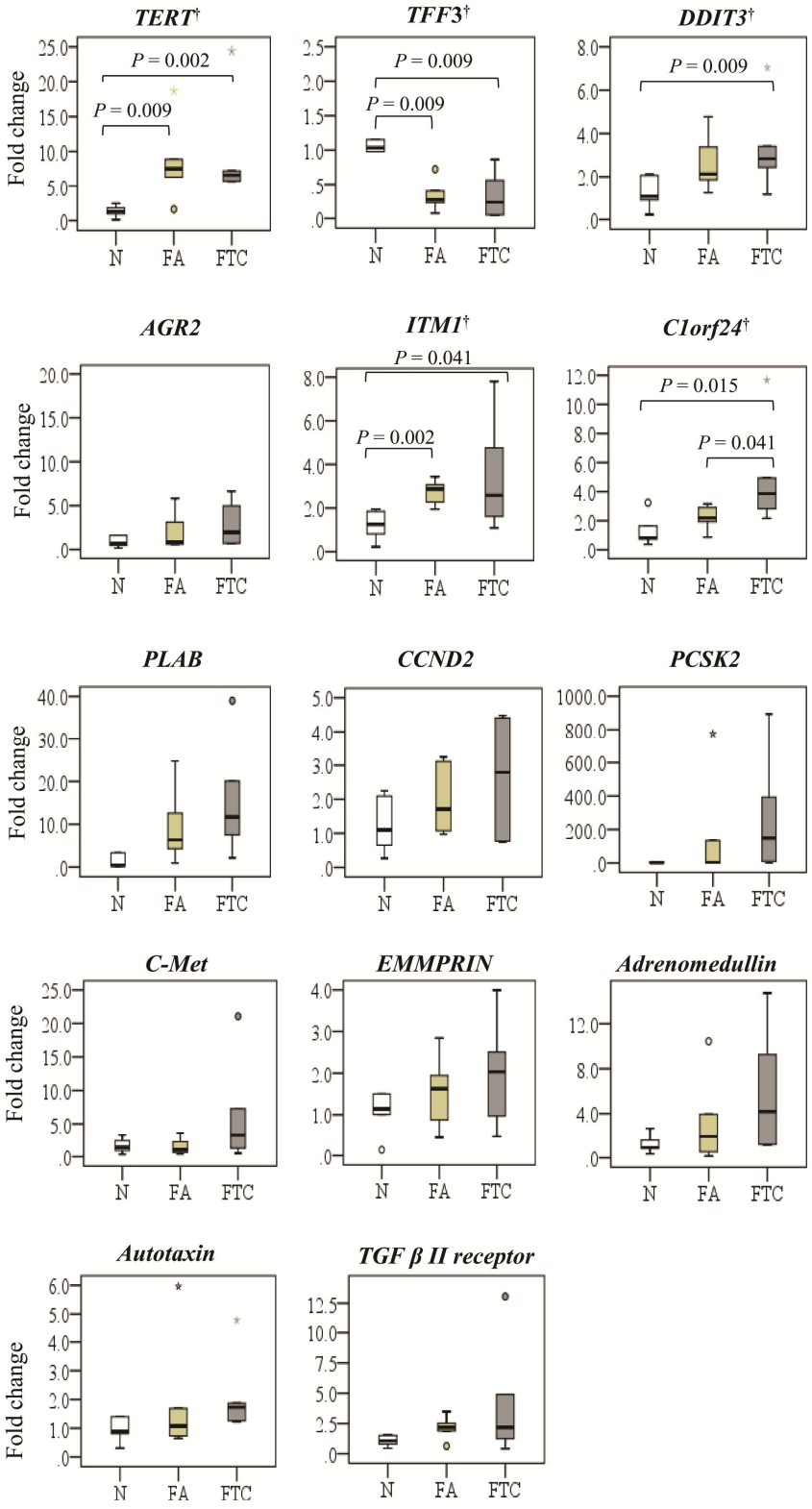


Figure 2. Boxplots illustrating fold changes of expression of 14 candidate genes in normal thyroid tissue (N), follicular adenoma (FA) and follicular thyroid carcinoma (FTC). The cross marks mean statistical significance ($P < 0.05$).

Pathology and immunohistochemical study

Table 4 shows pathologic features and results of immunohistochemical study in follicular neoplasm cases. We evaluated associations of pathologic features and results of immunohistochemical study with expression levels of the 5 genes (*TERT*, *TFF3*, *DDIT3*, *ITM1* and *C1orf24*) which were differently expressed in follicular neoplasm from normal thyroid tissue in real time RT-PCR. There were no statistical significance in correlation with gender, age and fold changes of gene expression. The fold change of *C1orf24* expression showed positive correlations with tumor size, number of capsular invasion sites and Ki-67 labeling index (Fig 3, 4). Positive galectin-3 staining cases showed significantly low fold changes of *TFF3* expression (Fig 5, 6). When the oncocytic change was present, the fold changes of *DDIT3* were increased (Fig 7). Expression levels of *TERT* and *ITM1* had not positive correlation with any pathologic feature or immunohistochemical staining. Although fold changes of *ARG2* and *PLAB* expressions failed to show statistical difference between the three groups in real time RT-PCR, they showed positive correlation with number of vascular invasion sites (Fig 8).

Table 4. Histologic features of follicular neoplasms and results of immunohistochemical study

Diagnosis	Gender	Age	Size (cm)	Nuclear atypia	Oncocytic change	Number of capsular invasion sites	Number of vascular invasion sites	HBME-1	Galectin-3	Ki67 index(%)
FA	F	50	1.5	Mild	Y	0	0	+	-	<1
FA	M	49	2.6	Mild	N	0	0	-	-	3
FA	F	28	3.3	Mild	Y	0	0	+	-	3
FA	M	73	1.2	Mild	N	0	0	-	-	<1
FA	M	49	0.8	No	N	0	0	-	-	<1
FA	F	68	4	No	Y	0	0	-	-	2
MI-FTC	F	32	4.5	No	N	2	0	-	-	2
MI-FTC	F	53	3.3	Moderate	N	2	0	+	-	5
MI-FTC	M	52	3.1	Mild	Y	5	1	-	+	5
MI-FTC	F	72	5	Mild	Y	3	6	-	+	3
MI-FTC	F	53	2.2	Moderate	Y	1	1	+	-	1
MI-FTC	F	24	4.2	Moderate	Y	1	1	+	-	5

FA, follicular adenoma; MI-FTC, minimally invasive follicular thyroid carcinoma

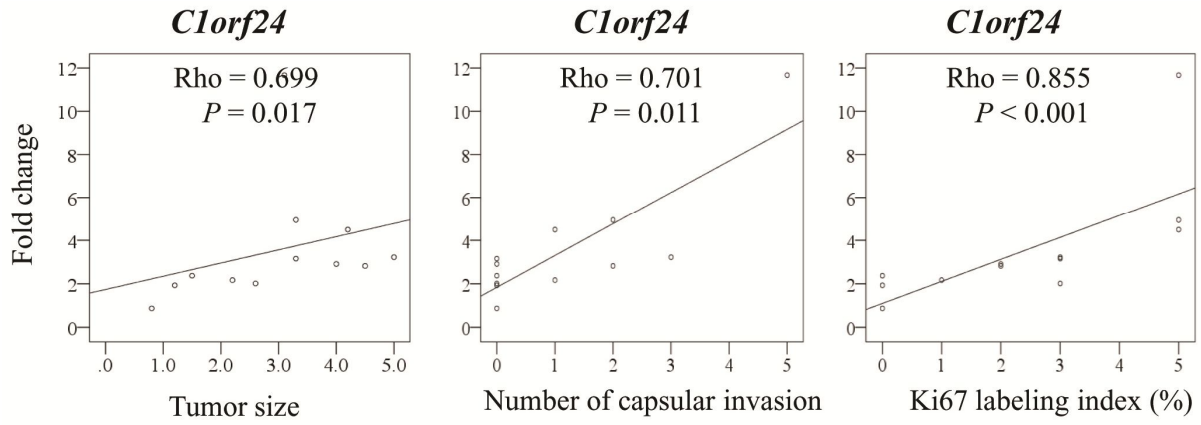
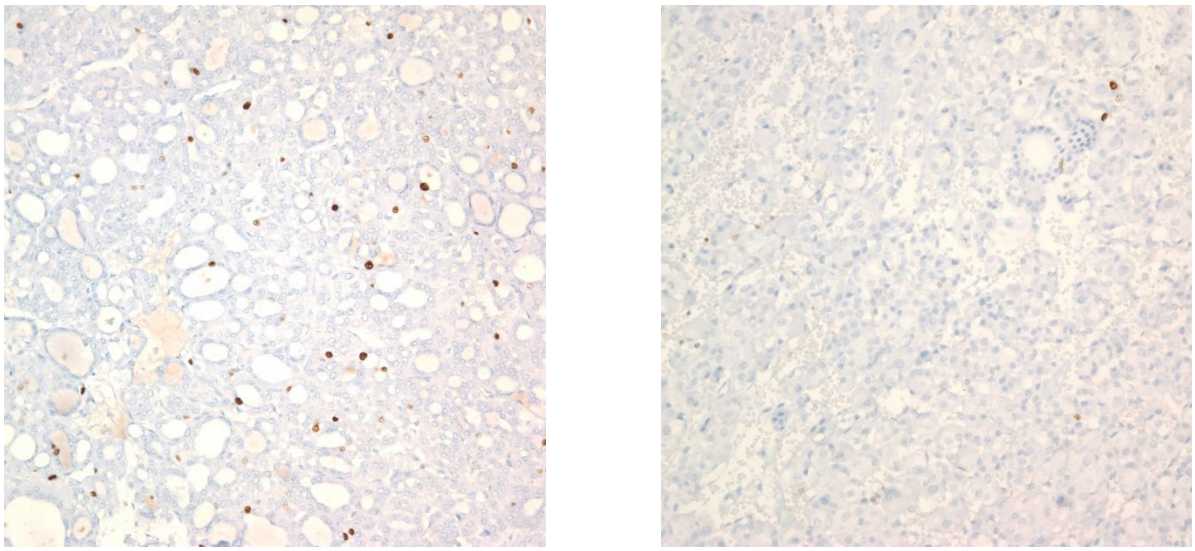


Figure 3. Positive correlations of expression of *C1orf24* with tumor size, number of capsular invasion sites and Ki-67 index



Figue 4. Left; High Ki-67 index case which showed high fold change of *C1orf24* expression, Right; Low Ki-67 index case which showed low fold change of *C1orf24* expression

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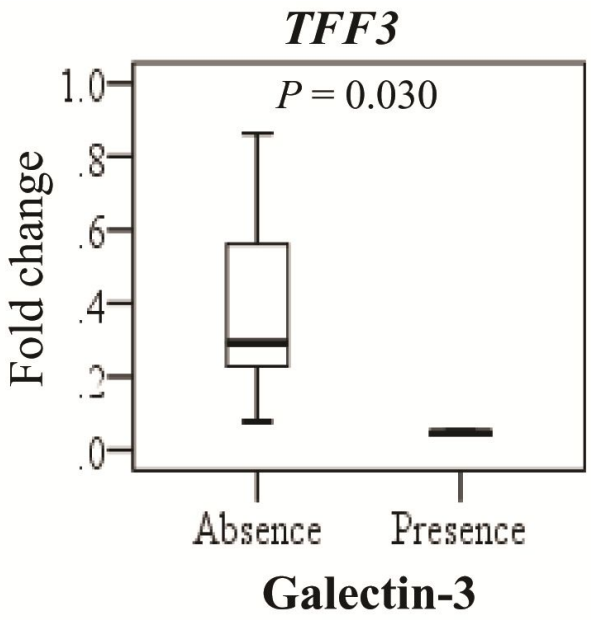

Figure 5. Negative correlation between expression of *TFF3* and galectin-3 positivity

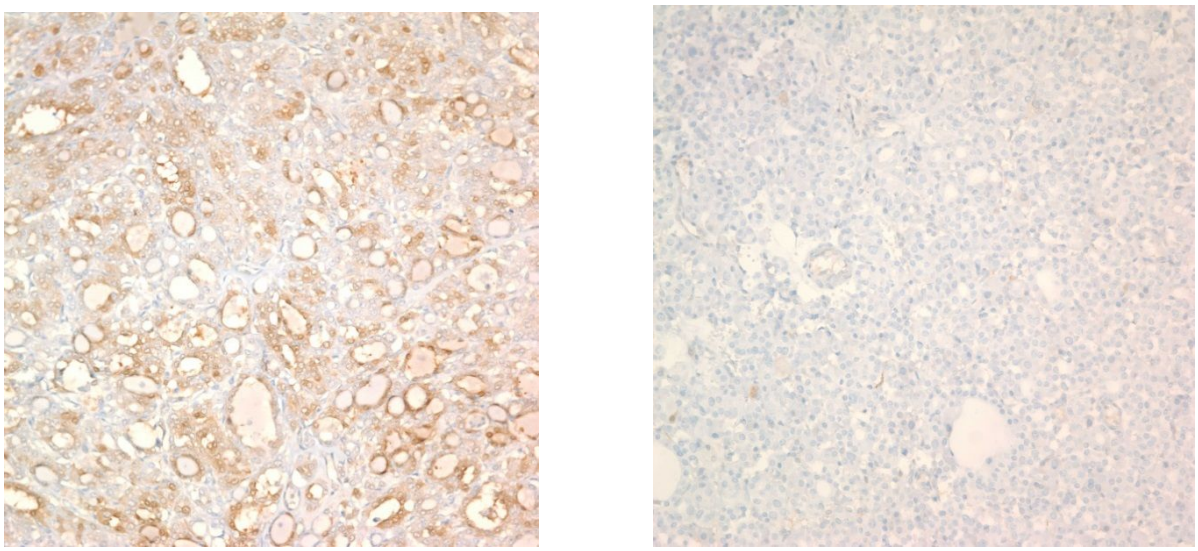

Figure 6. Left; Positive galectin-3 staining case which showed depressed *TFF3* expression, Right: Negative galectin-3 staining case which showed depressed *TFF3* expression

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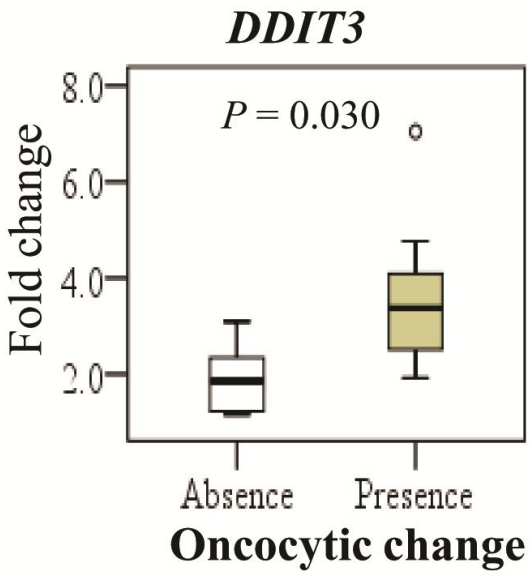

Figure 7. Positive correlation between presence of oncocytic change and fold change of *DDIT3*

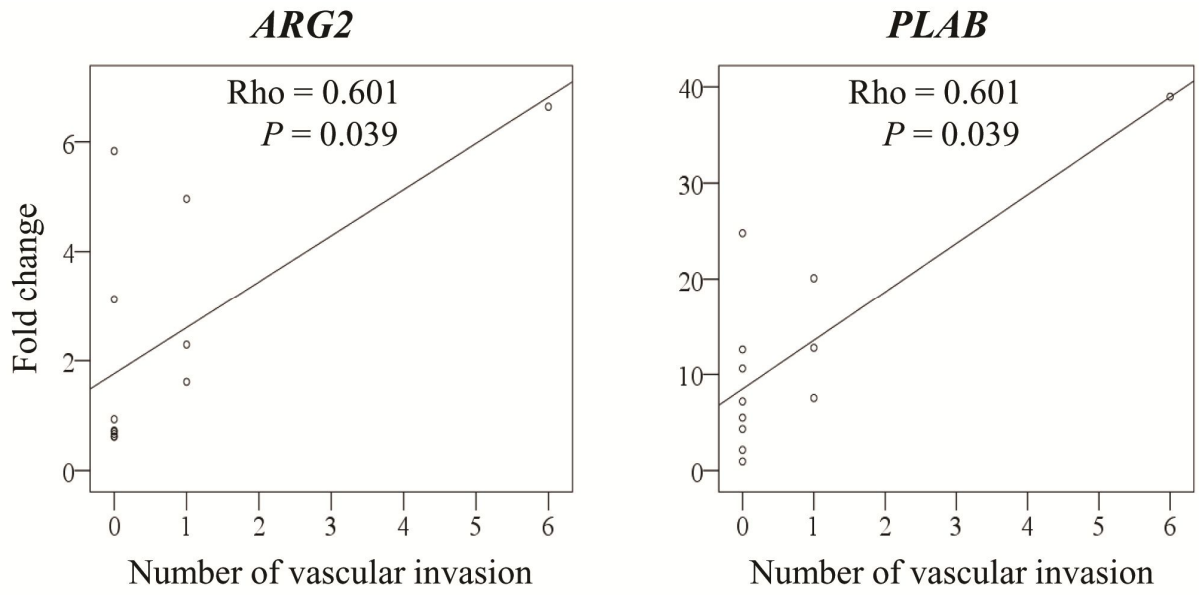

Figure 8. Positive correlations of expression of *ARG2* and *PLAB* with number of vascular invasion sites

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Discussion

Thyroid nodules are common, with a lifetime risk of developing a clinically significant thyroid nodule of 10% or higher. Preoperative diagnosis was greatly enhanced by the introduction of FNA in the 1970s, but there has been little advancement since that time. Discrimination between benign and malignant FN is currently not possible by FNA and can even be difficult after full pathologic review, because partial and not penetrated capsular invasion can also be observed in FA. Moreover, both FA and FTC may have various degrees of cellular atypia and extensive vascular invasion is not commonly detected in MI-FTC (16). The aggressiveness spectrum of FTC is wide. While patients with metastatic and/or widely invasive FTC have a relatively poor prognosis, patients with minimally invasive FTC rarely have an aggressive course (17). Sometimes histopathologically proven FA patient might present or develop metastasis during follow-up. These conditions bring the necessity of refinement of the diagnosis of FTC based not only on the histopathology but only on the molecular markers. Genome-wide scan using microarray is a powerful technology assessing the expression of numerous genes in parallel. Barden et al. conducted a microarray-based study to determine the genetic profiles of FTC and FTA (12). The group described a 105-gene expression classifier that differentiated between the two entities with high accuracy, although their model still failed to identify minimally invasive FTCs. Weber et al. performed gene expression profile of 12 FTA and 12 FTC (10). A new classification model, based on the expression levels of three genes, *PCSK2*, *CCND2* and *PLAB*, allowed the authors to discriminate between

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FTAs and FTCs with a high sensitivity of 100% and specificity of 96.7%. *PCSK2* and *CCND2* were highly expressed in FTAs whereas *PLAB* was highly expressed in FTC. A comprehensive approach for expression profiling is Serial Analysis of Gene Expression (SAGE), which provides digital information on transcript levels. The ability for evaluating the expression pattern of thousands of genes in a quantitative fashion without prior sequence information is one of the most attractive features of SAGE (18). SAGE produces a comprehensive profile of gene expression and can be used to search for candidate tumor markers (11). This approach has also been used to identify diagnostic and prognostic markers in thyroid (6,11,19). However, very few of these markers which were suggested after high throughput technique have been validated in other studies and no marker has been proven to be of practical value of for preoperative FNA diagnosis of FTC. In this study, we tried to validate these genetic markers by measuring the level of mRNA expression with RT-PCR in our FN fresh frozen tissue specimens rather than perform another genome-wide scan.

In this study, 14 molecular markers selected from other genome-wide methods were evaluated by real time RT-PCR in 18 thyroid tissues including 6 normal controls, 6 FA and 6 FTC. We could find five genes (*TERT*, *TFF3, DDIT3*, *ITM1* and *C1orf24*) whose expressions were different in FN from normal thyroid tissue.

TERT gene encodes telomerase reverse transcriptase which comprises the most important unit of the telomerase complex (20). Telomerase activation has been observed in ~90% of all human tumors and Saji et al. first suggested *TERT* gene as

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a potential marker of malignancy in follicular tumors by non-quantitative PCR methods (21). Recently, frequent *TERT* promotor mutations in advanced forms of thyroid carcinoma were reported by two different study groups (22,23).

TFF3 gene encodes trefoil factor 3, a family of peptides which are mainly synthesized and secreted by epithelial cells in the gastrointestinal tract. Takano et al. first identified that *TFF3* mRNA expression was suppressed in FTC compared with FA using SAGE (24) and microarray studies also demonstrated its reduced expression in FTC (12,25). The combination of *TERT* and *TFF3* was suggested as the most useful model for differentiating FN by Foukakis et al. after performing quantitative RT-PCR of 26 potential markers (26).

DDIT3 is a transcription factor being induced in response to cellular stress (27). Induction of DDIT3 leads to growth stimulation, differentiation, invasiveness and migration (28). *ITM1* encodes a conserved protein with 10-14 transmembrane domains without any enzymatic activity and this protein is regarded not to have any direct transmembrane signaling function (29). The function of C1orf24 and the pathways through which it acts also remain to be elucidated. C1orf24 does, however, contains a DnaJ domain, a feature of heat shock protein that was first described as an oncogene associated with renal carcinogenesis (30). Associations of gene expressions of *DDIT3*, *ITM1* and *C1orf24* with FTC proposed by Cerutti et al. that performed SAGE and confirming real time RT-PCR. They reported that a linear combination of expression levels of four genes (*DDIT3*, *ITM1*, *C1orf24* and *ARG2*) distinguished FTC from FA with an estimated accuracy of 0.83 (11).

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However, unlike previous studies of others, in our study, expressions of *TERT*, *TFF3*, *DDIT3* and *ITM1* just differed between FN and normal thyroid tissue, they were not helpful for differentiation of FTC from FA and *C1orf24* was the only gene whose expression was significantly different between FA and FTA.

There could be several reasons that can explain these results.

For one thing, not established and not uniformed bioinformatics methods for analysis of large amounts of sequencing data in genome-wide methods might lead selection of cancer genetic markers which cannot be applied to other settings. Genome-wide approaches are known to be very effective in detecting cancer genetic markers. However, they require specialized support for bioinformatics analyses of genomic data (31). Each different method of analysis of these large data can select different genetic markers. Validated genes in this study were selected from 3 independent genome-wide studies (10-12)and each study suggested totally different set of genes as genetic markers which might be caused by not established and not uniformed bioinformatics methods for analysis. That might be one of the reasons why none of these markers could be applied in our specimens.

For another, the difficulty in differentiating FA from minimally invasive FTC might affect the current results. The diagnosis of minimally invasive FTC is one of the most controversial issues in thyroid pathology (32). On the contrary, widely invasive FTC shows vivid characteristics such as extensive capsular and vascular invasion that can be differentiated from FA or minimally invasive FTC. Some

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studies have shown that molecular characteristics of widely invasive FTC are also quite different from FA or minimally invasive FTC without distant metastasis (24,26). Our FTC cases were all minimally invasive FTC and none of widely invasive FTC. That might cause the results that there was little difference in mRNA expression of genetic markers between FA and FTC.

For last thing, very small number of cases possibly prevent us from showing statistical difference and that is a considerable limitation of our study. Although FTC is known to comprise about 10–22% of all thyroid malignancies (33), it seems relatively rare in Korea because of high proportion of micropapillary carcinoma. Therefore, it is supposed that it is quite difficult to collect enough cases of FN to commit a molecular study for developing methods of differential diagnosis of FN in a single institute.

Based on our results, we could infer that to develop reliable genetic markers for differential diagnosis of FN that can be applied in general practice, there might be some requisites. There should be enough number of specimens from multicenter, collected under uniform condition and principle and genome-wide and high throughput technique with firmly established, uniform bioinformatics methods for analysis of large amounts of sequencing data.

Nevertheless, when we evaluated correlations of the molecular study results with pathologic characteristics and immunohistochemical staining results, some positive associations were detected. Ki-67 index, a cellular marker for proliferation has a marked correlation with the expression of *C1orf24*. Extent of capsular invasion has

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very strong correlations with the expression of *C1orf24*. Extent of vascular invasion has very strong correlations with the expression of *ARG2* and *PLAB*. These results suggest that for invasive FTC which have large extent of capsular and/or vascular invasion, not only *C1orf24* but also *ARG2* and *PLAB* would be useful markers that can differentiated FTC from FA.

In summary, among 14 molecular markers selected from other genome-wide methods, *TERT*, *TFF3*, *DDIT3*, *ITM1* and *C1orf24* genes were differently expressed between FN and normal thyroid tissue and *C1orf24* was the only genetic marker that expressed highly in FTC compared to FA. *ARG2* and *PLAB* were potential markers indicating vascular invasion in FN.

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국문초록

서론: 갑상선 여포종양은 미세침흡인세포검사와 수술 중 동결절편검사가 감별진단에 거의 도움이 되지 않기 때문에 다루기가 매우 까다롭다. 수술 전 미세침흡인검사에서 여포종양으로 진단된 병변은 실제로 결절성 증식증, 여포 혹은 허들세포선종, 여포 혹은 허들세포암, 갑상선유두암의 여포변종 등 다양한 병리를 포함할 수 있으며, 이러한 진단은 대부분 병변이 포함된 갑상선엽을 절제한 후 면밀한 검사 후에나 진단이 된다.

방법: 저자들은 타 genome-wide expression profiling 연구들에서 갑상선 여포종양의 감별진단을 위한 표지자로 제시된 14개의 유전자들 (*TERT, TFF3, DDIT3, ARG2, ITM1, C1orf24, PLAB, CCND2, PCSK2, c-Met, EMMPRIN, Adrenomedullin, Autotaxin, and TGF β II receptor*)을 선정하였다. 이 유전자 표자자들을 6개씩의 정상 갑상선조직, 갑상선 여포선종, 그리고 여포암의 신선 동결 조직 표본에서 real time RT-PCR을 시행하여, 이 유전자 표지자들의 여포종양의 감별진단을 위한 유용성을 평가하고 유전자 발현과 여포종양의 병리학적 특성과의 관련성을 조사하였다.

결과: *TERT, TFF3, DDIT3, ITM1 및 C1orf24* 의 다섯 개의 유전자에서 정상조직과 여포종양에서 발현의 차이를 나타내었다. 그 중 *Clorf24* 의 mRNA 발현이 여포선종에서 보다 여포암 조직에서 더 높게 나타났다 ($P = 0.041$). *Clorf24* 의 발현은 종양 세포의 피막 침범의 정도 및 Ki-67 지수와 양의 상관관계를 보였으며, *ARG2* 와 *PLAB* 의 발현은 종양 세포의 혈관 침범 정도와 상의 상관관계를 보였다.

결론: 서로 다른 각 기관에서 여포종양에서 genome-wide expression profiling 을 시행한 후 여포종양의 감별진단을 위한 표지자로 제시된 유전자들 중 *Clorf24* 이

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여포선종과 여포암에서 유의한 발현의 차이를 나타내었으며, *ARG2* 와 *PLAB* 도 여포종양의 혈관침범을 예측할 수 있는 유용한 인자로 판단된다.

주요어: 갑상선 여포선종, 갑상선여포암, 유전자 표지자

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