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Microarray Analysis of Papillary Thyroid Cancers in Korean

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Background/Aims: Papillary thyroid cancer (PTC) is the most common malignancy of the thyroid gland. It involves several molecular mechanisms. The *BRAF* V600E mutation has been identified as the most common genetic abnormality in PTC. Moreover, it is known to be more prevalent in Korean PTC patients than in patients from other countries. We investigated distinct genetic profiles in Korean PTC through cDNA microarray analysis.
Methods: Transcriptional profiles of five PTC samples and five paired normal thyroid tissue samples were generated using cDNA microarrays. The tumors were genotyped for *BRAF* mutations. The results of the cDNA microarray gene expression analysis were confirmed by real-time PCR and immunohistochemistry analysis of 35 PTC patients.
Results: Four of the five patients whose PTC tissues were subjected to microarray analysis were found to carry the *BRAF* V600E mutation. Microarrays analysis of the five PTC tissue samples showed the expression of 96 genes to be increased and that of 16 genes decreased. Real-time reverse transcription-polymerase chain reaction (RT-PCR) confirmed increased expression of *SLC34A2, TM7SF4, COMP, KLK7*, and *KCNJ2* and decreased expression of *FOXA2, SLC4A4, LYVE-1*, and *TFCP2L1* in PTC compared with normal tissue. Of these genes, *TFCP2L1, LYVE-1*, and *KLK7* were previously unidentified in PTC microarray analysis. Notably, *Foxa2* activity in PTC was reduced, as shown by its cytoplasmic localization, in immunohistochemical analyses.
Conclusions: These findings demonstrate both similarities and differences between our results and previous reports. In Korean cases of PTC, *Foxa2* activity was reduced with its cytoplasmic accumulation. Further studies are needed to confirm the relationship between *FOXA2* and *BRAF* mutations in Korean cases of PTC. **(Korean J Intern Med 2010;25:399-407)**

Keywords: BRAF mutation; Oligonucleotide array sequence analysis; *FOXA2* protein, human; Thyroid cancer

INTRODUCTION

Papillary thyroid cancer (PTC) is the most common thyroid cancer in iodine-sufficient regions [1]. It is believed to account for more than 80% of all thyroid cancers [2,3]. The clinical characteristics of PTC are diverse, ranging from slow, progressive micro-PTC to anaplastic cancers [4,5].

Although the pathogenetic mechanisms behind PTC remain unknown, multiple genes and environmental factors have been implicated in its development [3,6]. *RET/PTC* rearrangements, *RAS* mutations, and *BRAF* mutations are known genetic abnormalities in PTC. The incidences of *RET/PTC* rearrangements, *RAS* mutations, and *BRAF* mutations in PTC are 13-43%, 0-21%, and 29-69%, respectively [7]. In Korean PTC patients, *RAS* mutations are rare, and *BRAF* mutation more common [8].

The gold standard method for diagnosing PTC is fine-needle aspiration biopsy (FNAB) [9,10]. Nevertheless, 10-

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15% of FNAB results are inadequate, and 10-20% indeterminate, making accurate diagnosis of the thyroid nodules difficult [11].

To manage the limitations of FNAB in PTC, many researchers have attempted to identify useful genetic tools. One such tool, DNA microarray analysis, is expected to provide information about the pathogenetic mechanism and genetics of PTC. According to a meta-analysis of 21 published thyroid cancer gene expression profiling studies, in addition to genes such as *MET, TFF3, SERPINA1, TIMP1, FN1*, and *TPO* that have previously been linked to PTC, other genes such as *TGFA, OPCT, CRABP1, FCGBP, EPS8*, and *PROS1* showed different expression between PTC samples and normal tissues [12]. In a separate microarray analysis, PTC cases with *RET/PTC* rearrangements, *RAS* mutations, and *BRAF* mutations displayed distinct gene expression profiles [13].

The present study was undertaken in Korean PTC patients, who showed a high prevalence of BRAF mutations. Our objective was to identify, in microarray analyses, genes whose expression was altered in Korean PTC as candidate diagnostic markers in PTC.

METHODS

Subjects

PTC tissue samples were obtained intra-operatively from 35 patients during thyroidectomy procedures performed between January 2008 and December 2008. Normal tissue samples were collected from the same patients for pair-wise analysis. The study protocol was approved by the Institutional Review Board, and all patients signed informed consent.

Isolation of RNA and DNA microarray analysis

Tissue samples obtained during thyroidectomy were immediately frozen in liquid nitrogen and stored. Total RNA was extracted using TRIzol (Invitrogen, Camarillo, CA, USA) and purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany). Sample purity was confirmed by measuring A_{260}/A_{280} ratios, and quality by 1% agarose gel electrophoresis. RNA samples from five PTC tissue/normal tissue pairs were chosen for microarray studies. Biotinylated cRNA was prepared from 0.55 μg of total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA). Following fragmentation, 0.75 μg of cRNA was hybridized to Illumina HumanHT-12 v3 Expression BeadChips (Illumina Inc., San Diego, CA, USA) according to protocols provided by the manufacturer.

Real-time RT-PCR analysis

The expression of genes identified in microarray analyses as being up-regulated (*TM7SF4*, *SLC34A2*, *KCNJ2*, *COMP*, and *KLK7*) and down-regulated (*TFCP2L1*, *LYVE-1*, *FOXA2*, and *SL4A4*) in PTC was examined by real-time reverse transcription-polymerase chain reaction (RT-PCR). A RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) was used to synthesize cDNA. PCR reactions (20 mL) contained total RNA (2 mg), oligo $(dT)_{18}$ primer (1 mL), 5 × reaction buffer (4 mL), RiboLock™ Ribonuclease Inhibitor (20 u/mL) (1 mL), 10 mM dNTP mix (2 mL), and RevertAid™ M-MuLV Reverse Transcriptase (200 u/mL) (1 mL). An ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and TaqMan probe PCR master mix (Applied Biosystems) were used in real-time PCR reaction. The PCR amplification procedure was carried out in 96-well plates in 20-mL reactions containing cDNA (1 μL), probe (1 μL), Master Mix (10 μL) and QW (8 μL). The following thermal conditions were applied: 50°C for 2 minutes; 95°C for 10 minutes; and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

BRAF mutation analysis

BRAF mutation analysis was performed using the five papillary thyroid cancer tissue samples subjected to microarray analysis. Genomic DNA was extracted using G-DEX (iNtRON Biotechnology Co. Ltd., Seoul, Korea). Exon 15 of *BRAF* was PCR-amplified using the following PCR primers: *BRAF* 15, 5′- ATGTTGCTCTGATAGGAAA -3′ (sense), 5′- GATTTTTGTGAATACTGGGAA -3′ (antisense).

Statistical analysis

Significant differences in gene expression between cancerous and normal tissues in the microarray analysis were identified using paired Student's *t* test, fold-change, and hierarchical clustering, performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when the false-discovery rate (FDR) was 10% of the control (*p* values were subjected to Benjamini-Hochberg FDR correction) and the fold-change was ≥ 2.

RESULTS

Clinical characteristics of the study patients

The mean patient age was 50.2 ± 13.2 years. The patient group comprised 3 men and 32 women. The classic PTC subtype was found in 34 patients, and the follicular variant in one patient. The mean tumor size was 1.3 ± 0.6 cm. Eighteen cases (50%) showed extrathyroidal invasion, and 22 cases (66%) showed lymph node metastasis.

BRAF mutations and gene expression in DNA microarray analysis

Four of the five PTC samples subjected to microarray analysis were positive for the T1799A *BRAF* mutation. DNA microarray analysis of 5 PTC samples identified 112 genes whose expression showed a more than 2-fold change and where FDR was 10% of the control in PTC tissue compared with paired normal tissue. Of these, 96 genes, including *TM7SF4, SLC34A2, KCNJ2, C7orf24, PROS1, SERPINA1, TUSC3, COMP,* kallikrein-related peptidase 7 (*KLK7*), and matrix metallopeptidase 7, showed more than 2-fold higher expression in PTC tissues compared with paired normal tissues (Table 1). Expression of the remaining 16 genes, which included metallothionein 1F (*MT1F*), *HBA2*, *ACACB*, *RYR2*, *OTOS, SLC4A4*, *TFCP2L1*, *LYVE-1*, and *FOXA2*, was decreased more than 2-fold in PTC tissues compared with paired normal tissue (Table 1).

Of the genes previously linked to *BRAF* mutation-associated PTC, *TM7SF4*, *SLC34A2, PDE5A, TPD52L1, FN1, PLXNC1, TMPRSS6*, and *ERBB3* displayed more than 2-fold increased expression in PTC tissues, whereas the expression of *ARNTL, PERP, GATA3, IRS2, CLECSF2*, and *STAT1* was increased less than 2-fold in PTC tissues compared with paired normal tissues (Table 2). The expression of *TFF3, ARGBP2, HGD, FHL1, IRS1*,

Table 1. Up-regulated or down-regulated genes in microarray analysis of papillary thyroid cancer

Gene	Fold change
Transmembrane 7 superfamily member 4 (*TM7SF4*)	20.22
Solute carrier family 34 (sodium phosphate), member 2 (*SLC34A2*)	14.56
Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1 (*SERPINA1*)	13.25
Cartilage oligomeric matrix protein (*COMP*)	11.08
Matrix metallopeptidase 7	8.67
Kallikrein-related peptidase 7 (*KLK7*)	7.01
Protein S (alpha) (*PROS1*)	5.73
Potassium inwardly-rectifying channel, subfamily J, member 2 (*KCNJ2*)	5.69
Chromosome 7 open reading frame 24 (*C7orf24*)	4.27
Tumor suppressor candidate 3 (*TUSC3*)	4.21
Metallothionein 1F	-4.32
Otospiralin (*OTOS*)	-4.11
Forkhead box A2 (*FOXA2*)	-4.04
Lymphatic vessel endothelial hyaluronan receptor 1 (*LYVE-1*)	-3.82
Tanscription factor CP2-like 1 (*TFCP2L1*)	-3.76
Solute carrier family 4, sodium bicarbonate cotransporter, member 4 (*SLC4A4*)	-3.13
Ryanodine receptor 2 (cardiac) (*RYR2*)	-2.79
Hemoglobin, alpha 2 (*HBA2*)	-2.51
Acetyl-Coenzyme A carboxylase beta (*ACACB*)	-2.04

Table 2. Genes showing different expressions in this study among known distinct genes for *BRAF* mutant papillary thyroid cancer

Gene	Fold change
Transmembrane 7 superfamily member 4 (*TM7SF4*)	20.22
Solute carrier family 34 (sodium phosphate), member 2 (*SLC34A2*)	14.56
Transmembrane protease, serine 6 (*TMPRSS6*)	7.65
Fibronectin 1 (*FN1*)	5.99
V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (*ERBB3*)	4.41
Aryl hydrocarbon receptor nuclear translocator-like (*ARNTL*)	2.84
Tumor protein D52-like 1 (*TPD52L1*)	2.64
Phosphodiesterase 5A (*PDE5A*)	2.53
Plexin C1 (*PLXNC1*)	2.26
TP53 apoptosis effector (*PERP*)	1.86
Insulin receptor substrate 2 (*IRS2*)	1.60
Signal transducer and activator of transcription 1, 91kDa (*STAT1*)	1.43
GATA binding protein 3 (*GATA3*)	1.08
C-type lectin domain family 2, member B (*CLECSF2*)	1.07
Trefoil factor 3 (intestinal) (*TFF3*)	-8.50
Sorbin and SH3 domain containing 2 (*SORBS2: ARGBP2*)	-3.67
Homogentisate oxidase (*HGD*)	-3.25
Four and a half LIM domains 1 (*FHL1*)	-2.76
Insulin receptor substrate 1 (*IRS1*)	-2.50
Hepatic leukemia factor (*HLF*)	-2.01
Matrix metallopeptidase 15 (*MMP15*)	-1.48
Slit homolog 3 (*SLIT3: ARHN*)	-1.47
Collagen, type IV, alpha 5 (*COL4A5*)	-1.16
Vav 3 guanine nucleotide exchange factor (*VAV3*)	-1.15
Phospholipase A2, group IVB (*PLA2G4B*)	-1.15
Hairy and enhancer of split 1, (Drosophila) (*HES1*)	-1.04
Dual oxidase 1 (*DUOX1*)	-1.04
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 10 (*GALNT10*)	-1.03

and *HLF* was decreased more than 2-fold in PTC tissues compared with paired normal tissues, and that of *GALNT10, HES1, VAV3, MMP 15, PLA2G4B, COL4A5, ARHN*, and *DUOX1* less than 2-fold (Table 2).

Functional classification of differently expressed genes

We summarized the functional classifications of genes that were differently expressed in PTC tissues and paired

Table 3. Functional classification of genes showing altered expression in papillary thyroid cancer

Function	Genes
Cell cycle	Up-regulated: *KLK7, TGFA, Cyclin D1*
	Down-regulated: *FOXA2*
Cell adhesion molecule	Up-regulated: *COL8A1,* cadherin 3*, NRCAM,* periostin
Ion channel	Up-regulated: *KCNJ2, SCN-1B, SLC34A2,* claudin 16
	Down-regulated: *Ryanodine receptor-2* (*RYR2*)
Nuclear acid binding	Up-regulated: *RXRG, BHLHB3, IRX2, ZMAT3*
	Down-regulated: *forkhead box A2* (*FOXA2*)
Receptor	Up-regulated: *LPAR5, ADORA1, XPR1, RXRG*
	Down-regulated: *RYR2*
Signaling molecule	Up-regulated: *MDK, TGFA, COMP, CBLN1*
	Down-regulated: *SEMA6A*
Transcription factor	Up-regulated: *IRX2, RXRG, RUNX1, ETV5, TSC22D1*
	Down-regulated: *TFCP2L1*
Transporter	Up-regulated: *SLC34A2*

normal tissues (Table 3). Of the genes involved in cell-cycle control, the expression of *KLK7*, *TGFA*, and cyclin D1 was increased, whereas that of *FOXA2* decreased. Meanwhile, of the ion-channel genes, the expression of *SLC34A2, KCNJ2*, and claudin 16 was increased, and that of *RYR2* decreased. Expression of the adhesion molecules cadherin 3, *NRCAM, POSTN*, and *ITGA2* was increased.

Real-time RT-PCR analysis of gene expression

From the genes displaying large differences in expression between PTC and normal tissues in the microarray analysis, we selected 9 for which information was available in PubMed. Microarray analysis showed the expression of *TM7SF4, SLC34A2, KCNJ2, COMP*, and *KLK7* to be increased, and that of *TFCP2L1, LYVE-1, FOXA2*, and *SLC4A4* to be decreased. Real-time RT-PCR analysis confirmed all these findings (Figs. 1 and 2).

Immunohistochemical analysis of *FOXA2, COMP*, and *LYVE-1*

We studied the products of the *FOXA2, COMP*, and *LYVE-1* genes immunohistochemically using commercially available antibodies. LYVE1 and COMP were found to be only weakly expressed and could not be fully analyzed. *Foxa2* exhibited cytoplasmic expression in PTC tissues and nuclear expression in paired normal tissues (Fig. 3).

DISCUSSION

PTCs exhibit different patterns of gene expression according to the presence of *RET*/*PTC* rearrangements, *RAS* mutations, and *BRAF* mutations. In the present study, we used DNA microarrays to analyze gene expression in Korean PTC patients, in whom the prevalence of *BRAF* mutations is approximately 80% [8]. The results showed that the expression of 96 genes, including *TM7SF4, SLC34A2, KCNJ2*, and *KLK7*, was increased more than 2-fold in PTC tissues compared with paired normal tissues, and that of 16 genes, including *MT1F, FOXA2*, *TFCP2L1*, and *LYVE-1*, decreased more than 2-fold. Eszlinger et al. [14] previously identified shared and distinct gene expression in PTC subtypes characterized by *RET/PTC, BRAF*, and *RAS* mutations. They identified 20 up- and down-regulated genes in *BRAF*

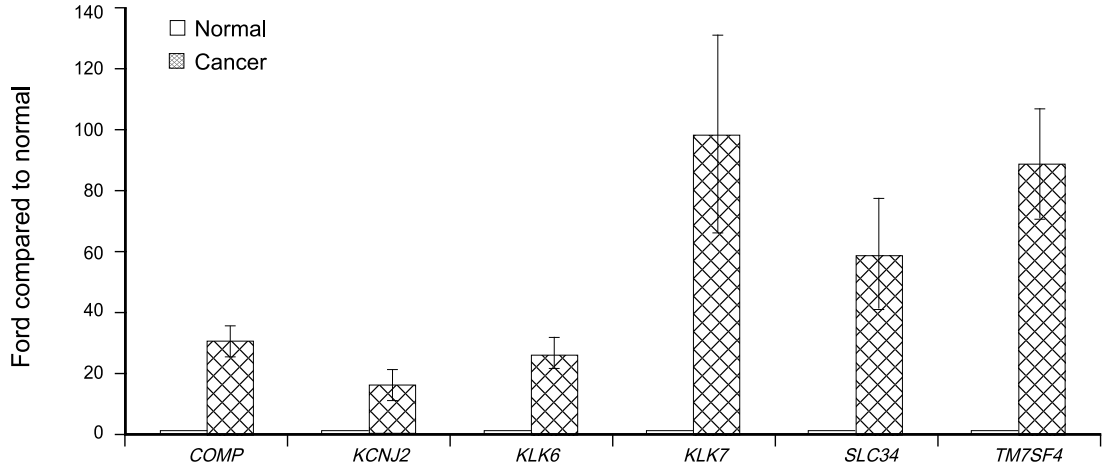

Figure 1. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of genes that were up-regulated in papillary thyroid cancer tissues compared with paired normal tissues. *COMP*, cartilage oligomatrix protein; *KCNJ2*, potassium inwardly-rectifying channel, subfamily J, member 2; *KLK-6*, kallikrein-related peptidase 6; *KLK-7*, kallikrein-related peptidase 7; *SLC 34*, solute carrier family 34 (sodium phosphate); *TM7SF4*, transmembrane 7 superfamily member 4.

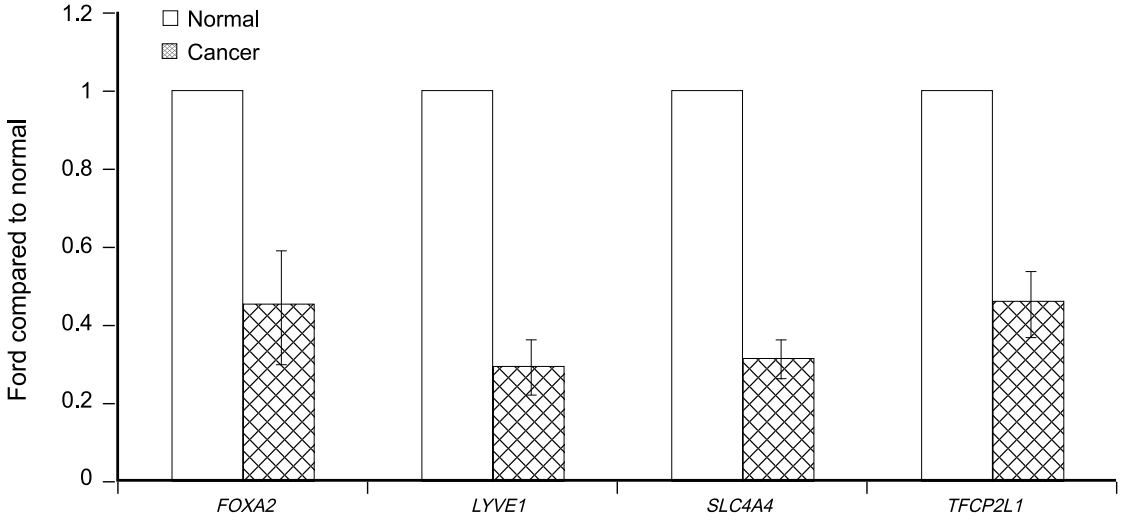

Figure 2. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of genes that were down-regulated in papillary thyroid cancer tissues compared with paired normal tissues. *FOXA2*, forkhead box A2; *LYVE-1*, lymphatic vessel endothelial hyaluronan receptor 1; *SLC4A4*, solute carrier family 4, sodium bicarbonate cotransporter, member 4; *TFCP2L1*, transcription factor CP2-like 1.

mutation-associated PTC. Of these genes, we confirmed the expression of *TM7SF4, SLC34A2, PDE5A, TPD52L1, FN1, PLXNC1, TMPRSS6*, and *ERBB3* to be increased more than 2-fold in PTC tissues compared with paired normal tissues, but found the expression of *ARNTL, PERP, GATA3, IRS2, CLECSF2*, and *STAT1* to be increased less than 2-fold in PTC tissues (Table 2). We also found similar > 2-fold down-regulation of *TFF3, ARGBP2, HGD, FHL1, IRS1*, and *HLF* in PTC tissues compared with paired normal tissues, but found the

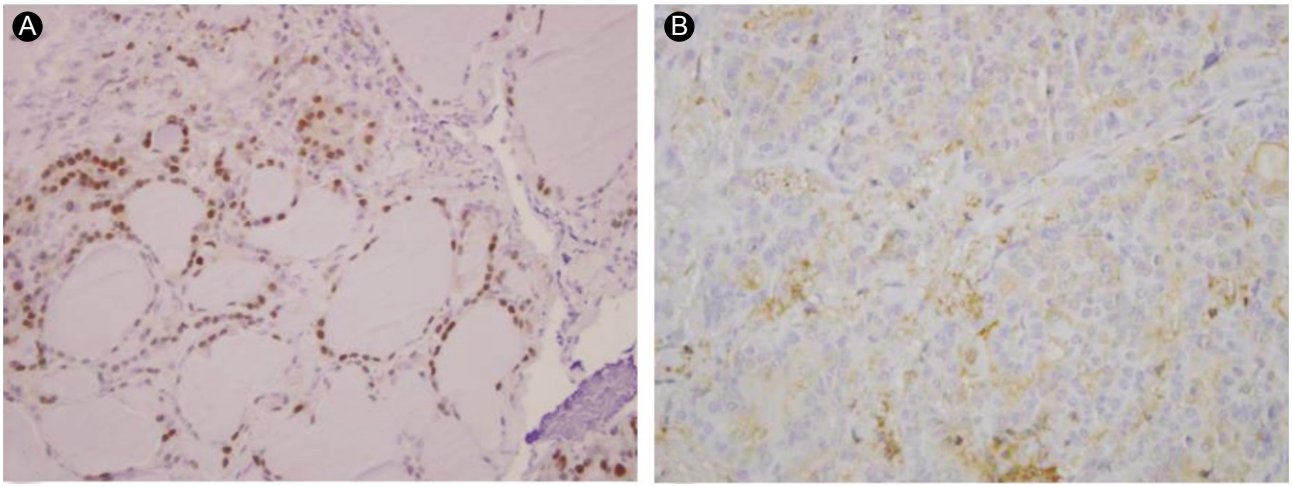

Figure 3. Cytoplasmatic accumulation of forkhead box A2 (*FOXA2*) in papillary thyroid cancer (PTC). (B) PTC tissues show especially cytoplasmatic accumulation, but (A) paired normal tissues show nuclear accumulation in *Foxa2* antibody staining (×100).

expression of *GALNT10, HES1, VAV3, MMP 15, PLA2G4B, COL4A5, ARHN*, and *DUOX1* to be decreased less than 2-fold in PTC tissues compared with paired normal tissues (Table 2). Moreover, although we found that the *LYVE1, MT1F, OTOS,* and *FOXA2* expression was significantly decreased in PTC tissues compared with normal tissues, these genes were not among those showing different expression in the study of *BRAF* mutation-associated PTC by Eszlinger et al. [14].

Our study has certain limitations. We did not specifically measure gene expression in *BRAF* mutation-positive PTC. However, four of the five PTC samples that we subjected to microarray analysis were positive for *BRAF* mutations. We therefore consider that our microarray results show characteristic gene expression profiles for Korean *BRAF* mutant PTC.

To confirm our initial findings, we analyzed a subset of genes showing altered expression in the microarray analysis by real-time RT-PCR. The results of this real-time RT-PCR confirmed the findings of the microarray analysis, showing the expression of *TM7SF4, KCNJ2, SLC34A2, KLK7*, and *COMP* to be increased in PTC tissues compared with normal tissues and that of *FOXA2, LYVE-1, TFCP2L1*, and *SLC4A4* to be decreased.

First identified in dendritic cells [15], *TM7SF4* (*DC-STAMP*) is also expressed in osteoclasts and is required for osteoclast cell fusion and monocyte-derived multinucleated giant cell formation [16]. A previous study employing the GeneFishing method reported that expression of the *TM7SF4* and *COL1A1* genes was significantly different in PTC tissues compared with normal tissues [17]. In addition, Galeza-Kulik et al. [18] analyzed tissue from 38 PTC patients by quantitative real-time PCR and identified several genes involved in the transport of ions whose expression was altered in PTC. They reported increased *SLC34A2* and *KCNJ2* expression and reduced *SLC4A4* expression in PTC tissues. The results of our microarray and real-time RT-PCR analyses confirmed the expression of *SLC34A2* and *KCNJ2* to be increased and that of *SLC4A4* decreased in PTC tissues. The kallikrein (KLK) family comprises 15 structurally homogeneous, trypsin-like serine protease genes [19]. *KLK4* and *KLK11* are known to be overexpressed in ovarian cancer and prostate cancer, and *KLK6* to be down-regulated in breast cancer and prostate cancer [20]. Talieri et al. [21] found that *KLK7* was more highly expressed in colon cancer than in normal tissues, and they proposed that it may be a prognostic factor in colon cancer patients. In the present study, microarray analysis showed *KLK7* expression to be 7-fold higher in PTC tissues than in normal tissues, a result that was confirmed by real-time RT-PCR. Differences in KLK family gene expression in PTC have not previously been studied and warrant further research.

Members of the forkhead box-O (FOXO) family of proteins are known to regulate important cellular events such as differentiation, DNA repair, cell cycle arrest, and apoptosis [22,23]. Karger et al. [24] reported the cytoplasmic accumulation of Foxo3a in differentiated thyroid cancer, in contrast to its nuclear accumulation in normal thyroid tissue and follicular adenoma. Cytoplasmic accumulation of Foxo3a results in increased phospho-activation of Akt, decreased transcription of the Foxo3a target genes *p27kip* and *Bim*, and increased

expression of Gadd45a mRNA [24]. The authors concluded that the inactivation of Foxo3a may represent a pathogenetic mechanism for the avoidance of cancer cell apoptosis in thyroid follicular carcinoma. Separately, Akagi et al. [25] reported decreased expression of *FOXA2* in PTC cell lines as a result of methylation of CpG islands in the *FOXA2* promoter. They also reported that forced expression of *FOXA2* inhibited cancer cell growth in PTC. Similarly, in the present study, we showed through microarray analysis and real-time RT-PCR that *FOXA2* expression was decreased in PTC tissues compared with normal tissues. In immunohistochemical analyses, we showed that *Foxa2* accumulated in the cytoplasm of PTC tissues and in the nucleus in normal tissues. This suggests that *Foxa2* is transported from the nucleus to the cytoplasm in PTC. Further study is required to determine whether the cause of decreased *FOXA2* expression is marked cytoplasmic accumulation of *FOXA2* or the methylation of CpG islands in the promoter region of *FOXA2*.

This study has certain limitations. First, the number of subjects was small. Second, the microarray analysis may not have been optimal. It is still not clear which preprocessing algorithm is the most appropriate, and some researchers argue that the statistical tools used to analyze microarray analysis results remain insufficient. Third, we did not test whether gene expression changes translated into changes in protein expression. Thus, additional studies that include a larger number of subjects and that analyze expression at the protein level are necessary.

In conclusion, our study confirmed some results of previous studies results and yielded others that were different. Our results show that *FOXA2* is down-regulated in Korean cases of PTC as a result of the transport of *Foxa2* from the nucleus to the cytoplasm. These findings provide insights into the molecular pathways involved in PTC in the Korean population.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

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