Int Surg 2017;**102:**39–46
DOI: 10.9738/INTSURG-D-17-00049.1

Gene Expression Profiles of Papillary Thyroid Microcarcinoma

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The objective was to identify gene expression profile of papillary thyroid microcarcinoma. To help improve diagnosis of papillary thyroid microcarcinoma, we performed gene expression profiling and compared it to pair normal thyroid tissues. We performed microarray analysis with 6 papillary thyroid microcarcinoma and 6 pair normal thyroid tissues. Differentially expressed genes were selected using paired *t* test, linear models for microarray data, and significance analysis of microarrays. Real-time quantitative reverse transcription–polymerase chain reaction was used to validate the representative 10 genes (MET, TIMP1, QPCT, PROS1, LRP4, SDC4, CITED1, DPP4, LRRK2, RUNX2). We identified 91 differentially expressed genes (84 upregulated and 7 downregulated) in the gene expression profile and validated 10 genes of the profile. We identified a significant genetic difference between papillary thyroid microcarcinoma and normal tissue by 10 upregulated genes greater than 2-fold ($P < 0.05$).

Key words: Microarray analysis – Papillary thyroid microcarcinoma – Gene expression profile

Thyroid carcinomas less than or equal to 1 cm are almost exclusively papillary and are termed papillary thyroid microcarcinoma (PTMC).[1] It is likely that the number of PTMC observed recently in many countries will continue to increase. Although some of the risk factors of PTMC include lymph node metastasis, extrathyroidal extension, and so on, PTMC has an indolent clinical course and generally has a better prognosis than papillary thyroid carcinoma (PTC). Recent studies showed

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that gene expression data from microarray analysis hold promise for understanding the molecular basis of thyroid carcinogenesis and thus improving tumor classification and predicting response to therapy.[2,3] The first array investigation on PTC (including PTMC) was performed by Huang *et al*[4] in 2001 using U95A Affymetrix GeneChips containing more than 12,000 transcripts. In their study of 8 PTC tumors, which were compared with normal surrounding tissues from the same 8 individuals, the authors specified 50 genes with the most distinct gene expression changes and identified a number of additional PTC-specific genes, many of which were associated with cell cycle or mitogenic control and some previously found in other cancers. However, to our knowledge, no study has identified the gene expression profile (GEP) of PTMC only and compared it with that of normal thyroid tissue of PTMC. We aimed to characterize the GEP of PTMC and compare it to that of pair normal thyroid tissues. We validated the microarray technique by selecting 10 upregulated genes and analyzing them by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Material and Methods

Tissue samples

The thyroid tissues were obtained from 6 PTMC patients who underwent total thyroidectomy between January 2010 and June 2011. Six PTMC fresh frozen tissues and 6 normal fresh frozen thyroid tissues from contralateral lobes of the patients were included in the study. Normal tissue samples were collected from the same patients for pairwise analysis. The medical records of the patients were reviewed to determine their clinical characteristics. Informed consent was obtained from all patients. This study was approved by the Institutional Review Board.

RNA extraction and microarray

Total RNA extraction was done with Trizol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. After homogenization, 1 mL of solution was transferred to a 1.5-mL Eppendorf tube and centrifuged at 12,000 ×g for 10 minutes at 4°C to remove insoluble material. The supernatant containing RNA was collected, mixed with 0.2 mL of chloroform, and centrifuged at 12,000 ×g for 15 minutes at 4°C. RNA in the aqueous phase was transferred into a new tube, precipitated by mixing 0.5 mL of isopropyl alcohol, and recovered by centrifugation at 12,000 ×g for 10 minutes at 4°C. The RNA pellet was washed briefly in 1 mL of 75% ethanol and centrifuged at 7500 ×g for 5 minutes at 4°C. Finally, total RNA pellet was dissolved in nuclease water, and its purity and integrity were assessed with an Agilent Bioanalyzer 2100. Gene expression was analyzed with GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA), which consist of 54,675 probe sets representing approximately 38,500 well-characterized human genes. For each gene, 11 pairs of oligonucleotide probes were synthesized *in situ* on the arrays. Biotinylated cRNA was prepared according to the standard Affymetrix protocol from 250 ng of total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). After fragmentation, 15 μg of aRNA was hybridized for 16 h at 45°C on a GeneChip Human Genome array. GeneChips were washed and stained in Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analyzed with Microarray Suite version 5.0 (MAS 5.0) using Affymetrix default analysis settings and global scaling as normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log-transformed intensity values were then analyzed using GeneSpring GX 11.5.1 (Agilent Technologies, CA). Fold-change filters included the requirement that the genes be present in at least 200% of controls for upregulated genes and lower than 50% of controls for downregulated genes.

Statistical and bioinformatics analysis

Data files (.CEL) were generated in GeneChip Operating Software (GCOS; Affymetrix), using default settings for scaling and detection call parameters. The Robust Multichip Average (RMA) Express 1.0.4 program (Bolstad BM, University of California, Berkeley, CA) was used for background adjustment and quantile RMA normalization of the 54,675 probe sets encoding human genome transcripts. Differentially expressed genes (DEGs) between the 2 groups were identified using linear models for microarray data (LIMMA) in R using Bioconductor, and significance analysis of microarrays (SAM, version 4.0, 2011, www-stat.stanford.edu/~tibs/SAM/) was used for overall and pairwise comparisons between the groups on RMA-normalized signal intensities. LIMMA uses Bayes method, which is a different algorithm for identifi-

cation of significantly expressed genes. Both SAM and LIMMA methods yielded large numbers of DEGs. DEGs were ranked on moderated t statistics, and those with *t* scores >5 were followed up further. The moderated *t*-statistic score is based on the ratio of the log2 fold change to its standard error. Similar to traditional *t*, moderated *t* ranks genes on a combination of magnitude and consistency of differential expression but downgrades values where exceptionally small variance elevates the ratio in the absence of detectable difference in expression level. False discovery rates (FDRs) were also calculated for all comparisons. To screen for the genes that have larger effects on the traits and to reduce the number of less relevant genes, we compared the lists of top-ranked genes from the LIMMA analysis with the lists obtained from the SAM analysis. Genes significant ($P < 0.01$) from the LIMMA lists and ranking high on the SAM lists were selected. SAM was applied to log2 transformed data of the filtered data set to compare the PTMC and normal groups. The principal component analysis (PCA) is an exploratory tool to characterize predominant gene expression patterns. Through PCA, it is possible to identify whether samples from the same conditions have similar patterns of gene expression. Hierarchical clustering data were clustered groups that behave similarly across experiments using GeneSpring GX 11.5.1 (Agilent Technologies, CA). Clustering algorithm with Euclidean distance and average linkage was used. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analyses were then carried out for DEGs using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.7, david.abcc.ncifcrf.gov) to identify the most significant biological processes and signaling pathways.

Real-time quantitative PCR

To further validate the results of the microarray test, we used qRT-PCR to examine the relative expression of 10 genes (MET, TIMP1, QPCT, PROS1, LRP4, SDC4, CITED1, DPP4, LRRK2, RUNX2) among the DEGs selected by integration of the results from the LIMMA and SAM analyses. We designed and synthesized 2 oligonucleotide primers based on the published genomic sequence of the 10 selected genes: MET (hepatocyte growth factor receptor, GenBank accession no. BG170541), TIMP1 (TIMP, metallopeptidase inhibitor 1, NM_003254), QPCT (glutaminyl-peptide cyclotransferase, NM_012413), PROS1 (protein S [alpha], NM_000313), LRP4 (low-density lipoprotein receptor-related protein 4, AA584297), SDC4 (syndecan 4, NM_002999), CITED1 (CBP/p300-interacting transactivator, with Glu/Asp-rich carboxyl-terminal domain, 1, NM_004143), DPP4 (dipeptidyl-peptidase 4, M80536), LRRK2 (leucine-rich repeat kinase 2, AK026776), and RUNX2 (runt-related transcription factor 2, AL353944). Amplification of transcripts was performed with 3 μg/μL total RNA and reverse transcriptase-polymerase chain reaction (RT-PCR), using high-capacity cDNA reverse transcription kit (ABI Applied Biosystems, CA, USA). Real-time PCR primers were designed and synthesized by Macrogen Corporation (Seoul, Korea). The following gene-specific primers were used: MET, 5′-GAAGGAGGGA CAAGGCTGAC-3′ and 5′-TGTTATTGTG′; TIMP1, 5′-TCTGGCATCCTGT TGTTGCT-3′ and 5′-TGTGCATTCCTCACAGCCAA-3′; QPCT, 5′-TCCCAGACAGACTCGGAGAG-3′ and 5′-GCCCCTCCAA AGAGTGATCC-3′; PROS1, 5′-GTGTGCCTTCCCTTGAACCT-3′ and 5′-GGCAAGCATAACA CCAGTGC-3′; SDC4, 5′-GTCCTGGCAGCTCTGATTGT-3′ and 5′-GGACT CCACACAACATCCGT-3′; CITED1, 5′-G′ and 5′-TCCCGAGGAACTAGTGGGAG-3′; DPP4, 5′-TGTTTAACTCGG GGCCGAAA-3′ and 5′-CGTTTGGAGACCACCACAGA-3′; LRP4, 5′-GAG GACGACTGTGCAGACAA-3′ and 5′-GGTCGGGC CGTAGTTCATA G-3′; LRRK2, 5′-ATGCACTCACGAGCTTTCCA-3′ and 5′-CGGGACCTGGTAG GTACTGA-3′; RUNX2, 5′-GACCAGTCTTACCCCTC CTACC-3′ and 5′-CTGCCTGGCTCTTCTTACTGAG-3′; and β-actin, 5′-GCAGAAGGAGATC ACTGCCCT-3′ and 5′-GCTGATCCACATCT GCTGGAA-3′. For the quantification of gene amplification, real-time PCR was performed using an ABI 7300 Sequence Detection System with SYBR Green. Target sequences were amplified at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. β-Actin was used as an endogenous normalization control. Experiments were independently repeated 3 times and were calculated by the ΔΔCt method. The n-fold change in mRNAs expression was determined according to the $2^{-\Delta\Delta Ct}$ method.

Results

To explore novel genes involved in PTMC, we investigated the genome-wide expression profiles of PTMCs and pair normal thyroid tissues. Using

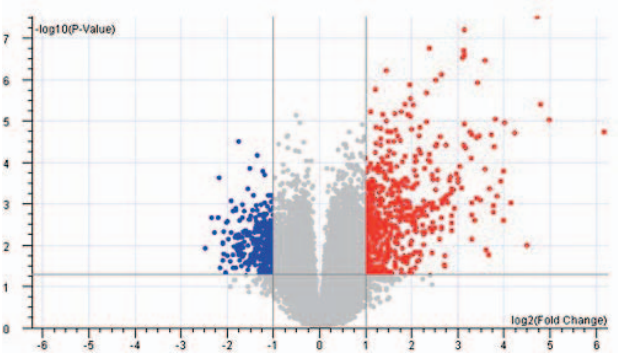

Fig. 1 Volcano plot of probe sets differing between PTMCs and normal tissues. Fold change (x axis) is plotted against statistical significance (y axis) for each probe set. Genes upregulated with a fold change >2 and $P < 0.05$ are depicted in red, and those downregulated with a fold change >2 and $P < 0.05$ are shown in blue. Gray represents genes in the arrays that were not found to differ significantly between 2 groups.

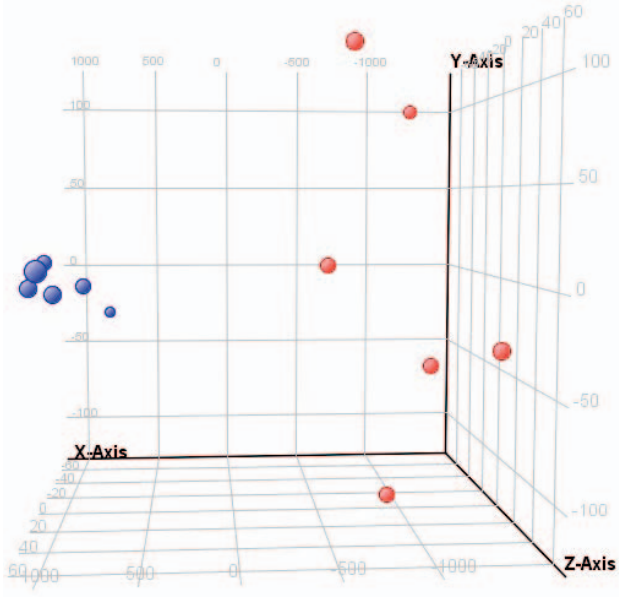

Fig. 2 Principal component analysis (PCA) was applied to 12 thyroid samples that were characterized by the gene expression of all probes on the GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA), including the control group (blue) and PTMC group (red). DEGs selected by LIMMA, SAM, and paired t test are shown.

GeneChip Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA), we compared the expression profiles of PTMCs and pair normal tissues. After performing paired t test analyses, we identified 878 genes whose expression differed significantly at $P < 0.05$ with a fold change of >2 and <–2. Genes differing in expression were represented in a volcano plot (Fig. 1). Among these, 591 genes were upregulated and 287 genes were downregulated in PTMCs compared with pair normal tissues. Then, we used LIMMA software and SAM in screening the DEGs, and we selected those genes with adjusted P values <0.01 and fold change > 2 and <–2 as the threshold cutoff. We identified 91 DEGs in PTMC (Table 1). The expressions of 84 genes, including HMGA2, GABRB2, COL10A1, COMP, and LRP4, was increased more than 2-fold in PTMC tissues than that in pair normal tissues, and the expressions of 7 genes, including CITED2, FOXA2, LOC201651, and ANKRD37, decreased more than 2-fold than that in pair normal tissues.

As shown in Fig. 2, the relationship between the two groups is apparent in the PCA. The patterns that are grouped in similar areas within the 3-dimensional condition scatterplot demonstrate the similarity between members of each sample group, as well as differences between groups (Fig. 2), suggesting that the samples used in this study were appropriately prepared and selected. Genes differing in expression were subjected to hierarchical clustering analysis; the heatmap shows that PTMCs are distinctly different from pair normal tissues (Fig. 3). By using significantly upregulated and downregulated genes, the altered GO categories were identified from DAVID. Thus, we were able to show the processes significantly linking cell adhesion, biological adhesion, wound healing, regulation of body fluid levels, endochondral ossification, coagulation, endochondral bone morphogenesis, hemostasis, bone morphogenesis, cell–cell adhesion, apoptosis, programmed cell death, cell–substrate adhesion, focal adhesion, membrane organization, blood vessel morphogenesis, fat cell differentiation, glycosylation, biopolymer glycosylation, protein amino acid glycosylation, and skeletal system development. The genes examined were also submitted to the DAVID in an attempt to identify significantly dysregulated pathways. The KEGG pathway analysis for the 91 genes showed that the 2 significant pathways involved extracellular matrix (ECM)-receptor interactions and cell adhesion molecules.

To verify the data obtained from microarray analysis, we selected 10 genes (MET, TIMP1, QPCT, PROS1, LRP4, SDC4, CITED1, DPP4, LRRK2, RUNX2) for qRT–PCR analysis. The results showed that the gene expression of the detected 10 genes was in accordance with the microarray data, as

Table 1 List of differentially-expressed genes of PTMC in microarray analysis

Gene symbol	Accession no. *	Fold-change (T/N)	Adjusted *P* value
COMP	NM_000095	26.18	1.74E-07
HMGA2	NM_003483	35.33	4.49E-07
LAMB3	L25541	8.82	6.43E-07
FN1	AF130095	8.59	1.55E-06
DTX4	AV728526	5.82	1.55E-06
GABRB2	BC036592	30.91	1.55E-06
PLAU	NM_002658	6.24	6.28E-06
SCEL	NM_003843	11.91	6.28E-06
CPNE4	BE645967	3.81	1.61E-05
PRR15	AI347918	18.97	1.63E-05
MET	BG170541	4.02	1.67E-05
PROS1	NM_000313	5.03	1.75E-05
CITED1	NM_004143	17.69	1.75E-05
TACSTD2	J04152	14.48	1.80E-05
LIPH	AA088857	6.54	2.36E-05
CLDN1	AF101051	8.75	2.55E-05
MPZL2	NM_005797	4.18	3.30E-05
KCNJ2	AF153820	10.97	3.30E-05
LRRK2	AK026776	6.170	3.30E-05
DPP4	M80536	6.139	3.30E-05
NGEF	AV703769	5.61	3.30E-05
TNFRSF12A	NM_016639	4.65	3.41E-05
QPCT	NM_012413	9.34	5.55E-05
GABBR2	AF056085	7.19	5.62E-05
XPR1	AF089744	2.72	5.71E-05
SDC4	NM_002999	3.58	6.25E-05
FLJ25076	H17038	4.96	8.06E-05
SYTL5	AW263497	9.806	8.21E-05
UNC5B	AK022859	3.44	9.41E-05
P4HA2	NM_004199	2.75	9.79E-05
PDZK1IP1	NM_005764	10.81	0.000104
SLC34A2	AF146796	14.57	0.000134
CDH3	NM_001793	5.96	0.000138
ENTPD1	U87967	4.28	0.000138
LRP4	AA584297	19.65	0.00014
NPC2	NM_006432	2.368	0.000155
IGSF3	AB007935	2.58	0.000176
CLDN16	NM_006580	6.04	0.000179
MRC2	AB014609	2.88	0.00018
LGALS3	BC001120	2.31	0.000204
GALE	NM_000403	2.99	0.000215
COL10A1	X98568	28.64	0.000238
GALNT7	NM_017423	2.96	0.000246
ZMAT3	NM_022470	3.14	0.000273
NRCAM	NM_005010	4.90	0.000317
UPP1	NM_003364	2.76	0.000332
LOC201651	BC014344	-3.35	0.000332
COL13A1	M33653	4.37	0.000333
SHROOM4	AI005420	3.16	0.00034
ADORA1	NM_000674	2.83	0.000413
PCSK2	NM_002594	4.51	0.000523
AMIGO2	AC004010	3.13	0.000564
IGF2BP2	NM_006548	2.50	0.000662
GGCT	AK021779	3.25	0.000829
FGF1	X59065	2.52	0.00086
CAMK2N1	NM_018584	7.68	0.000866
MAMLD1	NM_005491	2.33	0.000898
FAM176A	AV700753	3.69	0.00099
LPAR5	AW183080	5.26	0.001181
FABP4	AI766029	-2.55	0.001227
CHI3L1	M80927	15.65	0.001227
CITED2	NM_006079	-4.20	0.001238
CCND1	M73554	2.12	0.001392
SFTPB	J02761	7.61	0.001392
PVRL4	AF160477	2.93	0.001656
FRMD3	BF589413	3.83	0.001656
CORO2A	AL515381	2.81	0.001661
CFI	BC020718	3.12	0.001925
ERP27	AI051248	2.63	0.002348
STMN2	NM_007029	3.02	0.002423
B3GNT7	CA503291	2.36	0.002434
C8orf4	NM_020130	3.37	0.002493
TUSC3	U42349	3.05	0.002526
NRIP1	NM_003489	2.67	0.002607
NOD1	NM_006092	3.43	0.00296
BID	NM_001196	2.79	0.003588
TGFBI	NM_000358	2.98	0.00446
NAB2	BF337329	2.21	0.004474
ITGA3	NM_002204	2.18	0.004493
RAB27A	U38654	2.19	0.004559
RUNX2	AL353944	4.01	0.005421
FOXA2	AB028021	-3.73	0.005743
ANKRD37	AA886870	-3.00	0.005926
C4orf48	AI341602	2.1	0.006887
TIMP1	NM_003254	3.25	0.006921
CTSC	NM_001814	2.48	0.007056
KCNN4	NM_002250	3.90	0.0073
CENPJ	AF139625	-2.63	0.0077
SLC25A33	BC004991	-2.16	0.008658
TNIK	AF172268	2.16	0.008669
KDELR3	NM_006855	2.24	0.00876

shown in Fig. 4. The qRT–PCR results further confirmed the microarray data, with all of the tested genes showing statistically significant upregulation greater than 2-fold ($P < 0.05$).

Discussion

In this study, we performed GEP analysis using oligonucleotide microarray and identified 91 DEGs (84 upregulated and 7 downregulated) between PTMCs and pair normal thyroid tissues. The criteria of selection for DEGs were rather strict that, whereas other microarray studies on thyroid carcinomas used a *P* value of <0.05, we adopted an adjusted *P* value of <0.01 in LIMMA.

To know the similarity of GEP between PTMC and PTC, we compared them and we found significant genes of PTC in 2 meta-analysis studies. Griffith *et al* conducted a comprehensive meta-

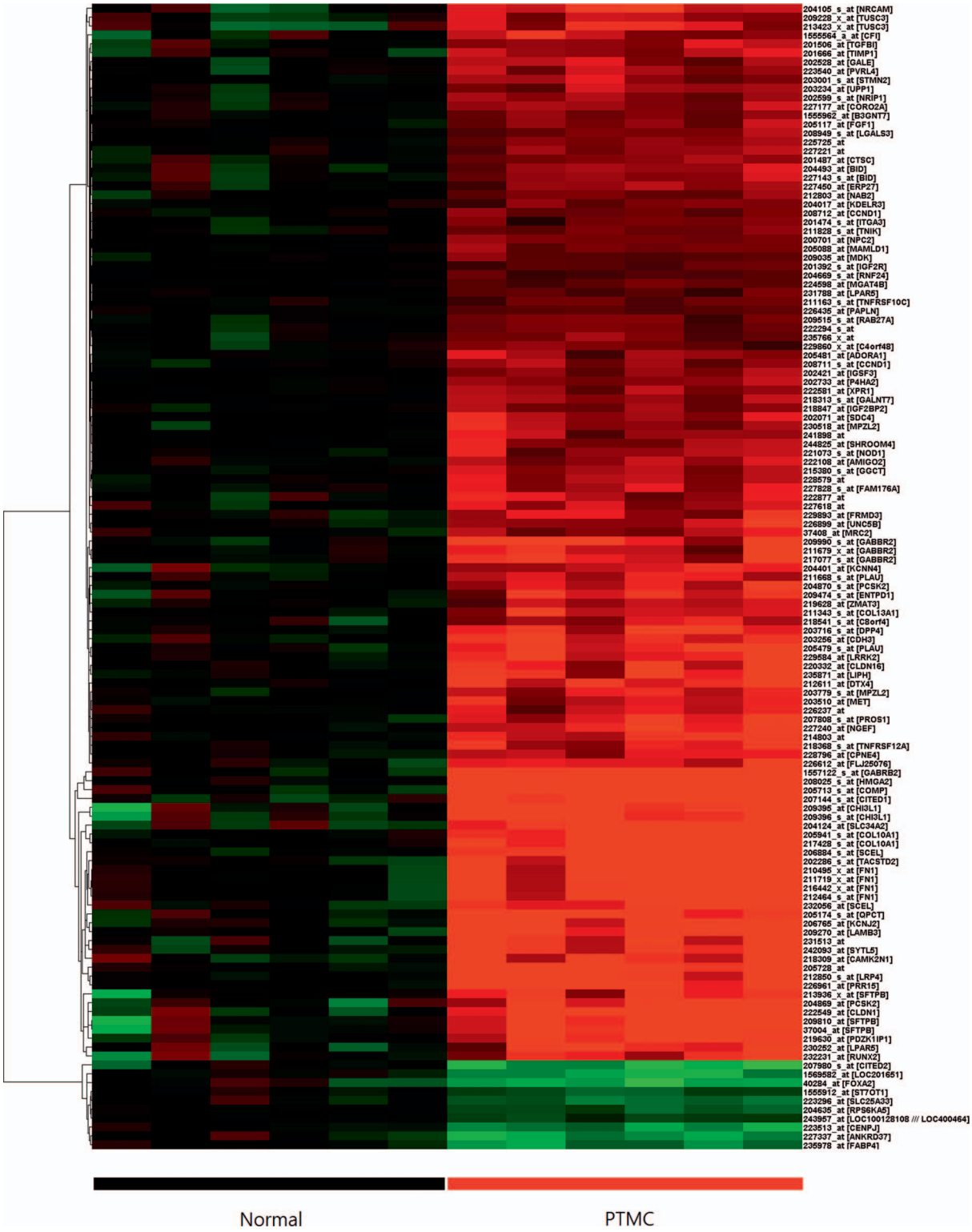

Fig. 3 Cluster analysis of the probes that were increased and decreased significantly in PTMC compared with normal tissue. A dendrogram of the cluster correlation is shown on the left. DEGs selected by LIMMA, SAM, and paired *t* test are shown. Pseudocolors indicate differential expression (red indicates transcript levels greater than the median; black indicates transcript levels equal to the median; green indicates transcript levels below the median; distance metric was Pearson centered and the average linkage rule was used).

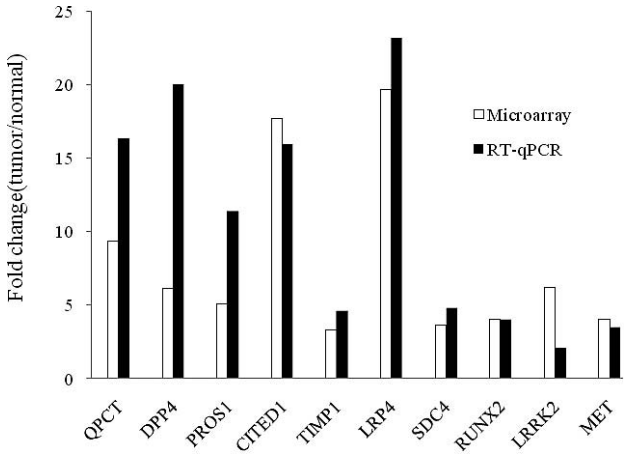

Fig. 4 Validation of array-based gene expression profiles by quantitative real-time PCR.

analysis of PTC gene expression profiling studies to identify meaningful biomarkers and reviewed 21 published studies, in which 34 comparisons were performed from 10 different expression platforms of microarrays; the analysis showed that 39 genes (23 upregulated and 16 downregulated in PTC) showed the same expression patterns among thyroid carcinoma, regardless of the tumor type, in an overlap of 3 or more studies.[5] Among the 23 upregulated genes in PTC from their study, 13 genes (MET, TIMP1, QPCT, PROS1, FN1, LRP4, SDC4, CITED1, LGALS3, DPP4, TUSC3, P4HA2, CCND1) corresponded with those that were selected among the 84 upregulated genes in our study. In addition, Vierlinger *et al* adopted a meta-analysis approach for 4 publicly available microarray data sets on PTC.[6] Among the 17 upregulated genes they identified, 13 genes (PROS1, LRP4, NPC2, LAMB3, DPP4, SDC4, QPCT, TIMP1, MET, CDH3, GGCT, CITED1, CHI3L1) overlapped with the genes identified in our study. Eight genes (MET, TIMP1, QPCT, PROS1, LRP4, SDC4, CITED1, DPP4) in this study were selected in the previous 2 meta-analyses. In the pathway analysis, the most obvious feature of PTMC, similar to PTC, is upregulation of extracellular activities, such as cell communication and adhesion and ECM-receptor interactions (Table 2). Up-regulation of ECM microenvironment genes has been described as an effect of BRAF mutations through phospho-ERK1/2 signaling.[7] In addition, recent studies have shown the role of ECM in tumor progression and invasion through ECM remodeling and stiffening of the tissue stroma.[8] These data and those from our study suggest dysregulation of the ECM-epithelial cell interactions as an early event, even before any evidence of invasion becomes visible in PTMC. Similarly, Birnie *et al*[9] reported dysregulated cell adhesion and ECM-receptor interaction pathways in their comparison of prostate cancer stem cells and their normal and differentiated counterparts.

Table 2 Pathways for 10 selected genes

Genes	Pathways
MET	Adherens junction
	Focal adhesion
	Pathways in cancer
TIMP1	Akt signaling pathway
QPCT	NF-κB-dependent pathway
PROS1	Complement and coagulation cascades
LRP4	Wnt signaling
SDC4	Cell adhesion molecules (CAMs)
	ECM-receptor interaction
CITED1	Nuclear SMAD2/3 signaling
DPP4	Protein digestion and absorption
LRRK2	MAPK Signaling Pathway
RUNX2	MAPK Signaling Pathway

PTMC can remain biologically silent with minimal morbidity for decades. However, a significant percentage of PTMC may show lymph node metastasis and a small number may even have distant metastases. Clinical characteristics or parameters such as tumor size, age, sex, tumor multifocality, vascular or capsular invasion, extrathyroidal extension, lymph node metastasis, and histological variants of PTC need to be considered in risk-adapted management of PTMC to achieve minimal morbidity. In addition, molecular markers, combined with these clinical risk factors, should be taken into account. For example, the presence of the BRAF mutation in PTMC has been shown as associated with increasingly aggressive features, such as extrathyroidal extension, lymph node metastasis, multifocality, and high stage.[10] The identification and investigation of GEP associated with risk factors of PTMC might improve the diagnosis, treatment, and prognosis of PTMC.

We identified 91 differentially expressed genes (84 upregulated and 7 downregulated) in the gene expression profile and validated 10 genes of the profile.

Conclusion

The present study presented a comprehensive analysis of gene expression profiling in PTMC with the use of microarray technology, which identified 91 DEGs (84 upregulated and 7 downregulated) between PTMCs and normal thyroid glands. To

verify the reproducibility of these genes, we performed qRT–PCR using the same RNA used for microarray analysis. The qRT-PCR results were similar to those obtained from the oligonucleotide microarray. Ten representative genes of PTMC were analyzed; MET, TIMP1, QPCT, PROS1, LRP4, SDC4, CITED1, DPP4, LRRK2, and RUNX2.

Acknowledgments

The authors report no declarations of interest. This study was sponsored by Korea University Grants.

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