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Original article

Identification of key genes involved in papillary thyroid cancer by bioinformatics tools

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Abstract. *Study Objectives:* Thyroid cancer is the sixth most common type of cancer among women worldwide, with an increasing incidence. It is the most common endocrine cancer and it is seen in 1.7% of all cancers. We aimed to detect genes whose expression level varies in this pathology by using gene expression data obtained from papillary thyroid cancer tissue. *Methods:* Microarray data selected for bioinformatic analysis is Gene Expression data stored with GSE35570 code in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. Thyroid cancer and healthy control groups were compared, then variance filtering was applied and as a result, gene lists with different expression levels were obtained between the compared groups. Totally, 1209 genes were differentially expressed between these two groups. *Results:* We have determined that *SFTPB, HMGA2, ARHGAP36, SYTL5, LRRK2, PRR15, DPP4, TENM1, SCEL* genes were upregulated in papillary thyroid cancer group and *CCL21, COL9A3, FBLN1, LRP1B, PROM1, NEB, CDH16, TFCP2L1* genes were downregulated. *Conclusion:* We have concluded that these identified genes can be used as candidate biomarker genes for diagnosis of papillary thyroid cancer.

Keywords: Papillary thyroid cancer, gene expression, bioinformatics

Introduction

Thyroid cancer is the sixth most common type of cancer among women worldwide, with an increasing incidence and it is the most common endocrine cancer and it is seen in 1.7% of all cancers (1). Histopathologically, there are four subtypes. These are indicated as Papillary, Follicular, Medullary and Anaplastic (2). 85-90% of all thyroid cancers are papillary thyroid cancers (3). In America, 77276 cases of thyroid cancer were diagnosed between 1974 and 2013, of which 64625 are the most common papillary thyroid (PTC) cancer (4). According to the American Cancer Society, the survival rate of early stage (stage I and stage II) PTC patients is about 100%, but the same situation drops to 55% in Stage 4 (5).

PTC patients develop about 1.7% to 15% of distant metastases, leading to a significant reduction in patients' survival (6). Distant metastasis is one of the most important prognostic factors for PTC patients. Distant metastases are considered a high risk for PTC patients and often require more aggressive treatments (7). Dedifferentiated primary tumor is also an important problem in papillary thyroid cancer. This gives the tumor an aggressive feature and affects survival rates negatively.

New molecular tests, such as ThyroSeq, can improve the management of thyroid nodules, but the cost and availability of these tests may be difficult at times (8). B-mode ultrasound (US) technology is an important factor in evaluating thyroid nodules. Cutting wave elastography and the addition of three-dimensional (3D) US imaging may improve the risk classification for thyroid cancer (9).

Mutations in many genes have been identified in thyroid cancers. The most common mutations were

detected in the *BRAF, RAS* and *RET* genes found in the MAPK signal pathway (10). In addition, some studies have also found that mutations occur frequently in galectin-3 (*LGALS3*), platelet-derived growth factor (*PDGF*) and epithelial muctin-1 (*MUC1*) genes (11). The methylation rate of the *TSHR* gene is high in PTC patients. This is associated with age, lymph node metastasis and tumor size, suggesting that the *TSHR* gene may be a biomarker for PTC diagnosis (12). It is very important to find the molecular mechanisms that play a role in the emergence and progression of thyroid cancer. It is known that there is a need for biomarkers that can be used in the diagnosis of the disease. We designed such a study to determine the molecular mechanisms involved in the occurrence of thyroid cancer and to obtain information about the molecular profile of the papillary thyroid cancer subtype.

In this study, we aimed to detect genes whose expression level varies in this pathology by using gene expression data obtained from papillary thyroid cancer tissue. In this way, we aimed to identify candidate biomarker genes that can be used to diagnose this pathology by a noninvasive molecular method.

Material and Methods

Microarray Data: Microarray data selected for bioinformatics analysis is Gene Expression data stored with GSE35570 code in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (13). Gene expression data of 17 papillary thyroid carcinomas and 21 normal thyroid tissue were used in this dataset. Raw gene expression data [HG-U133_Plus_2] was created with Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix Inc. Santa Clara, California, USA).

Analysis of Differentially Expressed Genes (DEG Analysis): All analyzes on raw data were done with Transcriptome Analysis Console 4.0 (Applied Biosystem). Preprocessing was performed with the robust multi-array average (RMA) method on the original expression data belonging to the thyroid cancer and healthy control group. After the expression values were calculated, group comparison was performed. Thyroid cancer and control groups were compared, then variance filtering was applied and as a result, gene lists with different expression levels were obtained between the compared groups.

Functional Clustering and Gene-Set Enrichment Analysis: The gene list is loaded into the DAVID Bioinformatics Tools program to perform functional cluster analysis, enrichment analysis and pathway analysis (14,15). With this program, cluster analysis was performed first and the genes in the list were clustered according to their functions. The program calculates an enrichment score for each cluster during analysis. Clusters with an enrichment score greater than 1.3 are considered significant and important (14, 15). Again, heatmap was created as a result of the list hierarchical cluster analysis given in the same program. Heatmap shows whether our samples have been separated from each other using this gene list. The program also made pathway analyzes. As a result of these analyzes, the molecular pathways of the genes in the significant clusters in our list were determined and the relationship of these molecular pathways with the disease was evaluated.

Results

In the bioinformatics analysis, we applied principle component analysis to determine the quality control and the general distribution of the data we used in the first stage. As a result of this analysis, we obtained the distribution graph of the sample group we used in three-dimensional space. As seen in Figure 1, the sample group we used includes thyroid cancer samples and healthy control samples, it was determined that these sample group showed a distribution as two different groups as we expected.

Figure 2 shows that our samples signal as a result of our bioinformatics analysis. This box plot showing the magnitude of the signals obtained from each sample. Scatter plot and Volcano plot graphics in Figure3 and Figure 4 are given. In the Scatter plot chart, the mean expression levels of the differently expressed genes obtained as a result of cancer and control group comparisons are shown in the log2 base. On the

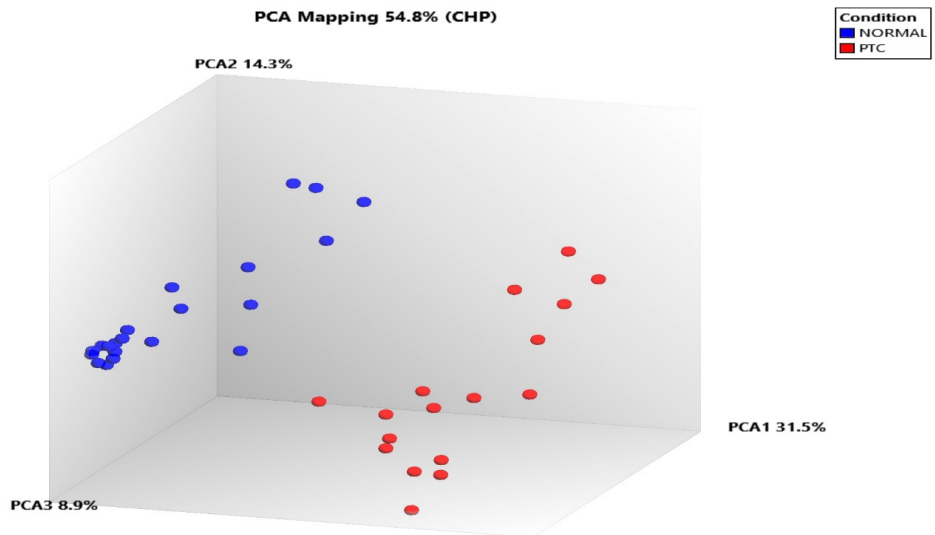

Figure 1. Principle Component Analysis results of Thyroid cancer and Healthy Control samples.

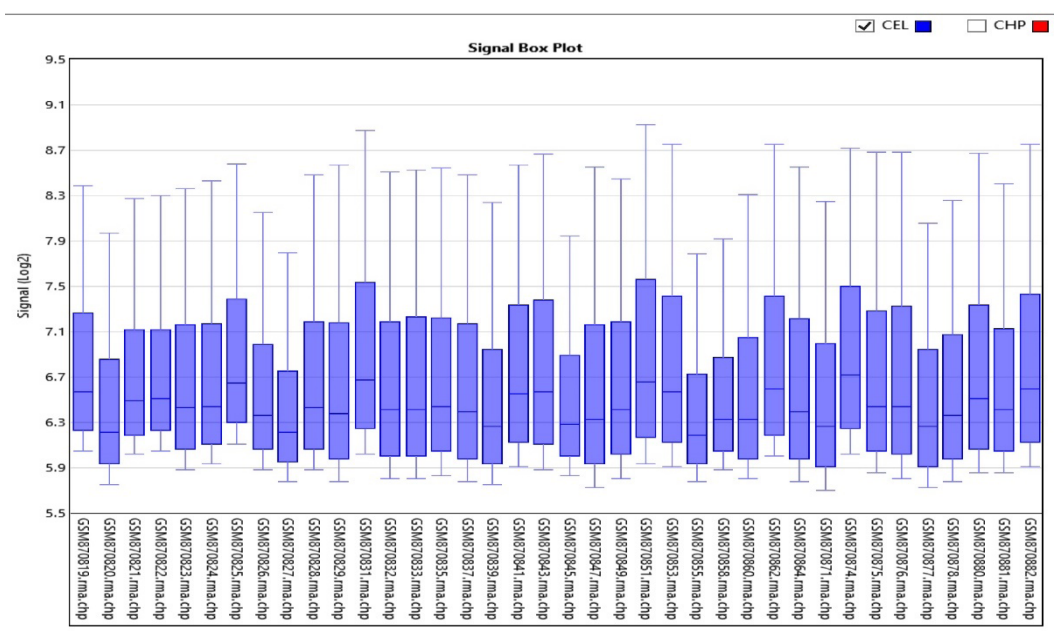

Figure 2. Signal Box Plot of Thyroid cancer and Healthy Control samples.

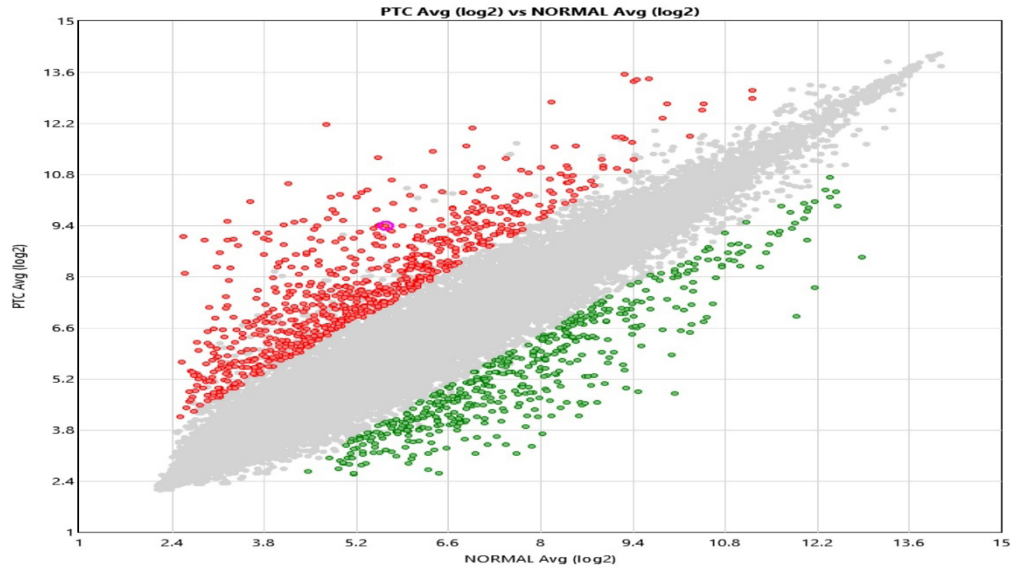

Figure 3. Scatter plot of obtained differentially expressed genes with comparison of Thyroid Cancer and Healthy group. Red dots represent upregulated genes; green dots represent downregulated genes.

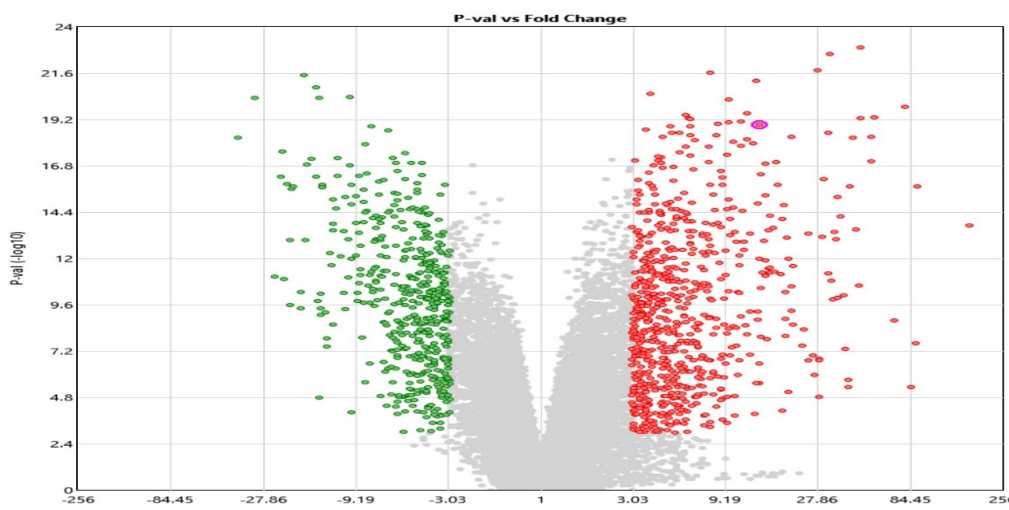

Figure 4. Volcano plot of differentially expressed genes obtained from Thyroid Cancer and Healthy group comparison. Red dots represent upregulated genes; green dots represent downregulated genes.

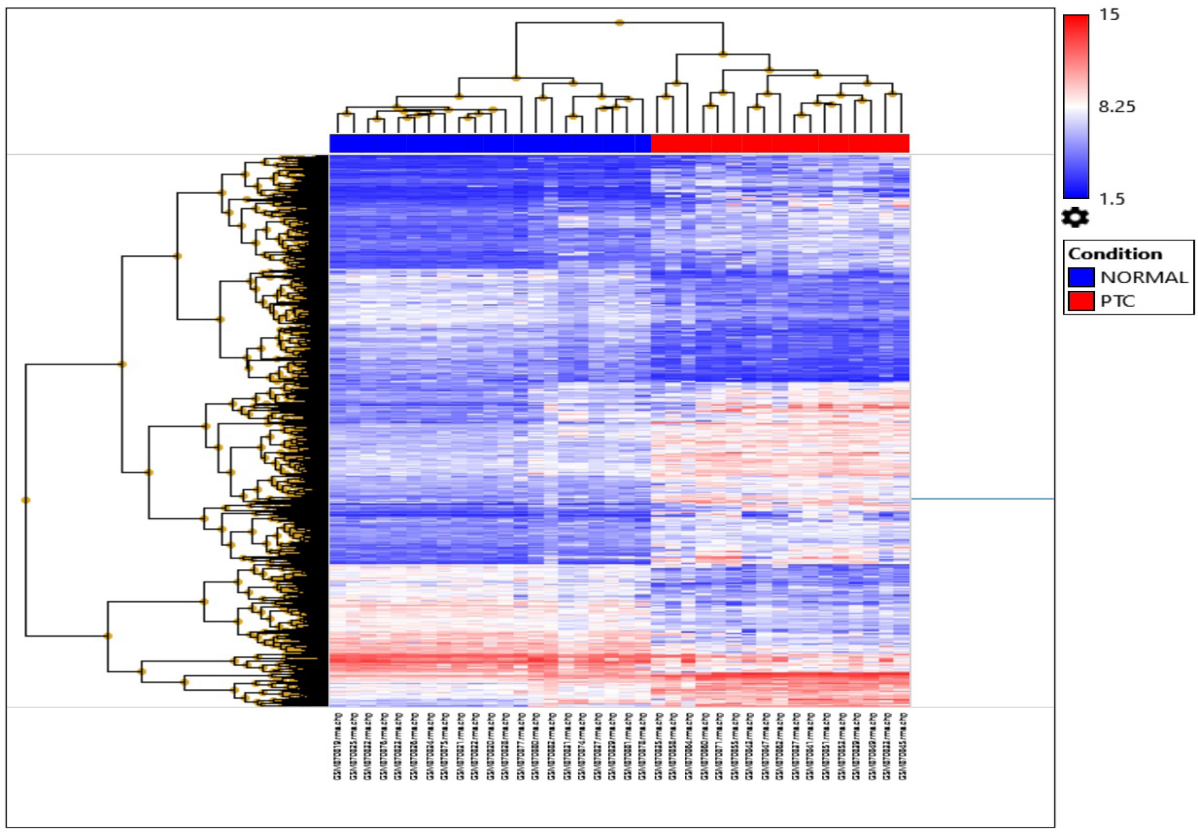

Figure 5. HeatMap of obtained from Hierarchical Cluster Analysis of 1209 differentially expressed genes between Thyroid and Healthy group.

Volcano plot chart, the distribution of the p value calculated for each gene and the fold change value shown is given.

It is clearly seen that differently expressed genes obtained from the comparison of cancer and control group are differentiated from each other in the hierarchical cluster analysis (Figure 5). The cancer group and the control group display a unique molecular pattern in terms of gene expression profile in the heatmap.

In Table 1 and Table 2, as a result of the comparison, the first 25 genes showing the highest fold change as upregulated from 1209 genes, whose expression level varies between these two groups, and the first 25 genes showing the highest fold change as downregulated are listed. In these tables, the calculated expression level of each gene in the cancer and control group, the fold change value it shows, the calculated p value and the FDR statistical value are given.

1209 genes obtained as a result of group comparison were used for gene set enrichment analysis using DAVID Bioinformatics Tools algorithm. The genes in the given list are grouped according to their functions and are divided into clusters. An enrichment score was calculated for each cluster. Clusters with an enrichment score greater than 1.3 were considered significant. The most important 5 clusters obtained as a result of the functional cluster analysis made in Table 3 are given. Also, using the same algorithm, pathway analysis was performed on the KEGG database. The molecular pathways that come forward as a result of this analysis are given in Table 4.

Table 1. Top 25 upregulated genes in comparison of cancer and healthy group

Gene Symbol	*PTC Avg (log2)*	*NORMAL Avg (log2)*	*Fold Change*	*P-value*	*FDR P-value*
ZCCHC12	12.18	4.75	172.14	1.84E-14	4.30E-12
GABRB2	9.1	2.58	92.14	1.83E-16	9.36E-14
CHI3L1	10.06	3.58	89.12	2.43E-08	7.53E-07
SFTPB	10.56	4.16	84.65	4.69E-06	6.73E-05
HMGA2	9.52	3.23	78.2	1.39E-20	5.83E-17
ARHGAP36	9	2.89	69.11	1.63E-09	7.38E-08
SYTL5	9	3.24	54.51	4.73E-20	1.61E-16
LRRK2	11.26	5.53	52.97	8.49E-18	7.73E-15
PRR15	9.03	3.33	52.25	4.87E-19	7.40E-16
DPP4	8.65	3.13	46.07	1.11E-23	6.08E-19
TENM1	9.2	3.67	46.02	5.37E-20	1.65E-16
LOC101930164	8.11	2.59	45.71	2.54E-11	2.08E-09
SCEL	8.87	3.41	43.88	2.97E-14	6.49E-12
LRP4	10.19	4.81	41.86	5.19E-19	7.64E-16
GABRB2	9.21	3.87	40.46	1.76E-16	9.14E-14
ALOX5	9.43	4.1	40.11	1.97E-06	3.24E-05
SFTPB	10.27	4.96	39.63	4.63E-06	6.67E-05
CHI3L1	9.06	3.79	38.61	4.66E-08	1.32E-06
PCSK2	9.49	4.26	37.6	7.56E-11	5.22E-09
SCEL	8.26	3.07	36.43	6.18E-15	1.75E-12
PLAU	9.55	4.4	35.39	1.01E-10	6.64E-09
SERPINA1	12.07	6.96	34.5	9.84E-14	1.79E-11
CLDN1	11.43	6.36	33.71	4.19E-14	8.63E-12
ECM1	10.31	5.27	33	1.23E-10	7.82E-09
PLAU	10.39	5.37	32.57	1.42E-11	1.28E-09

Discussion

In this study, we performed bioinformatics analysis using the gene expression data of 21 normal thyroid tissues and 17 papillary thyroid cancer tissues. We compared the cancer and healthy control groups and identified genes whose expression levels differed between these two groups. As a result of this analysis, we determined that a total of 1209 genes were expressed differently between these two groups. We determined that the expression levels of these genes, galectin-3 (*LGALS3*) and epithelial muctin-1 (*MUC-1*) genes, increased 4.84 and 5.03 times in the papillary thyroid cancer group, respectively. We have stated that these genes are upregulated in papillary thyroid cancer and potential biomarker candidate genes.

The *ZCCHC12* (zinc finger, CCHC domain containing 12) gene, which ranks first in our list of differently expressed genes and has increased 172-fold expression levels, has been suggested to be upregulated in the papillary thyroid cancer tissue and this gene is oncogene (16). The *GABRB2* (gamma-aminobutyric acid (GABA) A receptor, beta 2) gene is in our gene list, and its expression level is 93 times more in the

Table 2. Top 25 downregulated genes in comparison of cancer and healthy group

Gene Symbol	*PTC Avg (log2)*	*NORMAL Avg (log2)*	*Fold Change*	*P-value*	*FDR P-value*
CCL21	6.47	10.27	-13.92	6.57E-11	4.62E-09
COL9A3	5.45	9.27	-14.06	3.61E-10	1.98E-08
FBLN1	4.57	8.39	-14.09	8.42E-10	4.17E-08
LRP1B	2.63	6.45	-14.17	4.72E-21	2.34E-17
PROM1	3.17	7	-14.22	1.61E-05	0.0002
NEB	3.91	7.76	-14.47	1.49E-10	9.18E-09
CDH16	4.41	8.3	-14.83	1.33E-21	1.04E-17
TFCP2L1	5.08	8.97	-14.85	5.42E-17	3.48E-14
LYVE1	4.19	8.16	-15.62	6.75E-18	6.58E-15
PLA2R1	4.56	8.59	-16.38	1.30E-17	1.04E-14
LYVE1	3.4	7.46	-16.7	1.09E-13	1.95E-11
IPCEF1	5.87	9.98	-17.19	2.96E-22	3.23E-18
GPM6A	4.48	8.62	-17.63	3.73E-10	2.04E-08
DGKI; LOC100128727	4.54	8.68	-17.65	5.13E-11	3.78E-09
DPP6	4.56	8.85	-19.52	1.82E-16	9.36E-14
ADH1B	3.71	8.02	-19.83	2.42E-16	1.20E-13
TPO	8.53	12.88	-20.37	1.13E-13	1.99E-11
PKHD1L1	4.94	9.29	-20.38	2.52E-10	1.45E-08
DGKI	4.18	8.57	-20.94	1.29E-16	7.25E-14
DIO1	7.71	12.16	-21.94	1.14E-11	1.07E-09
MMRN1	3.17	7.64	-22.17	2.66E-18	3.09E-15
ADH1B	3.35	7.85	-22.69	5.75E-17	3.65E-14
PKHD1L1	4.86	9.47	-24.46	8.18E-12	7.95E-10
TFF3	6.91	11.88	-31.16	4.42E-21	2.34E-17
CRABP1	4.79	10.03	-37.65	5.32E-19	7.64E-16

cancer group. Studies have shown that this gene is upregulated in papillary thyroid cancer (17). These results are completely correlated with our results. According to our results, it has been shown by some researchers that *CHI3L1* (chitinase 3-like 1 (cartilage glycoprotein-39)), a gene whose expression level increased 89-fold, was associated with poor prognosis in papillary thyroid cancer (18).

In addition, *SFTPB*, *HMGA2*, *ARHGAP36*, *SYTL5*, *LRRK2*, *PRR15*, *DPP4*, *TENM1*, *SCEL*, *LRP4*, *ALOX5*, *SFTPB*, *PCSK2*, *SCEL*, *PLAU*, *SERPINA1*, *CLDN1*, *ECM1 and PLAU* genes are listed in Table 1 were determined as expression levels increasing genes. The genes listed in Table 2 are genes whose expression levels decreased in the papillary thyroid cancer group. These genes are *CCL21*, *COL9A3*, *FBLN1*, *LRP1B*, *PROM1*, *NEB*, *CDH16*, *TFCP2L1*, *LYVE1*, *PLA2R1*, *LYVE1*, *IPCEF1*, *GPM6A*, *DPP6*, *ADH1B*, *TPO*, *PKHD1L1*, *DGKI*, *DIO1*, *MMRN1*, *ADH1B*, *PKHD1L1*, *TFF3 and CRABP1*. It has been suggested that *MMRN1* gene is expressed differently in papillary thyroid cancer and this gene may be a diagnostic biomarker (19).

Table 3. The most important (High Enrichment Score) five clusters obtained with Functional Cluster Analysis which performed with 1209 differentially expressed genes

Annotation Cluster 1		***Enrichment Score: 30.07***	***Count***	***P-value***	***Benjamini***
	UP_SEQ_FEATURE	signal peptide	276	4.40E-41	1.10E-37
	UP_KEYWORDS	Disulfide bond	256	8.50E-32	3.60E-29
	UP_KEYWORDS	Signal	290	1.10E-31	2.30E-29
	UP_KEYWORDS	Glycoprotein	301	4.80E-29	6.80E-27
	UP_KEYWORDS	Secreted	174	7.70E-29	8.20E-27
	UP_SEQ_FEATURE	disulfide bond	224	1.60E-27	2.00E-24
	UP_SEQ_FEATURE	Glycosylation site: N-linked (GlcNAc...)	282	1.50E-25	1.30E-22
Annotation Cluster 2		***Enrichment Score: 15.68***	***Count***	***P-Value***	***Benjamini***
	GOTERM_CC_DIRECT	extracellular matrix	49	9.30E-17	2.30E-14
	GOTERM_CC_DIRECT	proteinaceous extracellular matrix	46	2.30E-16	3.10E-14
	UP_KEYWORDS	Extracellular matrix	43	4.20E-16	3.80E-14
Annotation Cluster 3		***Enrichment Score: 8.09***	***Count***	***P-Value***	***Benjamini***
	UP_SEQ_FEATURE	topological domain: Extracellular	171	3.80E-11	2.40E-08
	GOTERM_CC_DIRECT	integral component of plasma membrane	108	1.10E-10	7.90E-09
	UP_KEYWORDS	Membrane	360	1.20E-10	7.20E-09
	UP_SEQ_FEATURE	topological domain:Cytoplasmic	198	2.60E-10	1.30E-07
	UP_KEYWORDS	Transmembrane	274	6.50E-08	3.10E-06
	UP_KEYWORDS	Transmembrane helix	273	7.40E-08	3.20E-06
	UP_SEQ_FEATURE	transmembrane region	254	1.10E-07	3.80E-05
	GOTERM_CC_DIRECT	integral component of membrane	251	2.80E-04	1.10E-02
Annotation Cluster 4		***Enrichment Score: 4.46***	***Count***	***P-Value***	***Benjamini***
	UP_KEYWORDS	GPI-anchor	20	6.40E-07	1.80E-05
	GOTERM_CC_DIRECT	anchored component of membrane	18	2.70E-06	1.20E-04
	UP_SEQ_FEATURE	propeptide:Removed in mature form	21	6.70E-04	5.50E-02
	UP_KEYWORDS	Lipoprotein	50	1.30E-03	1.90E-02
Annotation Cluster 5		***Enrichment Score: 4.36***	***Count***	***P-Value***	***Benjamini***
	INTERPRO	EGF-like, conserved site	28	1.50E-08	1.90E-05
	INTERPRO	Epidermal growth factor-like domain	29	9.60E-08	6.20E-05
	UP_KEYWORDS	EGF-like domain	28	2.10E-07	7.60E-06
	INTERPRO	Insulin-like growth factor binding protein, N-terminal	20	1.80E-06	5.80E-04

	SMART	EGF	25	3.30E-06	9.00E-04
	INTERPRO	EGF-like calcium-binding	18	8.40E-06	1.30E-03
	UP_SEQ_FEATURE	domain:EGF-like 4	12	1.10E-05	3.00E-03
	INTERPRO	EGF-type aspartate/asparagine hydroxylation site	16	1.30E-05	1.80E-03
	INTERPRO	EGF-like calcium-binding, conserved site	15	3.90E-05	4.50E-03
	UP_SEQ_FEATURE	domain:EGF-like 1	16	4.40E-05	8.00E-03
	SMART	EGF_CA	18	4.80E-05	6.60E-03
	UP_SEQ_FEATURE	domain:EGF-like 2; calcium-binding	11	5.40E-05	9.10E-03
	INTERPRO	Complement Clr-like EGF domain	8	7.50E-05	6.80E-03
	UP_SEQ_FEATURE	domain:EGF-like 3	12	1.20E-04	1.50E-02
	GOTERM_MF_DIRECT	calcium ion binding	50	1.30E-04	1.50E-02
	UP_SEQ_FEATURE	domain:EGF-like 3; calcium-binding	7	2.70E-03	1.50E-01
	UP_SEQ_FEATURE	domain:EGF-like 5; calcium-binding	5	5.30E-02	8.60E-01
	UP_SEQ_FEATURE	domain:EGF-like 6; calcium-binding	4	7.20E-02	9.00E-01
	UP_SEQ_FEATURE	domain:EGF-like 4; calcium-binding	4	1.30E-01	9.70E-01

Table 4. Molecular pathways obtained from KEGG Pathway Analysis

Category	*Term*	*Count*	*P-value*	*Benjamini*
KEGG_PATHWAY	Complement and coagulation cascades	14	7,0E-6	1,7E-3
KEGG_PATHWAY	Staphylococcus aureus infection	12	1,7E-5	2,0E-3
KEGG_PATHWAY	Malaria	11	4,0E-5	3,2E-3
KEGG_PATHWAY	ECM-receptor interaction	12	1,3E-3	7,8E-2
KEGG_PATHWAY	PI3K-Akt signaling pathway	28	2,2E-3	1,0E-1
KEGG_PATHWAY	Transcriptional misregulation in cancer	17	2,5E-3	9,7E-2
KEGG_PATHWAY	Cell adhesion molecules (CAMs)	15	3,6E-3	1,2E-1
KEGG_PATHWAY	Pathways in cancer	30	3,7E-3	1,1E-1
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	21	4,7E-3	1,2E-1
KEGG_PATHWAY	p53 signaling pathway	9	8,4E-3	1,8E-1
KEGG_PATHWAY	Phagosome	14	1,4E-2	2,7E-1
KEGG_PATHWAY	Proteoglycans in cancer	17	1,4E-2	2,5E-1
KEGG_PATHWAY	Protein digestion and absorption	10	1,4E-2	2,3E-1
KEGG_PATHWAY	Pertussis	9	1,6E-2	2,4E-1
KEGG_PATHWAY	Tyrosine metabolism	6	1,7E-2	2,4E-1
KEGG_PATHWAY	Focal adhesion	17	1,8E-2	2,4E-1
KEGG_PATHWAY	TGF-beta signaling pathway	9	3,0E-2	3,5E-1
KEGG_PATHWAY	Natural killer cell mediated cytotoxicity	11	4,1E-2	4,3E-1
KEGG_PATHWAY	Influenza A	14	4,1E-2	4,1E-1
KEGG_PATHWAY	Tuberculosis	14	4,6E-2	4,3E-1

(continued)

Table 4. (continued)

KEGG_PATHWAY	African trypanosomiasis	5	5,4E-2	4,7E-1
KEGG_PATHWAY	Inflammatory bowel disease (IBD)	7	6,0E-2	4,9E-1
KEGG_PATHWAY	Rap1 signaling pathway	15	7,6E-2	5,6E-1
KEGG_PATHWAY	Proximal tubule bicarbonate reclamation	4	7,7E-2	5,5E-1
KEGG_PATHWAY	Small cell lung cancer	8	7,8E-2	5,4E-1
KEGG_PATHWAY	Chagas disease (American trypanosomiasis)	9	8,5E-2	5,6E-1
KEGG_PATHWAY	Legionellosis	6	8,6E-2	5,5E-1
KEGG_PATHWAY	Rheumatoid arthritis	8	9,0E-2	5,5E-1
KEGG_PATHWAY	NOD-like receptor signaling pathway	6	9,7E-2	5,7E-1

As a result of the pathway analysis, the genes of the differently expressed we detected were generally there in molecular pathways such as the Complement and coagulation cascades, ECM-receptor interaction, PI3K-Akt signaling pathway, Transcriptional misregulation in cancer, Cell adhesion molecules (CAMs), Pathways in cancer, Cytokine receptor interaction, p53 signaling pathway, Phagosome, Proteoglycans in cancer, Protein digestion and absorption, Tyrosine metabolism, Focal adhesion, TGF-beta signaling pathway and Natural killer cell mediated cytotoxicity have been determined. Among these molecular pathways, PI3K-Akt signaling pathway, Transcriptional misregulation in cancer, Cell adhesion molecules (CAMs), Pathways in cancer, Cytokine receptor interaction, p53 signaling pathways are very important. The genes in these pathways that are play a role in the organization of the tumor microenvironment, the connection of tumor cells with the extracellular matrix, and the emergence of the inflammatory response. How these molecular pathways are affected also determines the prognosis of the disease and the differentiation and metastatic features of the tumor. Spirina and colleagues detected some changes in the expression of transcription and growth factors and AKT/m-TOR signaling pathway components (20). We have also show that PI3K-Akt pathway plays important role in the emergence of papillary thyroid cancer.

Conclusion

We investigated which genes and molecular pathways are affected in papillary thyroid cancer compared to normal thyroid tissue. The results we found correlated with other studies. The candidate biomarker genes we have identified planned to be validated in different patient groups. The genes we identified in this way are intended to be transformed into a diagnostic gene panel. In this way, it is aimed to diagnose thyroid cancer by looking at the expression profile of this gene panel with a single blood sample without any invasive intervention. Our results have a potential to transform clinical use. We have detected some candidate biomarker genes and molecular pathways. These genes and pathways can be used for diagnostic tools or new drug target molecules in the future.

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