Identification of Key Genes in Papillary Thyroid Cancer by Transcriptome Analysis

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ABSTRACT

Papillary thyroid cancer (PTC) is the most common type of thyroid malignancies. PTC has good prognosis, but it can dedifferentiate into aggressive forms. In this study, we aimed to identify differentially expressed genes (DEGs) between PTC samples and normal controls. We used gene expression microarrays to identify DEGs between 20 PTC samples and 10 normal controls. We performed enrichment analysis to discover biological processes and signalling pathways associated with PTC and construct protein-protein interaction (PPI) networks to find out key genes for the disease. We identified 1554 up-regulated and 912 down-regulated DEGs in PTC samples compared to normal controls. The coagulation system was the most significant pathway and *SERPINA1* was the most up-regulated gene of this pathway. *CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU and MDM2* were key nodes in PPI networks. Causal network analysis revealed that *SFN*, which was one of the up-regulated DEGs found in our study, was the most causative upstream regulator for PTC. In conclusion, deregulation of *SERPINA1, CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU and MDM2* genes and coagulation system pathway may contribute to PTC development. *SFN* may be an important gene in diagnosis, prognosis and novel anticancer drug approaches for PTC. Further experiments are required to confirm the functions of identified DEGs in our study.

Keywords: Coagulation system, Differentially expressed genes, Gene expression profiling, Papillary thyroid cancer, Stratifin

ÖZET

Papiller Tiroid Kanserinde Anahtar Genlerin Transkriptom Analizi ile Saptanması

Papiller tiroid kanseri (PTK), tiroid kanser tipleri arasında en yaygın olanıdır. PTK' lerin prognozları oldukça iyidir fakat agresif formlara da dönüşebilirler. Bu çalışmada PTK örnekleri ile normal kontroller arasında diferansiyel olarak eksprese olan genleri (DEG) tanımlamayı amaçladık. 20 PTK numunesi ve 10 kontrol arasındaki DEG'leri tanımlamak için gen ekspresyon mikrodizinlerini kullandık. PTK ile ilişkili biyolojik süreçleri ve sinyal yolaklarını bulmak için zenginleştirme analizleri yaptık ve hastalıkta rol oynayan anahtar genleri saptamak amacıyla da protein-protein etkileşim (PPE) ağları oluşturduk. PTK örneklerinin normal kontroller ile karşılaştırılması sonucu 1554 ekspresyonu artan ve 912 ekspresyonu azalan gen tespit ettik. Koagülasyon sistemi en anlamlı yolak olarak saptanırken, *SERPINA1* bu yolakta ekspresyonu en fazla artan gen olarak tanımlandı. *CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU ve MDM2,* PPE ağlarında anahtar genler olarak tespit edildi. Nedensel ağ analizleri, çalışmamızda ekspresyonu artan genlerden biri olarak saptanan *SFN*'nin, PTK için en nedensel upstream regülatör olduğunu ortaya koydu. Sonuç olarak, *SERPINA1, CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU* ve M*DM2*'nin ekspresyon değişimleri PTK gelişimine neden olabilir. *SFN*, PTK için tanıda, prognozda ve yeni antikanser ilaç geliştirme yaklaşımlarında kullanılabilir. Çalışmamızda saptanan DEG'lerin fonksiyonlarını tanımlamak için ileri çalışmalar gerekmektedir.

Anahtar Kelimeler: Koagülasyon sistemi, Diferansiyel olarak eksprese olan genler, Gen ekspresyon profili, Papiller tiroid kanseri, Stratifin

INTRODUCTION

Thyroid cancer is the most common endocrine malignancy and accounts for about 0.5-1% of all human malignant tumors.[1,2] Histologically, thyroid cancer can be divided into four types: papillary thyroid carcinoma (PTC); follicular carcinoma; medullary carcinoma; and undifferentiated carcinoma. Of these, PTC is the most common, accounting for 80% of all thyroid malignancies.[3] PTC has good prognosis and 5-year survival has increased above 95% with the use of neck ultrasound and fine needle aspiration biopsy (FNAB). However, PTC can dedifferentiate into aggressive forms which present clinical characteristics including invasion and metastasis.[4,5] Five percent of patients with distant metastasis do not benefit from conventional therapies such as radioiodine remnant ablation (RAI). In addition, postoperative complications are very common after thyroidectomy surgery and drug treatments have many side effects.[5] Therefore, there is much interest in identifying genetic molecules that can be used both as diagnostic and prognostic biomarkers and as novel therapeutic targets for more effective drug development with fewer side effects.

There have been many attempts to identify molecular markers for PTC. The suggested markers have included *LGALS3, KRT19, FN1, BRAF, RET/PTC, RAS, HBME-1, MET, DPP4, SERPINA1, MUC1, NTRK* and *TIMP1* and genetic variation in *BRAF, RAS, RET/PTC* and *NTRK* have been reported in over 70% of PTCs.[6,7] Activation of the mitogen-activated protein kinase (MAPK) pathway by rearrangements in *RET/PTC* and *NTRK* and point mutations in *RAS* and *BRAF* are thought to be important steps in the development of PTC.[3] Despite the many studies which have been conducted in an attempt to identify genes and/or pathways associated with PTC development, there remains significant uncertainty as to its molecular etiology.

In this study, we aimed to identify differentially expressed genes (DEGs) between PTC samples and normal controls using gene expression profiling microarrays. We performed enrichment analysis to discover the biological processes and signaling pathways associated with PTC and construct protein-protein interaction (PPI) networks to identify key genes for the disease.

MATERIALS and METHODS

Tissue Samples

PTC samples from 20 patients were obtained from the General Surgery Department of Kocaeli University at the time of initial surgery between June 2009 and March 2010. Obtained tissues were snap-frozen immediately after tumor removal and stored at -80^{0}C until RNA isolation procedure. All tumor tissue samples were reviewed by an experienced endocrine pathologist to confirm the diagnosis. Distribution of diagnosed PTC variants were: classic variant PTC (cPTC) in 14 patients; follicular variant (fvPTC) in four patients; and the oncocytic variant in two patients. The control group comprised histologically confirmed normal tissues taken from the opposite, unaffected lobe of ten of the PTC patients.

This study was approved by the Human Subjects Research Ethical Committee of Kocaeli University (Project Number: 2008/77, IAEK: 11/10). All procedures followed were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all patients for inclusion in the study.

Total RNA Preparation

Total RNA was isolated from cells for each patient using RNeasy Mini Kit (Qiagen, Hilden, Germany) following DNase I treatment. Sample purity was confirmed by measuring A260/A280 ratios. The quality of the RNA was assessed using the 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). An RNA integrity value (RIN) of ≥ 7.0 was considered acceptable.

Microarray and Differential Expression Analysis

Microarray analysis was performed using the Whole Human Genome Oligo Microarray (Agilent Technologies, Waldbronn, Germany) which contains 26.083 Entrez Genes. 50 ng of total RNA per sample was processed according to manufacturer's instructions. After scanning arrays with Agilent Technologies Scanner (model G2505B), numerical results were extracted with Feature Extraction

Table 1. Primer sequences of selected genes for qRT-PCR validation

Gene	Forward Primer	Reverse Primer
ß2 microglobulin	5' TGA CTT TGT CAC AGC CCA AGA TA 3'	5' AAT CCA AAT GCG GCA TCT TC 3'
CCND1	5' GAG ACC ATC CCC CTGACG GC 3'	5' TCT TCC TCC TCC TCGGCG GC 3'
CDKN1A	5' TGA GCG ATG GAA CTT CGA CT 3'	5' GAC AGT GAC AGG TCC ACA TGG 3'

version 9.5.1.1 (Agilent Technologies, Santa Clara, CA) using 014850_D_F_20060807 grid, GE1-v5_95_Feb07 protocol and GE1_QCM_Feb07 QC metric set.

The GeneSpring software version 14.9 (Agilent Technologies, Santa Clara, CA) was used to obtain the differentially expressed genes (DEGs) by comparing PTC tissue and normal tissues. Thresholding of the signal values were set to 1.0. The program normalized the data to 75th percentile. Raw data were then normalized to 75th percentile using Percentile shift normalization and baseline transformation to median of all samples was performed. Samples were grouped into Tumor and Normal as experimental parameters. To visualize the data, we checked the multidimensional scaling (MDS) plot that was generated by GeneSpring. DEGs were identified by filtering the dataset using p-value < 0.05 and a signal-to-noise ratio ≥ 2 for use in T-test unpaired statistical analysis. A fold change (FC) of > 2.0 was set as the cutoff value. Moderated t-test with Benjamin-Hochberg multiple testing corrections was used to calculate the p-value for the volcano plots in GeneSpring software.

Functional Enrichment and Protein-Protein Interaction (PPI) Network Analysis

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA) was used to construct gene networks and relevant pathways. Core analysis was run selecting human species and direct interactions. Key genes were identified by evaluating the interaction degree of nodes, according to network topology. Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool (https://david.ncifcrf.gov/) was used to enrich Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the threshold p≤ 0.05. Gene Ontology (GO) terms including three categories including biological process (BP), cellular component (CC) and molecular function (MF) were identified using DAVID with the thresholds p< 0.05.

Causal Network Analysis

IPA provides causal network analysis to identify upstream molecules which control the expression of the genes in the input dataset. To generate causal networks, we selected "causal networks" in the Networks section of the core analysis and added "papillary thyroid cancer" from the pull-down menu.

qRT-PCR Validation

CCND1 and *CDKN1A* were selected randomly for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) validation. cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Relative expression levels of selected genes were determined using LightCycler® 480 *SYBR* Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) using the LightCycler® 480 Instrument (Roche Diagnostics GmbH, Mannheim, Germany). Gene specific primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Primer sequences are listed in Table 1. β2 microglobulin was used as endogenous control. Gene expression levels were calculated using Relative Expression Software Tool (REST) (QIAGEN).

RESULTS

Differentially Expressed Genes

Multidimensional scaling (MDS) allowed assessment of the similarity in gene expression among the samples. MDS analysis revealed that controls

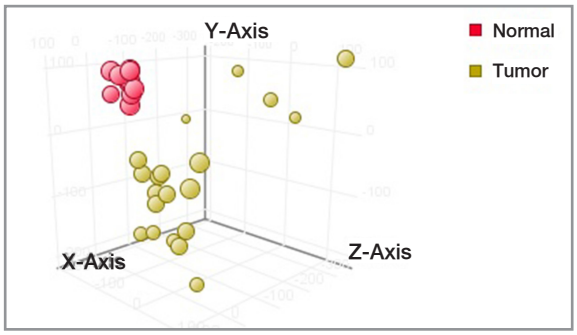

Figure 1. Multidimensional scaling plot of 10 controls and 20 tumors in three-dimensional space. Plot is generated using Gene Spring 14.9.

and tumors were positioned in different coordinates within the planes (Figure 1). A total of 2466 DEGs were identified in PTC samples compared to normal thyroid tissues, using GeneSpring with the FC of > 2.0 cutoff, including 1554 up-regulated and 912 down-regulated DEGs. The distributions of the fold changes and p-values of genes in each subgroup were shown in Figure 2 as volcano plots.

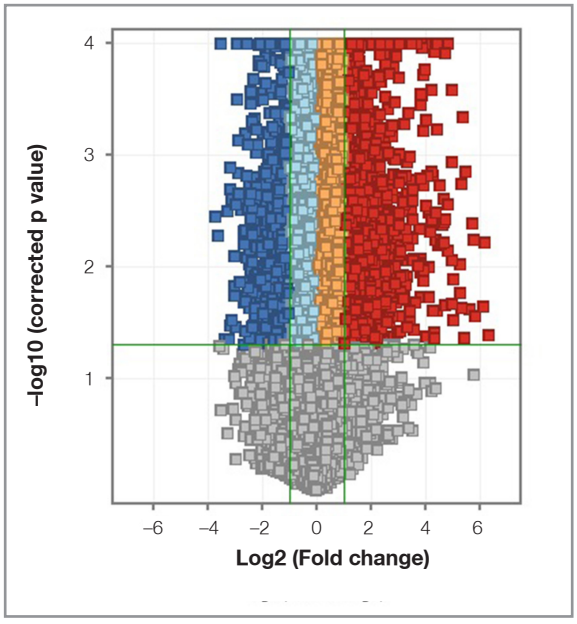

Figure 2. Volcano plot. The distribution of the gene expression fold changes and corrected p-values in PTC compared with normal controls was shown. A total number of 2466 genes with p-value < 0.05 and fold change > 2.0 were used for the analysis. Down regulated genes are indicated in dark blue, up-regulated genes are indicated in red. Plot is generated using GeneSpring 14.9 with moderated t-test and Benjamini-Hochberg testing correction.

Table 2. Top 10 significantly up- and down-regulated genes.

Up-regulated genes		Down-regulated genes	
Gene	**Fold Change**	**Gene**	**Fold Change**
KLK11	79.286	ARX	13.155
LIPH	73.467	LOC105376351	12.402
DCSTAMP	69.306	C11orf88	10.378
ST6GALNAC5	54.354	GTSF1	9.297
TMPRSS4	52.214	AGR3	8.951
SYT12	48.968	FKSG29	8.876
ARHGAP36	43.834	IPGK3	8.688
CHI3L1	43.150	CCBE1	8.199
FN1	40.454	TBX22	8.165
KLK10	40.073	ERICH3	8.002

The top 10 significantly up-regulated and down-regulated genes are shown in Table 2. Most significantly up-regulated DEGs were *KLK11, LIPH, DCSTAMP, ST6GALNAC5, TMPRSS4, SYT12, ARHGAP36, CHI3L1, FN1* and *KLK10*, whereas most significantly down-regulated DEGs were *ARX, LOC105376351, C11orf88, GTSF1, AGR3, FKSG29, IPGK3, CCBE1, TBX22* and *ERICH3*.

PPI Network Analysis

IPA constructed 5 interaction networks of DEGs with the filters human species and direct interactions were created. The most significant network was associated with Endocrine System Disorders, Gastrointestinal Disease and Immunological Disease (Figure 3). By evaluating the interaction degrees of nodes in the network, *CCND1* was defined as a major hub gene. Other identified hub genes were *PGR, CEBPA and CDKN1A, SPDEF* and *PLAU* and *MDM2*.

Causal Network Analysis

Causal network analysis was performed in order to identify papillary thyroid cancer-related upstream regulators that control expressions of the genes in our dataset. Table 3 lists the five most significant upstream regulators. Among these, *SFN* was

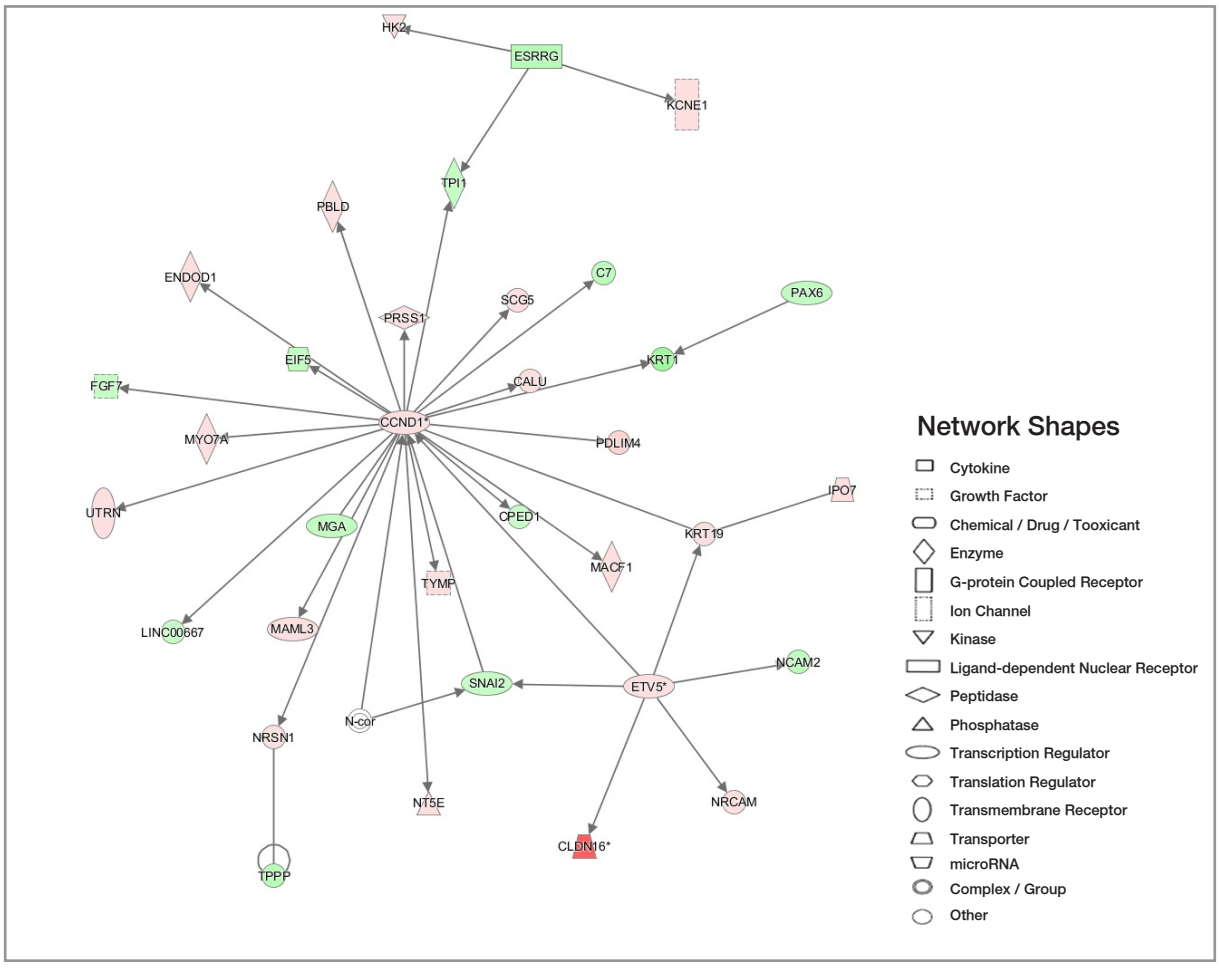

Figure 3. Interaction Network of Endocrine System Disorders, Gastrointestinal Disease, Immunological Disease related genes constructed using IPA.

the most significant regulator and was found to be 3.162 fold up-regulated in our study. Other papillary thyroid cancer related upstream regulators were *TP53, MYC, CDC25B and LEF1*, but they were not in our DEGs dataset. IPA predicted activation of *MYC, CDC25B* and *LEF1* regulators according to expression changes of their target genes in our dateset.

Functional and Pathway Enrichment Analysis

KEGG pathway enrichment analysis using DAVID showed that DEGs were enriched significantly in hsa04610: Complement and coagulation cascades, hsa04512:ECM-receptor interaction, hsa05200:Pathways in cancer, hsa04151:PI3K-Akt signaling pathway and hsa04510:Focal adhesion

Table 3. The most significant upstream regulators identified in causal network analysis

Upstream regulator	p-value of overlap	Molecular type	Predicted activation
SFN	8.77E-13	Other	
TP53	2.17E-12	Transcription regulator	
MYC	1.42E-10	Transcription regulator	Activated
CDC25B	5.42E-10	Phosphatase	Activated
LEF1	9.12E-10	Transcription regulator	Activated

Table 4. Themost significant canonical pathways identified using DAVID and IPA

Pathways identified using DAVID			
Term	**Genes**	**p-value**	**FDR[a]**
hsa04610:Complement and coagulation cascade	F12, C3AR1, C7, CR1, F10, C5AR1, MASP1, C6, PLG, PLAUR, PROC, C8A, F5, FGA, SERPINA5, SERPINE1, TFPI, SERPINA1, CFI, CFD, PROS1, PLAU	4.88E-9	6.37E-6
hsa04512:ECM-receptor interaction	TNXB, TNC, ITGA2, SDC4, LAMA2, LAMA1, SDC1, LAMB3, LAMA3, CD36, ITGB8, COMP, COL27A1, ITGB6, COL1A2, RELN, LAMC2, COL1A1, THBS1, COL11A1, FN1	1.79E-6	2.33E-3
hsa05200:Pathways in cancer	F2RL3, FGF8, FGF7, PDGFA, ADCY8, PPARG, CXCL12, GLI1, AGTR1, CDKN2A, WNT3, CDKN2B, CASP8, TGFA, BIRC8, HHIP, FGF1, PLCB2, PTGER3, CYCS, RXRG, RUNX1T1, FGF22, CCND1, LPAR5, PDGFRA, MDM2, LAMC2, FGFR2, CXCL8, LAMB3, KRAS, BCL2, PIK3R5, AXIN2, FN1, CEBPA, FZD8, KLK3, TGFBR1, MET, BIRC7, ITGA2, HGF, WNT2B, LAMA2, LAMA1, CBLC, CDKN1A, LAMA3, BAX, ABL1	1.21E-5	1.58E-2
hsa04151:PI3K-Akt signaling pathway	FGFR2, FGF8, FGF7, PDGFA, TNC, LAMB3, KRAS, ITGB8, BCL2, COL27A1, COMP, ITGB6, CREB3L1, PDGFC, PIK3R5, PPP2R2B, FGF1, THBS1, PPP2R2C, COL11A1, GHR, FN1, TNXB, SGK2, MET, ITGA2, FGF22, CREB5, HGF, EPHA2, GH2, LAMA2, LAMA1, CCND1, CDKN1A, YWHAG, LAMA3, LPAR5, CCND2, COL1A2, PDGFRA, MDM2, EFNA5, LAMC2, RELN, COL1A1	3.60E-5	4.70E-2
hsa04510:Focal adhesion	PDGFA, TNC, LAMB3, ITGB8, PAK3, BCL2, COMP, COL27A1, ITGB6, PDGFC, PIK3R5, SHC3, THBS1, COL11A1, FN1, TNXB, MET, ITGA2, HGF, FLNC, VASP, LAMA2, LAMA1, CCND1, LAMA3, RASGRF1, CCND2, COL1A2, PDGFRA, RELN, LAMC2, COL1A1	4.24E-5	5.53E-2

[a]FDR= false discovery rate

Pathways identified using IPA		
Pathway	**p-value**	**Molecules**
Atherosclerosis signaling	6.00E-08	↑ALOX5, ↑ALOX15B, ↑APOA4, ↓APOD, ↓CCR2, ↓ CD36, ↑CLU, ↑CMA1, ↑COL10A1, ↑ COL1A1, ↑COL1A2, ↑CXCL8, ↓CXCL12, ↑IL1RN, ↑IL36A, ↑ IL36RN, ↑MMP3, ↑ MSR1, ↑PDGFA, ↑ PDGFC, ↑PLA2G10, ↑PLA2G16, ↑PLA2G2E, ↓PLA2G4C, ↓PLA2R1, ↓PLB1, ↑PON1, ↑SERPINA1, ↑TNFRSF12A
Intrinsic protrombin activation pathway	1.94E-07	COL10A1 ↑ , COL1A1 ↑ , COL1A2 ↑ , F5 ↓ , F10 ↓ , F12 ↑ , FGA ↓ , KLK2 ↓ , KLK3 ↑ , KLK7 ↑ , KLK10 ↑ , KLK11 ↑ , KLK12 ↑ , PROC ↑ , PROS1 ↑
Agranulocyte adhesion and diapedesis	2.07E-07	ACTG2 ↓ , AOC3 ↓ , C5AR1 ↑ , CCL7 ↑ , CCL16 ↓ , CCL17 ↑ , CKLF ↑ , CLDN1 ↑ , CLDN10 ↑ , CLDN12 ↑ , CLDN16 ↑ , CXCL1 ↑ ,CXCL2 ↑ , CXCL3 ↑ , CXCL8 ↑ , CXCL12 ↓ , CXCL17 ↑ , FN1 ↑ , HRH1 ↑ , IL1RN ↑ , IL36A ↑ , IL36RN ↑ , ITGA2 ↑ , MMP3 ↑ , MMP7 ↑ , MMP10 ↑ , MMP11 ↑ , MMP16 ↑ , MMP17 ↑ , MSN ↑ , MYH10 ↑ , MYH11 ↓ , PF 4 ↓ , PPBP ↓ , SDC4 ↑
Coagulation system	1.04E-06	F5 ↓, F10 ↓, F12 ↑, FGA ↓, PLAU ↑, PLAUR↑, PLG↓, PROC ↑, PROS1 ↑, SERPINA1 ↑, SERPINA5 ↓, SERPINE1 ↑, TFPI ↓
Granulocyte adhesion and diapedesis	1.39E-06	C5AR1 ↑ , CCL7 ↑ , CCL16 ↓ , CCL17 ↑ , CKLF ↑ , CLDN1 ↑ , CLDN10 ↑ , CLDN12 ↑ , CLDN16 ↑ , CXCL1 ↑ , CXCL2 ↑ , CXCL3 ↑ , CXCL8 ↑ , CXCL12 ↓ , CXCL17 ↑ , HRH1 ↑ , IL1RAP ↑ , IL1RN ↑ , IL36A ↑ , IL36RN ↑, ITGA2 ↑ , MMP3 ↑, MMP7 ↑ , MMP10 ↑ , MMP11 ↑ , MMP16 ↑ , MMP17 ↑ , MSN ↑ , PF4 ↓ , PPBP ↓ ,SDC1 ↑ , SDC4 ↑

↑= up-regulated; ↓= down-regulated

Table 5. GO analysis results for the DEGs

Term	Count	P-value	FDR[a]
Biological process			
GO:0007155~cell adhesion	78	4.46E-11	8.30E-8
GO:0030198~extracellular matrix organization	41	9.03E-9	1.68E-5
GO:0042060~wound healing	23	8.80E-8	1.64E-4
GO:0051965~positive regulation of synapse assembly	20	1.00E-7	1.87E-4
GO:0001525~angiogenesis	39	2.56E-6	4.76E-3
GO:0007399~nervous system development	46	3.296E-6	6.12E-3
GO:0006508~proteolysis	67	9.561E-6	1.78E-2
Cellular component			
GO:0005576~extracellular region	222	1.24E-19	1.89E-16
GO:0005615~extracellular space	188	3.72E-17	5.44E-14
GO:0005578~proteinaceous extracellular matrix	52	7.45E-10	1.09E-6
GO:0005887~integral component of plasma membrane	163	4.05E-8	5.92E-5
GO:0009986~cell surface	77	1.20E-7	1.75E-4
GO:0031093~platelet alpha granule lumen	17	1.88E-6	2.75E-3
Molecular Function			
GO:0005509~calcium ion binding	99	2.48E-9	4.00E-6
GO:0004252~serine-type endopeptidase activity	46	4.69E-8	7.56E-5
GO:0008083~growth factor activity	30	8.34E-6	1.35E-2

[a]FDR= false discovery rate

(Table 4). The complement and coagulation cascades pathway was the most enriched pathway.

Atherosclerosis signaling, intrinsic protrombin activation pathway, agranulocyte adhesion and diapedesis, coagulation system and granulocyte adhesion and diapedesis were the most significant canonical pathways identified using IPA (Table 4). The coagulation system was common to both IPA and DAVID. *SERPINA1* was the most up-regulated gene with the fold-change of 14.128 in this pathway.

GO analysis using DAVID revealed that the biological process of cell adhesion, molecular function of calcium ion binding and the cellular component of the extracellular region were the most significant terms (Table 5).

qRT-PCR Validation

CCND1 and *CDKN1A* gene expressions obtained from the qRT-PCR validation study showed consistent expression differences with our microarray data. Gene expression results of two genes from qRT-PCR were 3.276 fold up-regulation for *CCND1* and 10.483 fold up-regulation for *CDKN1A*.

DISCUSSION

In the present study, 1554 up-regulated and 912 down-regulated DEGs were identified in PTC samples compared to normal thyroid tissues. Among the DEGs, *KLK11* was the most up-regulated, whereas ARX was the most down-regulated gene. The coagulation system was determined as the most significant pathway and *SERPINA1* was the most up-regulated gene of this pathway. *CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU* and *MDM2* were the key nodes in the PPI networks.

Kallikrein-related peptidases (*KLKs*) function in many physiological and pathological processes, like skin desquamation, semen liquefaction, immune system regulation and oncogenesis.[8] Several *KLKs* are known to be dysregulated in differ-

ent solid cancers, including ovarian, gastric, lung, prostate, breast, larynx, stomach, colorectum and kidney cancers.[8,9] Overexpression of *KLK4* and *KLK11* in ovarian cancer and prostate cancer, and downregulation of *KLK6* in breast cancer and prostate cancer have been reported previously.[9] High expression of *KLK7* has been found in colon cancer compared with normal tissues, and *KLK7* has been suggested to be a prognostic factor for colon cancer patients.[9] *KLK11* is expressed mostly in prostate, stomach, trachea, skin, and colon. High levels of *KLK11* have been reported to be associated with poor prognosis in ovarian cancer, lung cancer and gastric cancer.[8] Kallikrein 11 expression in PTC has not previously been studied. Kim et al. showed 7-fold higher expression levels of *KLK7* in PTC tissues than in normal tissues.[9] Here, we reported high levels of *KLK11* in PTC samples compared to normal tissues. Thus, *KLK11* may have an important role in development of PTC and may be used as a diagnostic factor for PTC.

Coagulation is a dynamic and complex process that responds to injury by the rapid formation of a clot.[10] Disruption of the coagulation system is common in cancer development and metastasis.[10] Deregulation of the coagulation cascade has been reported in PTC.[1] In our study *SERPINA1* (α1-AntiTrypsin, AAT) was the most up-regulated gene enriched in the coagulation system pathway with the fold change of 14.127. Other up-regulated genes enriched in this pathway were *PROS1, PLAUR, PLAU, PROC, SERPINE1* and *F12* with the fold changes 9.168, 7.160, 5.914, 4.990, 3.469 and 2.202 respectively. *SERPINA1* is an inflammatory response molecule and acts on serine proteases.[11] It has been suggested as a biomarker for many cancers such as Cutaneous Squamous Cell Carcinoma, Non-Small Cell Lung Cancer (NSCLC), lung cancer and breast carcinoma as well as for papillary thyroid carcinoma.[1] *SERPINA1* has been reported as a potential diagnostic marker for papillary thyroid carcinoma and has been found activated or overexpressed in PTC previously.[12] Vierlinger et al. suggested that *SERPINA1* differentiates PTCs from benign nodules or healthy tissues with 99% accuracy.[13] We speculated that *SERPINA1* may play an important role in PTC development through the coagulation system pathway.

Cyclin D1 (*CCND1*), is a promoter of cell cycle progression and is overexpressed in many benign and malignant neoplasms, with an oncogenic role. It has been reported that there is a positive correlation between the overexpression of *CCND1* and cellular proliferation, the proliferation marker Ki-67, tumor stage and aggressive biological behavior.[14] Overexpression of *CCND1* has been reported in both benign and malignant thyroid tumors before.[15] *CCND1* upregulation has also been reported in a previous study and has been associated with poor prognosis.[16] We detected a 5.083 fold upregulation of *CCND1* compared to control samples in the current study which is consistent with the literature. Thus, *CCND1* may be used for PTC diagnosis as well as for assessing prognosis.

PGR, the progesterone receptor, encodes a member of the steroid receptor superfamily. Its protein mediates the physiological effects of progesterone. It has been used as a biomarker for ERα (estrogen receptor-α) function and breast cancer prognosis. ERα has proliferative and antiapoptotic activity and has been found more highly expressed in metastatic PTC than in the primary site and in PTC patients with a large tumour size. Many studies showed the expressions of *ER*-α and *PR* in PTC.[17] Dai et al. examined mRNA and protein expressions of *ERα, ERβ, PR, ERα36, EGFR* and *HER2* in PTCs, nodular hyperplasias and normal thyroid tissues using real time RT-PCR and immunohistochemical staining. The mRNA and protein expression of ERα and PR were increased in PTCs whereas ERβ was decreased.[18] In our study, increased expression of *PGR*, with the fold change 2.477, was found which is similar to the literature. Findings suggest that *PGR* may be a potential diagnostic and prognostic biomarker for PTC.

CEBPA (C/EBPα), is an intronless gene encoding a basic leucine zipper transcription factor. The protein product of this gene functions in normal tissue development, regulation of cell proliferation and cell differentiation. Approximately 10% of acute myeloid leukemia (AML) patients have loss-of-function mutations in C/EBPα, suggesting a tumor suppressor role. C/EBPα expression is deregulated in many neoplasias, such as liver, breast and lung cancer.[19] Interestingly we found

activation of C/EBPα, with the fold change 5.849, rather than inactivation in our study. Chapiro et al. suggested that C/EBPα which was found activated in precursor B-cell acute lymphoblastic leukemia has an oncogenic role.[20] C/EBPα may contribute to the development of papillary thyroid cancer via its oncogenic role.

CDKN1A (p21) is a cyclin-dependent kinase (cdk) inhibitor, and has a mediator role in p53-dependent cell cycle arrest after DNA damage. P21 is known as a tumor suppressor gene as it inhibits proliferation. However, p21 also acts as an oncogene since it exhibits procancer and antiapoptotic activities.[21] Varkondi et al. studied cyclin D1, p53 and p21 expressions using an immunohistochemical method in papillary thyroid cancer samples and found p21 production in 50% of tumour samples with cyclin D1 overexpression. As p21 is a cyclin dependent kinase (CDK) inhibitor, this association was suggested to be a modulatory role of p21 rather than its inhibitory role.[16] In our study both *CCND1* (fold change 5.083) and p21 (fold change 5.757) were found to be up-regulated in PTCs compared to normal controls. In light of this we suggest that p21 may be involved in PTC pathogenesis through modulating *CCND1* activity with its oncogenic role.

The prostate epithelium-specific Ets transcription factor, *SPDEF* (also termed *PDEF* or *PSE*), regulates gene expression in the prostate and goblet cell hyperplasia in the lung.[22] *SPDEF* mediates invasion and migration of immortalized mammary epithelial cells.[23] In tumor cells, activation of *SPDEF* blocks migration and invasion, however inhibition of *SPDEF* expression enhances migration, invasion, and metastasis.[24] It has been reported that *SPDEF* acts as a metastasis supressor gene in prostate cancer and its expression is inversely correlated with tumor aggressiveness and patient prognosis.[25] It has a critical role in estrogen receptor-positive (ER+) breast cancer risk and cancer progression.[26] Higher expression of *SPDEF* has been identified in brain, breast, prostate, lung and ovarian tumors previously.[27] It has been reported that SPDEF associates with tumors better than other cancer-related molecules.[27] There are no reports in the literature concerning *SPDEF* expression in papillary thyroid cancers. In our study *SPDEF* was found to be up-regulated with the fold change 2.055. According to the known role of *SPDEF* in cancer cells, we suggest that *SPDEF* may be used as a prognostic factor for papillary thyroid cancer to evaluate patients' metastasis risk. Further studies with metastatic PTC population should be performed to confirm this.

In 50% of human cancers, p53 is found mutated whereas in remainder wild-type p53 is inhibited with overexpression of Murine Double Minute 2 (*MDM2*). *MDM2* is involved in cancer in both a p53-dependent and p53-independent manner. *MDM2* targets p53 for ubiquitylation and proteasomal degradation. In the p53-independent mechanism, *MDM2* overexpression promote neoangiogenesis, tumor transformation, invasion and metastases. *MDM2* overexpression with gene amplification or other mechanisms have been observed in many cancers such as colorectal, esophageal, breast and colon cancers, melanoma, retinoblastoma and also in papillary thyroid cancer.[28] In our study, we detected high levels of *MDM2* expression with the fold change 2.295. We suggest that *MDM2* may play a role in pathogenesis of PTC and it may be a useful target to develop therapeutic approaches for controlling PTC progression.

PLAU (Plasminogen activator, urokinase) is a member of urokinase plasminogen activator (uPA) system, and codes for a serine protease.[28] The uPA system plays a critical role in inflammation, embryogenesis, tumor invasion, metastasis and tumour progression by inducing extracellular matrix degradation, activation of latent growth factors, malignant cell spread and tumour neoangiogenesis.[29] The system consists of urokinase-type plasminogen activator (uPA), the glycolipid-anchored cell membrane receptor for the uPA (uPAR) and plasminogen activator inhibitors (PAIs). Many human cancers, including thyroid malignancies show overexpression of uPA and/or uPAR when compared with normal tissue. It has been reported that high levels of uPA and uPAR are associated with lymph node metastases, advanced tumour stage and reduced disease-free interval.[29] *PLAU* was identified 5.914 fold overexpressed in our PTC group, suggesting *PLAU* to be a prognostic biomarker for PTC.

Causal network analysis in the present study revealed that *SFN*, which was one of the up-regulated DEGs, was the most causative upstream regulator for papillary thyroid cancer. *SFN* (stratifin or 14-3-3 sigma) is a major cell cycle regulator and an important component of signal transduction that belongs to the 14-3-3 protein family.[30] Among seven isoforms of this protein family, it is the only isoform which is induced by p53 after DNA damage. It has been referred to as a "double-edged sword of human cancers" because of acting both as a tumor suppressor with reduced expression in various malignancies, including breast, stomach, colon, liver, prostate, oral cavity and vulva cancers with hypermethylation of the CpG island present in the promoter area of the gene and acting as an oncogene with increased expression in cancers such as head and neck, stomach, pancreas and colorectum, associated with demethylation of the CpG island. Thus, its expression has been reported to be tissue specific and context-dependent.[30,31] Elevated expression of *SFN* was observed in previous PTC studies and has been suggested to play an important role in large tumor size, invasion and metastasis.[31] High expression of *SFN* has been reported in cPTC and fvPTC with advanced stage and poor differentiation, but was not found in follicular thyroid cancer (FTC) and normal thyroid tissues.[32] It has been reported that *SFN* has lower expression in fvPTC than in cPTC. In contrast anaplastic tumors have been reported showing the highest *SFN* expression levels.[33] Therefore it has been suggested that combination of *SFN* expression with FNA cytology might be used to differentiate malignant from benign tumors which have suspicious or indeterminate cytology.[34] Also we can conclude that, as *SFN* is not expressed in normal thyroid tissue, it may be involved in thyroid carcinogenesis.

Elevated levels of *SFN* has been reported to contribute to drug resistance in cancer treatment. Studies with drug resistant cell lines of breast cancer, prostate cancer and pancreatic cancer showed that knocking down *SFN* expression decreased resistance to anticancer drugs, while ectopic overexpression of *SFN* increased drug resistance in these cell lines again.[31] Thus, *SFN* has been accepted as a potential target for developing new therapeutic approaches via knocking down or reducing its expression. In further studies, papillary thyroid cancer cell lines should be treated with chemical agents and/or oligonucleotides targeting *SFN* to see investigate their effects on PTC. We can suggest that *SFN* may be a diagnostic and prognostic biomarker for PTC and inhibition or reduction of its expression using novel anticancer drug approaches may help to improve prognosis.

Compared to similar earlier studies, *KLK11, CEBPA* and *SPDEF* genes were never reported in the context of thyroid cancer in our study.[1,15,35] On the other hand, we observed similar findings to these studies. Liang and Sun integrated four gene expression datasets to identify novel, clinically relevant genes for PTC.[15] They reported six central genes: *BCL2; CCND1; FN1; IRS1; COL1A1;* and *CXCL12*. Among these genes, *BCL2, CCND1* and *COL1A1* were reported to be clinically relevant. These three genes were also reported as DEG in our study. They also identified DEGs enriched in PI3K–Akt signaling pathway, pathways in cancer, focal adhesion and proteoglycans in cancer. We also observed PI3K–Akt signaling pathway, pathways in cancer and focal adhesion in KEGG pathway enrichment analysis in the current study. Activation of PI3K–Akt signaling pathway is a common process in human cancers and also has been reported to be involved in thyroid cancer development. PI3K–Akt signaling pathway has been known as a representative, upstream factor of SFN, and associated with poor prognosis in lung cancer. It has been reported that SFN is activated by the PI3K/Akt signaling pathway in a p53-independent manner and thus SFN mediates cell cycle progression.[30] This mechanism, is supported by the results of causal network analysis in our study, suggest that SFN may promote PTC development via PI3K/Akt signaling pathway in a p53-independent manner. Zhao and Hehe aimed to identify gene alterations and biomarkers for PTC.[35] As a result of pathway analysis in their study, they found pathways in cancer, proteoglycans in cancer, focal adhesion, axon guidance and ECM-receptor interaction as the most enriched pathways. Among these pathways, pathways in cancer, focal adhesion and ECM-receptor interaction were also significant in our study. In consequence, deregulation of pathways in cancer, focal adhesion and ECM-receptor

interaction, the PI3K/Akt signaling pathway and coagulation system may be common phenomenon contributing to PTC development.

The present study has some limitations. First, we have analyzed gene expression profiles of 20 tumors and 10 controls. Further studies with large numbers of patients will be needed to confirm the gene profiles of our study. Second, we used 2-fold threshold to identify differentially expressed genes during data analysis using GeneSpring software. In this case, genes with low expression may be excluded.

In conclusion, deregulation of *SERPINA1, CCND1, PGR, CEBPA, CDKN1A, SPDEF, PLAU* and *MDM2* genes and pathways in cancer, focal adhesion, ECM-receptor interaction, the PI3K/Akt signaling and coagulation system pathways may contribute to PTC development. *KLK11, CEBPA* and *SPDEF* genes were never reported and suggested being involved in thyroid cancer development. *SFN* may promote PTC development via the PI3K/Akt signaling pathway in a p53-independent manner and may be an important gene in diagnosis, prognosis and novel anticancer drug approaches for PTC. Further experiments are required to confirm the functions of identified DEGs in our study.

Acknowledgements

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK; project number 108S415) and Scientific Research Projects Coordination Unit of Kocaeli University (project number 2009/024).

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