Key genes and pathways in thyroid cancer based on gene set enrichment analysis

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Abstract. The incidence of thyroid cancer and its associated morbidity has shown the most rapid increase among all cancers since 1982, but the mechanisms involved in thyroid cancer, particularly significant key genes induced in thyroid cancer, remain undefined. In many studies, gene probes have been used to search for key genes involved in causing and facilitating thyroid cancer. As a result, many possible virulence genes and pathways have been identified. However, these studies lack a case contrast for selecting the most possible virulence genes and pathways, as well as conclusive results with which to clarify the mechanisms of cancer development. In the present study, we used gene set enrichment and meta-analysis to select key genes and pathways. Based on gene set enrichment, we identified 5 downregulated and 4 upregulated mixed pathways in 6 tissue datasets. Based on the meta-analysis, there were 17 common pathways in the tissue datasets. One pathway, the p53 signaling pathway, which includes 13 genes, was identified by both the gene set enrichment analysis and meta-analysis. Genes are important elements that form key pathways. These pathways can induce the development of thyroid cancer later in life. The key pathways and genes identified in the present study can be used in the next stage of research, which will involve gene elimination and other methods of experimentation.

Introduction

Thyroid cancer is the most common endocrine neoplasm and accounts for ~1.7% of total cancer diagnoses; the incidence of thyroid cancer has increased the most rapidly of all cancers since the nuclear accident at Chernobyl. Currently, thyroid cancer ranks fifth among the most prevalent female cancers (1). Although numerous studies have been conducted to determine the genes that may influence the development of thyroid cancer, the results are not satisfactory. Many studies have applied real-time quantitative PCR and in situ hybridization to select possible key genes derived from cancer tissue sections; these genes include PDCD4 (2), PROM1, LOXL2, GFRA1 and DKK4 (3). Yet, thyroid cancer is not determined by a single gene; therefore, it is critical to elucidate the gene interactions involved in thyroid cancer and identify several key genes in the pathway that can effectively suppress cancer development.

The main issue with regards to genome-wide RNA expression analysis is how to obtain gene expression profiles. To solve this problem, genome-wide expression analysis with RNA microarray, which can identify predefined biological pathways associated with the phenotypic variations in many studies, have been performed (4). In this way, we can identify various biological pathways. Another issue is determining a method for identifying the entire range of pathways related to thyroid cancer.

To resolve this problem, Mootha et al (5) recommended gene set enrichment analysis (GSEA), which has been recognized as the most effective way for gene set analysis. It can be used to identify predefined gene sets that demonstrate differential expression levels in normal and abnormal tissue samples.

After performing GSEA, it is possible to identify the significant genes in the mixed pathways rather than in abnormal tissues only. By contrasting normal and abnormal tissues, we can easily and clearly determine the differentially expressed genes from both tissues. GSEA and meta-analysis were used to find the mixed pathways. Two gene analysis methods provided a systematic insight into the pathways that were altered during the mechanism of thyroid cancer. After identifying the genes that had significant differences in expression between normal and abnormal tissues and, thus may cause disease, we can use those genes for further research in clinical or animal experiments.

Materials and methods

Datasets. Using the GSEA method, thyroid cancer genes were searched for in GEO (http://www.ncbi.nlm.nih.gov/geo/) and
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ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Using the above sources, we designed Table I. The rows of Table I are as follows: row 1 is the GEO accession number; row 2 is the first author or contributor; row 3 is the year the gene was published; row 4 is the data platform; and row 5 states whether the tissue was paired or unpaired.

After searching, we determined 6 gene expression datasets which met the above standard. We collected the data and organized it into Table I. There were 2 paired and 4 unpaired datasets that contained 303 tissues in total.

Data processing of standardized microarray preprocessing. With the Bioconductor software (v2.10.1) (5), we preprocessed the data. The robust multichip average (RMA) algorithm in the Affy conductor package was used for each Affymetrix raw dataset to calculate the adjusted background, normalize and set the log2 probe intensities. We selected genes that met explicit KEGG pathways for further GSEA analysis and meta-analysis. The measure of variability was within the interquartile range (IQR) and a cut-off was set up to remove IQR values <0.5 for all the remaining genes. If one gene was targeted for multiple probe sets, we retained the probe set with the largest variability. Pathway analysis of each dataset was performed independently.

Data processing of GSEA. Using the category v2.10.1 package, which is used for GSEA, each pathway and the mean of the genes were calculated with the Student's t-test statistical score. A permutation test with n=1,000 was used to identify the significantly altered pathways. A P-value ≤0.05 was considered statistically significant.

Data processing of the meta-analysis. We calculated the Chi-square value of each gene remaining after the selection process based on the formula according to Brown (6). The formula is:

\[ X^2 = -2 \sum \log_2 p_i^f \]

The genes whose Chi-square values were <0.05 were eliminated and the remaining genes were used to obtain the pathways of the KEGG from DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/).

Results

Re-analyzing each dataset to produce differentially expressed pathways. There were 6 datasets containing 214 thyroid cancers and 89 normal tissues. We investigated each dataset using the same GSEA method. It should be mentioned that the GSE27155 data platform is different from other platforms, thus our software was unable to recognize the data generated from that platform. In addition, the tissues in the GSE6004 dataset were from thyroid cancer centers and thyroid cancer invasive areas, and, thus were not in fact normal tissues. The above 2 datasets were eventually discarded from the GSEA analysis.

Common significant pathways obtained from 4 thyroid cancer tissue datasets by GSEA. After performing the GSEA calculations, we obtained 4 upregulated and 5 downregulated pathways that existed in 4 thyroid cancers. From the results of the GSEA, we obtained information concerning the regulated pathways in KEGG (http://www.genome.jp/kegg/) (7).

Conclusion of the meta-analysis. With the paired t-test we obtained the P-value for each gene. We downloaded the gene probe platform to translate the gene probe number to the gene name (http://www.ncbi.nlm.nih.gov/geo/) and inserted the gene names into the SAS 9.13 software for total analysis. Finally, we selected 525 significant genes whose P-value was <0.05 and we found 266 genes in DAVID (http://david.abcc.ncifcrf.gov/). The identified genes exist in 18 pathways, and the main pathways are involved in ECM-receptor interaction, focal adhesion, and complement and coagulation cascades. The details are documented in Table II.

Common pathways between GSEA and meta-analysis. There was one intersecting pathway between the common significant pathways of GSEA and meta-analysis in cancer tissues. This was identified as the p53 signaling pathway a member of the regulatory pathways. The p53 signaling pathway contains 13 genes: Bid, PMAIP1, CCND1, CDKN1A, TNFRSF10B, CDKN2A, CCND3, Bax, RRM2, DDB2, Ras, THBS1 and insulin-like proteins.

<table>
<thead>
<tr>
<th>GEO accession no.</th>
<th>Contributor</th>
<th>Year</th>
<th>Chip</th>
<th>Experimental design (tissues)</th>
<th>Probes</th>
<th>Source</th>
<th>Disease</th>
<th>Normal</th>
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<td>GSE3678</td>
<td>Ismael Reyes</td>
<td>2005</td>
<td>HG-U133_Plus_2</td>
<td>Paired</td>
<td>54675</td>
<td>Homo sapiens</td>
<td>7</td>
<td>7</td>
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<tr>
<td>GSE3467</td>
<td>Sandya Liyanarachchi</td>
<td>2005</td>
<td>HG-U133_Plus_2</td>
<td>Paired</td>
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<td>Homo sapiens</td>
<td>9</td>
<td>9</td>
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<td>GSE33630</td>
<td>Gil Tomas</td>
<td>2011</td>
<td>HG-U133_Plus_2</td>
<td>Unpaired</td>
<td>54675</td>
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<td>45</td>
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<tr>
<td>GSE29265</td>
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<td>HG-U133_Plus_2</td>
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<td>29</td>
<td>20</td>
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<td>Rork Kuick</td>
<td>2011</td>
<td>HG-U133A</td>
<td>Unpaired</td>
<td>22283</td>
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<td>GSE6004</td>
<td>Sandya Liyanarachchi</td>
<td>2006</td>
<td>HG-U133_Plus_2</td>
<td>Unpaired</td>
<td>54675</td>
<td>Homo sapiens</td>
<td>14</td>
<td>4</td>
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</table>

Paired, compare thyroid cancer (TPC) to normal controls from the same patients with TPC; Unpaired, compare TPC from men to normal controls from men without TPC.
<table>
<thead>
<tr>
<th>Pathway entry</th>
<th>Pathway name</th>
<th>P-value</th>
<th>No. of genes included</th>
<th>Gene names (P-value)</th>
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<td>map04512</td>
<td>ECM-receptor interaction</td>
<td>1.23 E-08</td>
<td>27</td>
<td>TNC (0.00000), COL3A1 (0.01894), ITGB4 (0.00956), ITGB5 (0.00001), SDC4 (0.00000), SDC2 (0.00000), SDC3 (0.00013), LAMB3 (0.00000), CD44 (0.00000), COMP (0.00000), COL6A3 (0.04683), AGRN (0.00000), THBS1 (0.00035), THBS2 (0.01667), FN1 (0.00000), SPPI (0.00113), COL4A2 (0.01460), COL4A1 (0.00005), ITGA3 (0.00000), COL5A1 (0.00122), LAMA2 (0.00006), SDC1 (0.00009), ITGA7 (0.01369), COL1A2 (0.00004), LAMC2 (0.00006), COL1A1 (0.00001), LAMC1 (0.00000)</td>
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<tr>
<td>map04510</td>
<td>Focal adhesion</td>
<td>1.29 E-04</td>
<td>36</td>
<td>CAV2 (0.04769), CAV1 (0.00540), PGF (0.00002), TNC (0.00000), COL3A1 (0.01894), ITGB4 (0.00956), ITGB5 (0.00001), LAMB3 (0.00000), RAC2 (0.02943), PAK3 (0.00006), COMP (0.00000), BCL2 (0.00000), COL6A3 (0.04683), THBS1 (0.00035), THBS2 (0.01667), FN1 (0.00000), SPPI (0.00113), COL4A2 (0.01460), COL4A1 (0.00005), MET (0.00000), ACTN1 (0.00000), ITGA3 (0.00000), COL5A1 (0.00122), LAMA2 (0.00006), MAPK1 (0.00107), CCND1 (0.00000), CCND3 (0.00159), FYN (0.00081), JUN (0.00000), VEGFA (0.00850), ITGA7 (0.01369), COL1A2 (0.00004), LAMC2 (0.00006), LAMC1 (0.00000), COL1A1 (0.00001)</td>
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<td>map04610</td>
<td>Complement and coagulation cascades</td>
<td>3.85 E-04</td>
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<td>C3AR1 (0.02394), C7 (0.00962), A2M (0.00352), CFB (0.00126), C1S (0.01114), PLAUR (0.00001), C1QA (0.01674), C1QB (0.01345), CD55 (0.00003), THBD (0.01514), F5 (0.02164), TFPI (0.00000), SERPINA1 (0.00000), CFI (0.00000), CFD (0.00000), PROS1 (0.00000), PLAU (0.00000)</td>
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<td>11</td>
<td>C1QA (0.01674), EGR1 (0.01634), NCAM1 (0.00000), C1QB (0.01345), MAPK1 (0.00107), C7 (0.00962), FYN (0.00081), Bax (0.00000), LAMC1 (0.00000), PRKACB (0.03796), PRKX (0.00000)</td>
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<td>Pathways in cancer</td>
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<td>44</td>
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<td>Bid (0.00000), ICA1M (0.00000), CAV1 (0.00040), ITGB2 (0.02312), CD40 (0.00511), LAMA2 (0.00006), CCND1 (0.00000), CD86 (0.01044), CD55 (0.00003), RAC2 (0.2943), FYN (0.00081), MYH11 (0.00012), SGCD (0.00001), MYH10 (0.00400)</td>
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<td>map00910</td>
<td>Nitrogen metabolism</td>
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<td>map04115</td>
<td>p53 signaling pathway</td>
<td>0.01952</td>
<td>13</td>
<td>Bid (0.00000), PMAIP1 (0.00004), CCND1 (0.00000), CDKN1A (0.00000), TNFRSF10B (0.00000), CDKN2A (0.00019), CCND3 (0.00159), Bax (0.00000), RRM2 (0.00721), DDB2 (0.00000), Fas (0.00161), THBS1 (0.00035), IGFBP3 (0.01155)</td>
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<tr>
<td>map04710</td>
<td>Circadian rhythm</td>
<td>0.02567</td>
<td>5</td>
<td>CRY2 (0.00000), CSNK1E (0.04482), PER2 (0.01305), BHLHE40 (0.00000), ARNTL (0.0074)</td>
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<td>map04350</td>
<td>TGF-β signaling pathway</td>
<td>0.02598</td>
<td>15</td>
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<td>Fatty acid metabolism</td>
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</tr>
</tbody>
</table>
growth factor binding protein 3 (IGFBP3). The relationship among these genes is described in Fig. 1 from analysis with DAVID (http://david.abcc.ncifcrf.gov/conversion.jsp).

Discussion

Although the thyroid is a small and inconspicuous organ (8), its morbidity is ranked first of all the endocrine organs. The mechanism of thyroid cancer is not clear and a single theory cannot thoroughly explain it. As we know, the causes of cancer are multiple and complex. At the gene level, a large number of genes are thought to be associated with cancer, but which genes are most important is difficult to ascertain. Traditional methods have involved the study of one gene in one sample or one experiment alone. This causes obvious bias and can easily result in researchers ignoring the key gene and/or pathway. In addition, analyzing gene chip data with a single set t-test has certain limitations; for example, restrictions on the sample size may result in a suspect variation estimate that can produce high false-positive results (9). Finally, gene chip data may ignore differences in expression levels among different samples, which may result in various significant genes not being identified.

A gene that is truly significant should impact the key pathway that regulates the growth of the cancer or be involved in a mixed-pathway that leads to cancer formation. To identify these genes, a group of chip data that contains samples of 2 different biological states (such as normal and cancerous) must be analyzed based on the GSEA method (10). Analysis of the genes that have a common expression trend will reveal the genes and pathways that are associated with disease.

We combined the GSEA and meta-analysis methods to analyze 6 datasets in order to find the key pathways or genes in thyroid cancer. The GSEA analysis indicated differentially expressed genes between samples (the number of samples >2) and we clustered the samples to obtain sample classification of obvious gene expression differences. Using the software R language and statistical analysis we obtained 9 groups of data with expression level alterations in common pathways (4 upregulated and 5 downregulated pathways). Meta-analysis with a t-test was used for single data sets to determine differentially expressed genes; the pathways associated with the differentially expressed genes were determined using the DAVID website. Finally, we analyzed the pathways that overlapped to determine the genes with significant differences. We obtained one pathway with significant differences in gene expression between normal and thyroid cancerous tissues and that was the p53 signaling pathway.

p53 signaling pathway. The p53 gene is a tumor-suppressor gene. Its expression product is a gene regulatory protein (p53 protein). When the product of p53 gene expression is increased dramatically, it can inhibit the cell cycle. Once the p53 gene is mutated or the p53 protein is inactivated, control over cell division is lost and human cancers arise. Approximately half of human cancer is due to p53 gene mutations or protein inactivation (11). The protein encoded by the p53 gene is a transcription factor that controls initiation of the cell cycle (12). Many relevant signals that indicate the health of a cell are sent to the p53 gene for determining whether to initiate cell division. If the cell is damaged beyond repair, the p53 protein will participate in the initiation of cell death by apoptosis. Cells

Table II. Continued.

<table>
<thead>
<tr>
<th>Pathway entry</th>
<th>Pathway name</th>
<th>P-value</th>
<th>No. of genes included</th>
<th>Gene names (P-value)</th>
</tr>
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<td>map05222</td>
<td>Small cell lung cancer</td>
<td>0.04134</td>
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<td>COL4A2 (0.01460), COL4A1 (0.00005), RXRG (0.00000), BCL2L1 (0.00000), LAMA2 (0.00006), CCND1 (0.00000), LAMB3 (0.00000), BCL2 (0.00000), LAMC2 (0.00006), PIAS1 (0.00046), LAMC1 (0.00000), TRAF5 (0.01267), FN1 (0.00000)</td>
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<td>map05014</td>
<td>ALS</td>
<td>0.04991</td>
<td>10</td>
<td>Bid (0.00000), GPX1 (0.00000), TNFRSF1B (0.00290), DERL1 (0.00021), MAPK13 (0.00000), GRIN2C (0.00000), BCL2 (0.00000), Bax (0.00000), CCS (0.01625), BCL2L1 (0.00000)</td>
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<tr>
<td>map04514</td>
<td>CAMs</td>
<td>0.05605</td>
<td>19</td>
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<td>6</td>
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<td>8</td>
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<td>map00980</td>
<td>Metabolism of xenobiotics by cytochrome P450</td>
<td>0.09494</td>
<td>10</td>
<td>GSTM1 (0.00371), AKR1C3 (0.00087), GSTM2 (0.00100), AKR1C2 (0.00000), CYPIB1 (0.00000), ALDH1A3 (0.00000), ADH5 (0.00000), AKR1C1 (0.00000), ALDH3B1 (0.00000), GSTP1 (0.02676)</td>
</tr>
</tbody>
</table>

ALS, amyotrophic lateral sclerosis; CAMs, cell adhesion molecules.
without the p53 gene will continue to divide even in adverse conditions. Like all other tumor suppressors, the p53 gene plays a monitoring role in cell division. The p53 gene judges the degree of DNA variation, and if the variation is small, the gene will promote cell repair, whereas if the DNA variation is striking, p53 will induce apoptosis (13). Many animal experiments have confirmed this function of p53 (14).

The p53 signaling pathway was the only common intersecting pathway between the significant pathways found by GSEA and meta-analysis in cancer tissues. p53 activation is induced by a number of stress signals, including DNA damage, oxidative stress and activation of oncogenes. The p53 protein is employed as a transcriptional activator of p53-regulated genes that causes one of three outcomes: cell cycle arrest, cellular senescence or apoptosis. Other p53-regulated gene functions include communication with adjacent cells, repair of damaged DNA and establishment of positive or negative feedback loops that enhance or attenuate the functions of the p53 protein and integrate stress responses with other signal transduction pathways (15). The present study confirmed that thyroid cancer is associated with the p53 signaling pathway which is also closely related to ataxia with ocular apraxia (16), Li-Fraumeni syndrome (17), lymphangioleiomyomatosis (18), tuberous sclerosis complex (19), Seckel syndrome (20), choroid plexus papilloma (21), plasminogen activator inhibitor type-1 (PAI-1) deficiency (22) and VACTERL/VATER association (23). A previous mouse experiment indicated that inactivation of p53 is related to thyroid cancer (24). One study indicated that p53 expression was not observed in thyroid cancer specimens (25). However, additional studies indicate that abnormal p53 activation is closely related to the development of thyroid cancer (26). The p53 signaling pathway plays an important role in cancer cell apoptosis and DNA repair (27).

There are 13 genes in the p53 signaling pathway: Bid, PMAIP1, CCND1, CDKN1A, TNFRSF10B, CDKN2A, CCND3, Bax, RRM2, DDB2, Fas, THBS1 and IGFBP3. The mechanisms of some of the above genes are clear. For example, the Bid gene encodes a death agonist that heterodimerizes with either the Bax agonist or BCL2 antagonist, and the protein encoded by Bid is a member of the BCL-2 family of cell death regulators. The protein encoded by the CCND1 gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle and function as regulators of CDK kinases.

Studies indicate that tumor-specific pyruvate kinase M2 (PKM2) is required for the dissociation of HDAC3 from the CCND1 and MYC promoter regions (28). The CDKN1A gene (also called p21), which has been confirmed as a target gene in prostate cancer (29), encodes a potent cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2, whose expression reduction leads to the activation of CDK2- (30) and CDK4-associated kinases complexes, and thus, functions as a regulator of cell cycle progression during the G1 phase. The expression of CDKN1A is tightly controlled by the tumor-suppressor protein p53 and CDKN1A mediates p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. Studies indicate that G2-M cell cycle arrest is associated with upregulation of p21CIP1/WAF1 expression (31).

The protein encoded by the TNFRSF10B (also called DR5) gene is a member of the TNF-receptor superfamily and contains an intracellular death domain. This receptor can be activated by tumor necrosis factor-related apoptosis inducing ligand (TNFsf10/TRAIL/APO-2L) and transduces an apoptotic signal. Drug studies indicate that the upregulation of DR5 leads to tumor death (32).
The CDKN2A gene (also called p14 or ARF) generates several transcript variants that differ in their first exons. At least three alternatively spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. Some studies suggest that polymorphisms of MDM2 and p14ARF contribute to the interindividual differences in susceptibility to differentiated thyroid cancer (33).

The protein encoded by the CCND3 gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns that contribute to the temporal coordination of each mitotic event. Co-immunoprecipitation experiments indicate that the level of p27-bound cyclin D3 was much higher in oxyphilic neoplasias than in normal thyroids and other thyroid tumors (34).

The protein encoded by the Bax gene belongs to the BCL2 protein family. BCL2 family members form heterodimers or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. The Bax protein forms a heterodimer with BCL2 and functions as an apoptotic activator. Studies indicate that apoptosis is associated with a decrease in the level of Bcl-2 expression and an increase in the level of Bax expression (35) and that BCL2 may be associated with the multifocality and bilaterality of papillary thyroid cancer (36).

The RRM2 gene encodes one of two non-identical subunits for ribonucleotide reductase. This reductase catalyzes the formation of deoxyribonucleotides from ribonucleotides.

The DDB2 gene encodes a protein that is necessary for the repair of ultraviolet light-damaged DNA. This protein is the smaller subunit of a heterodimeric protein complex that participates in nucleotide excision repair, and this complex mediates the ubiquitylation of histones H3 and H4, which facilitate the cellular response to DNA damage.

The protein encoded by the Fas gene is a member of the TNF-receptor superfamily. This receptor contains a death domain, has been shown to play a central role in the physiological regulation of programmed cell death, and has been implicated in the pathogenesis of various malignancies and diseases of the immune system (37). The present study showed that FasL expression (positivity, staining and intensity) was high and increased in papillary microcarcinoma of the thyroid tissue.

The protein encoded by the THBS1 gene is a subunit of a disulfide-linked homotrimeric protein. This protein is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Some studies indicate that the THBS1 gene is related to thyroid cancer (38).

The IGFBP3 gene is a member of the IGFBP family and encodes a protein with an IGFBP domain and a thyroglobulin type-I domain. One study indicates that serum IGFBP-3 and TSH levels did not modulate these associations in thyroid cancer (39).

The mechanism of the p53 signaling pathway is not completely clear to date, and the interaction of genes in this pathway require further study. Of the 13 genes in the p53 signaling pathway, 8 have been confirmed to be related to thyroid cancer by one or more experiments. These 8 genes include CCND1, CCNK1A, TNFRSF10B, CDKN2A, CCND3, Bax, Fas and THBS1. The other genes, BID, PMAIP1, RRM2 and DDB2, have not been confirmed to be related to thyroid cancer and require further investigation. To date, studies of IGFBP-3 have indicated that it is not involved in the development of thyroid cancer, but additional experiments with larger samples are needed to confirm these results.

In conclusion, the pathogenesis of thyroid papillary cancer is quite complicated. We identified a significant pathway and 13 key genes based on gene set enrichment and meta-analysis. The clinical trials that we have mentioned in our discussion confirm that the p53 signaling pathway and the relevant genes play an important role in the development of thyroid cancer. Various mechanisms of this pathway and the genes are clear, but others require clarification. Further study concerning the specific role and interactions of the genes included in the related pathways are needed to improve the understanding of thyroid cancer, particularly the interaction of genes in the p53 pathway.

References


