Identification of Biomarkers Related To The Diagnosis And Prognosis of Thyroid Cancer Through Bioinformatics Analysis

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Abstract

Background: Thyroid cancer is the most common malignant tumor of the head and neck. In recent years, the incidence of thyroid cancer (THCA) worldwide has rapidly increased and shows a trend in the younger generation. This study attempted to screen key genes and potential prognostic biomarkers for thyroid cancer using bioinformatics analysis.

Methods: This study attempted to screen key genes and potential prognostic biomarkers for thyroid cancer using bioinformatics analysis. 101 cases of thyroid cancer and 78 cases of normal thyroid tissue were collected from three Gene Expression Omnibus (GEO) databases, then we identified the differentially expressed genes (DEGs) and conducted downstream analyses. Moreover, we screened hub genes by constructing a protein-protein interaction (PPI) network. Finally, we assessed the expression level of hub genes in thyroid cancer tissue and its normal tissue using GEPIA and qRT-PCR respectively.

Results: 159 upregulated and 251 downregulated genes were determined after gene integration of these three GEO data sets. Through PPI analysis, we consider the top 20 DEGs with high connectivity as the hub genes of THCA. After that, this study verified 20 central genes through the GEPIA database and found that only four hub genes (TOP2A, FN1, TIMP1, and MMP9) had significantly higher expression levels in thyroid cancer tissues than in normal thyroid tissues. We further analyzed the correlation between these four hub genes and the prognosis of patients with thyroid cancer, which suggests that FN1, MMP9, TIMP1 help assess the prognosis of patients with thyroid cancer. We performed GSEA analysis on these 4 hub genes simultaneously, found that the high expression of these 4 hub genes enriched the "cell cycle." Subsequently, we collected thyroid cancer tissue specimens, verified these four hub gene expression levels by RT-PCR, and found that only FN1 and TIMP1 genes in thyroid cancer tissues had significantly higher mRNA levels than normal tissues.

Conclusions: Our research has identified 20 hub genes that may be related to the occurrence and development of thyroid cancer through multiple gene expression profile data sets and a series of comprehensive bioinformatics analyses. Further database and tissue validation analysis revealed that only 2 hub genes may be considered as potential prognostic biomarkers, including FN1 and TIMP1. In addition, these two hub genes are involved in the cell cycle, suggesting that they may play a role in the occurrence and development of thyroid cancer.

1 Introduction

Thyroid cancer is a malignant tumor originating from thyroid follicular epithelium or para-follicular epithelial cells, which is also the most common malignant tumor of the head and neck. In recent years, the incidence of thyroid cancer worldwide has rapidly increased and shows a trend in the younger generation. The incidence of thyroid cancer among women in urban areas in China ranks fourth among all malignant tumors in women, and it continues to increase at an annual rate of 20%. Regarding the increase in the incidence of thyroid cancer year by year, some scholars have pointed out that the possible

reasons are the increase in the number of patients willing to go to the hospital for cancer screening or treatment, the increase in the level of cancer detection, and the increase in the population exposed to radiation^[1-2]. The prognosis of early thyroid cancer is generally good, but the prognosis of patients with advanced or recurrent metastatic thyroid cancer is poor^[3]. Therefore, there is an urgent need to find reliable diagnostic and prognostic biomarkers to improve the therapeutic effect. At present, bioinformatics research based on high-throughput sequencing technology can comprehensively analyze changes in gene expression and has become an essential method for the study of cancer pathogenesis. This study intends to screen the transcriptome gene microarray expression profiles of thyroid cancer and normal tissues by searching the microarray data set in the GEO database and perform the functional enrichment analysis of differential genes, screen out the core genes with clinical significance through PPI analysis, and provide the basis for research on the mechanism of thyroid cancer.

This study involved 101 cases of thyroid cancer and 78 cases of normal thyroid tissue from 3 GEO data sets (GSE33630, GSE65144, and GSE29265). Through the analysis of 3 differential gene data sets, a total of 410 identical differentially expressed genes were screened out, including 159 up-regulated genes and 251 down-regulated genes in tumor samples. Through PPI analysis, we consider the top 20 DEGs with high connectivity as the hub genes of THCA. After that, this study verified 20 central genes through the GEPIA database and found that only four hub genes (TOP2A, FN1, TIMP1, and MMP9) had significantly higher expression levels in thyroid cancer tissues than in normal thyroid tissues. We further analyzed the correlation between these four hub genes and the prognosis of patients with thyroid cancer, which suggests that FN1, MMP9, TIMP1 help assess the prognosis of patients with thyroid cancer. We performed GSEA analysis on these 4 hub genes simultaneously, found that the high expression of these 4 hub genes enriched the "cell cycle." Subsequently, we collected thyroid cancer tissue specimens, verified these four hub gene expression levels by RT-PCR, and found that only FN1 (fibronectin 1) and TIMP1 (TIMP metalloproteinase inhibitor 1) genes in thyroid cancer tissues had significantly higher mRNA levels than normal tissues. Therefore, we speculate that FN1 and TIMP1 may be potential therapeutic targets and prognostic markers of thyroid cancer.

2 Materials And Methods

2.1 Microarray data information

The GEO (<http://www.ncbi.nlm.nih.gov/geo/>) database is a free public database of microarrays and is used for gene expression data sets and platform records. The gene expression profiles of GSE33630, GSE65144, and GSE29265 were chosen from the GEO database. GSE33630 was based on the GPL570 platform (HG-U133_ Plus_2; Affymetrix Human Genome U133 Plus 2.0 Array), containing 11 anaplastic thyroid carcinomas (ATC), 49 papillary thyroid carcinomas (PTC), and 45 normal thyroids (N). GSE65144 was based on the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array, consisting of 12 ATC tissues and 13 normal thyroid tissues. The platform of GSE29265 was the GPL570 ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array) which included nine anaplastic thyroid

carcinomas (ATCs), together with twenty papillary thyroid carcinomas (PTCs) paired with their respective adjacent tissues (N). The data set information is shown in Table 1. The downloaded data was processed using the R package, then calibrated, standardized, and log2 transformation was performed on all data of gene expression.

Table 1
Details of the four Gene Expression Omnibus thyroid carcinoma data sets.

GEO	Tissue	Platform	Normal	Tumor
GSE33630	thyroid	GPL570	45	60
GSE65144	thyroid	GPL570	13	12
GSE29265	thyroid	GPL570	20	29

2.2 Screening for DEGs

After downloading the 3 sets of original data sets, use R software for data processing, merging, standardization, and calculation of expression values. The data uses Fold-change and T-test for differential gene screening. The DEGs with $|\log FC| \geq 1$ and $\text{adjust } P < 0.05$ were considered as the cut-off criteria.

2.3 GO term and KEGG pathway enrichment analysis of DEGs

Gene Ontology (GO) is a biological model framework composed of three major parts: molecular function (MF), cell composition (CC), and biological process (BP). Jingdong Genome Encyclopedia (KEGG) is an information resource for understanding biological systems and genome functions from the molecular level. Use KOBAS3.0 (<http://kobas.cbi.pku.edu.cn/kobas3/?t=1>) for KEGG pathway analysis of differentially expressed genes, and use R package clusterProfiler for GO analysis.

2.4 PPI network and module analysis

The STRING database is an analytical database used to predict gene interaction networks in EMBL, which aggregates the extraction results from the Protein Interaction Network (PPI) database. Cytoscape's MCODE plug-in was used to evaluate the network model, and the genes in the top 3 templates were selected for pathway enrichment analysis. The results obtained through PPI were imported into the cytaHuba package of Cytoscape 3.8.2 software for analysis, and then the hub genes were screened according to the degree.

2.5 Validation of the hub genes in GEPIA

GEPIA (<http://gepia.cancer-pku.cn/index.html>) is a newly developed interactive web server that can analyze RNA from TCGA (The Cancer Genome Atlas) and GTEx (Genotype-Tissue Expression) databases Sequencing expression data. GEPIA provides differential expression analysis of tumor and normal

tissues, analysis of the relationship between gene expression and pathological staging, analysis of patient survival, analysis of the correlation between genes, etc. In this study, GEPIA was used for hub gene expression analysis.

2.6 Analyze the correlation between hub gene and pathological stage by GEPIA as well as survival

GEPIA was used to analyze the correlation between hub gene and pathological staging, as well as survival.

2.7 GSEA analysis of hub gene

GSEA (GSEA v2.0, available online: <http://www.broad.mit.edu/gsea/>) is used to analyze the biological pathways involved in the hub gene in thyroid cancer, and calculate the standardized enrichment score (NES), NOM p -val <0.05 .

2.8 Verify the expression of hub gene in thyroid cancer tissue by qPCR

The expression level of the hub gene was verified by quantitative real-time PCR (qRT-PCR) in thyroid cancer tissues ($n = 10$) and surrounding normal tissues ($n = 10$).

2.9 Statistical analysis

The Kaplan-Meier (KM) curve was conducted to assess the association between the expression level of hub genes and the survival time of patients with thyroid cancer. The data were summarized by Excel and then statistically analyzed with GraphPad Prism5 (Graphpad Software, Inc, La Jolla, CA) software, and $P<0.05$ was considered as statistically significant.

3 Results

3.1 Microarray data information and identification of DEGs in ATC and PTC

Filter DEG through R software ($|\log FC| \geq 1$ and adjust $P<0.05$). In this study, 101 thyroid cancers and 78 normal thyroid tissues from 3 GEO data sets were involved. 1145 differential genes were screened from GSE33630; 2252 differential genes were screened by GSE65144; 792 differential genes were screened by GSE29265. Subsequently, 3 data sets of differential genes were analyzed, and a total of 410 identical differentially expressed genes were obtained, of which 159 genes were up-regulated in tumor samples and 251 genes were down-regulated. The DEGs of each gene expression profile data were visualized using a volcano plot (Figure 1). Subsequently, overlapping genes were screened in the thyroid cancer group compared with the control group using Venn software online (Figure 2).

3.2 GO functional enrichment analysis

To explore the biological functions of integrated DEG in ATC and PTC, we performed GO enrichment analysis on 410 DEGs (159 up-regulated genes and 251 down-regulated genes). The GO analysis results are divided into two parts. To explore the biological functions of integrated DEG in ATC and PTC, we performed GO enrichment analysis on 410 DEGs (159 up-regulated genes and 251 down-regulated genes). The GO analysis results are divided into two parts (up-regulated DEGs and down-regulated DEGs). In terms of biological processes, up-regulated DEGs are mainly abundant in the negative regulation of endoderm cell differentiation, endoderm formation, collagen fibril organization, endoderm development, and cell-matrix adhesion (Figure 3). In terms of cellular components, the upper DEGs mainly involve fibril collagen trimers, ribbon collagen fibrils, collagen trimer complexes, concentrated chromosomal microkinetochores, and extracellular matrix. In terms of molecular functions, the upward DEG mainly focuses on the combination of hyaluronic acid, extracellular matrix, proteoglycan, and the combination of extracellular matrix structural components that impart tensile strength and collagen. In the other part, in terms of biological processes, down-regulated DEGs are mainly abundant in the biological processes of thyroid hormone production, thyroid hormone metabolism, phenol-containing compound metabolism, hormone biosynthesis, and cold-induced thermogenesis (Figure 4). In terms of molecular functions, down-DEGs mainly focus on peroxidase activity, SH3/SH2 adaptor activity, RNA polymerase II activation of transcription factor binding, oxidoreductase activity with peroxide as the receptor, and signal adaptor active. Using the ggplot2.R package, the bubble chart shows significant GO enrichment items, $P < 0.05$.

3.3 KEGG pathway analysis

KEGG pathway analysis of the 159 up-DEGs was conducted in ATC and PTC using the KOBAS 3.0 online analysis tool, of which the top five (Figure 5) were “protein digestion and absorption,” “p53 signaling pathway,” “cytokine- cytokine receptor interaction,” “ECM-receptor interaction,” and “proteoglycans in cancer,” respectively. The top five KEGG pathways in the 251 down-regulated DEGs (Figure 6) are “thyroid hormone synthesis,” “metabolic pathways,” “TGF-beta signaling pathways,” “adipocytokine signaling pathways,” and “regulation of lipolysis in adipocytes.” The analysis indicated that the 410 dysregulated genes were mainly related to human cancer.

3.4 PPI network construction, module analysis, and hub gene selection

The PPI network was constructed for further investigation of the interaction among the integrated DEGs via Cytoscape software, based on the STRING database. The PPI network consisted of 376 nodes and 361 edges, among which including 159 upregulated and 251 downregulated genes (Figure 7). We regarded the top 20 DEGs with a high degree of connectivity as the hub genes of THCA (Table 2). The String database was used to construct an interactive network of all hit genes. Interestingly, all the hub genes formed a network of interactions (Figure 8).

Table 2 **Details of the 20 hub genes**

Gene	Degree	Type	MCODE Cluster
CCNB2	20	up	cluster1
TOP2A	19	up	cluster1
KIF20A	19	up	cluster1
NDC80	18	up	cluster1
DLGAP5	18	up	cluster1
NUSAP1	17	up	cluster1
BUB1B	17	up	cluster1
ASPM	17	up	cluster1
CENPF	16	up	cluster1
RRM2	16	up	cluster1
KIF4A	16	up	cluster1
CEP55	15	up	cluster1
TPX2	15	up	cluster1
PBK	15	up	cluster1
FN1	14	up	cluster 2
KIF15	14	up	cluster1
MELK	14	up	cluster1
SDC2	13	down	cluster 2
TIMP1	12	up	cluster 2
MMP9	12	up	cluster1

3.5 Validation of the hub genes in GEPIA

This study verified the expression level of hub genes in thyroid cancer tissue through GEPIA. The results showed that compared with normal thyroid tissues, TOP2A, FN1, TIMP1 and MMP9 were highly expressed in thyroid cancer tissues (Figure 9, $P < 0.05$), while the differences in other genes were not significant. At the same time, the correlation between the four central genes was analyzed. The results showed that except for the uncorrelated relationship between TOP2A and FN1, the expression of other hub genes was significantly positively correlated (Figure 10, $P < 0.05$).

3.6 Analyze the correlation between hub gene and pathological stage by GEPIA as well as survival

This study explored the relationship between the hub gene and Disease Free Survival (DFS) in patients with thyroid cancer. The results showed that, except for TIMP1, the DFS of the high expression group of TOP2A (DNA topoisomerase II alpha), FN1 and MMP9 were worse in the lower expression group (Figure 11, $P < 0.05$). In addition, the study also found that in addition to TOP2A, the expression of the other three hub genes (FN1, MMP9, and TIMP1) were all related to the clinical stage of thyroid cancer (Figure 12, $P < 0.05$).

3.7 GSEA analysis of hub genes

To further evaluate the hub genes, we used GSEA to analyze the biological pathways involved in the hub gene. We divided the patients between the data set into high expression groups and low expression groups according to the median of TOP2A, FN1, TIMP1, and MMP9. The results showed that the high expression of these three hub genes (FN1, TOP2A, MMP9) enriched the "cell cycle" (Figure 13, NOM p -val < 0.05).

3.8 qRT-PCR verifies the expression level of hub gene in thyroid cancer tissue

We performed RT-PCR verification on the collected thyroid cancer tissues and surrounding normal tissues. The results found that the mRNA level of FN1 and TIMP1 genes in thyroid cancer tissues was significantly higher than that of normal tissues ($P < 0.05$), while TOP2A and MMP9 did not find significant differences (Figure 14).

4 Discussion

Thyroid cancer is prevalent. The incidence of thyroid cancer is on the rise worldwide, and the number of cases has increased sharply in the past decade^[4]. Most oncologists support the use of next-generation sequencing to find potentially actionable mutations^[5-6]. Although these mutations are small, treatments that target these targets are generally easier to tolerate and may produce longer-lasting responses. BRAF V600E is the most common molecular change in PTC. It is highly specific for PTC and exists in 36% to 80% of papillary carcinomas^[7]. Researchers found that BRAF-mutated thyroid cancer has a higher lymph node metastasis and recurrence rate after resection, and radioactive iodine therapy is ineffective^[8]. Our results provide more treatment options for patients and perhaps improves the quality of life of patients. Bioinformatics combined with high-throughput sequencing can systematically analyze disease-related genes and is an important method for studying the pathogenesis of diseases.

In this study, 101 thyroid cancers and 78 normal thyroid tissues from 3 GEO data sets were involved. 1145 differential genes were screened from GSE33630; 2252 differential genes were screened by GSE65144; 792 differential genes were screened by GSE29265. Subsequently, 3 data sets of differential genes were analyzed, and a total of 410 identical differentially expressed genes were obtained, of which 159 genes were up-regulated in tumor samples and 251 genes were down-regulated. To reduce the error, the 3 GEO data sets we selected are all from the GPL570 platform. Through PPI analysis, we consider the top 20 DEGs with high connectivity as the hub genes of THCA. After that, this study verified 20 hub genes

through the GEPIA database and found that only 4 hub genes (TOP2A, FN1, TIMP1, and MMP9) had significantly higher expression levels in thyroid cancer tissues than in normal thyroid tissues. We further analyzed the correlation between these 4 hub genes and the prognosis of patients with thyroid cancer, and the results showed that in addition to TIMP1, the DFS of the high expression group of TOP2A, FN1, and MMP9 were all lacking in the low expression group. In addition, the study also found that in addition to TOP2A, the expression of the other three hub genes (FN1, MMP9, and TIMP1) are all related to the clinical stage of thyroid cancer ($P < 0.05$), which further suggests that FN1, MMP9, and TIMP1 are useful for evaluation. The prognosis of thyroid cancer patients is helpful. We performed GSEA analysis on these 4 hub genes (TOP2A, FN1, TIMP1, and MMP9) at the same time, and found that the high expression of these 4 hub genes enriched the "cell cycle." Subsequently, we collected thyroid cancer tissue specimens and verified the expression of these four hub genes by RT-PCR, and found that only FN1 and TIMP1 genes in thyroid cancer tissues had significantly higher mRNA levels than normal tissues. Therefore, we speculate that FN1 and TIMP1 may be potential therapeutic targets and prognostic markers of thyroid cancer.

FN1 is a member of the fibronectin (FN) family of interstitial markers. FN1 signal activation has been confirmed to be closely related to EMT to promote tumor invasion and metastasis^[9]. It is reported in the literature that FN1 is up-regulated in breast cancer, ovarian cancer, gastric cancer, and other human tumors^[10-12]. Similarly in PTC, FN1 is more highly expressed in cancerous tissues than in adjacent tissues. FN1 knockout inhibits PTC cell proliferation, adhesion, and metastasis^[13]. Some researchers pointed out that microRNA-139 can target FN1 to hinder PTC progress^[14]. Studies have found through bioinformatics analysis that FN1 can be used as a prognostic marker for sporadic medullary thyroid carcinoma^[15]. We found through bioinformatics that FN1 is highly expressed in thyroid cancer tissues and is related to the DFS of thyroid cancer, indicating that it is associated with the prognosis of thyroid cancer.

Tissue inhibitor matrix metalloproteinase 1 (TIMP1) belongs to the tissue inhibitor of the metalloproteinase family. Researchers found that by inducing TIMP1 to specifically regulate the FAK-PI3K/AKT and MAPK pathways, inhibiting TIMP1 expression can reduce proliferation and metastasis, and increase cell apoptosis, which can be used a prognostic indicator of colon cancer^[16]. Inhibition of TIMP1 expression can make pancreatic cancer sensitive to radiotherapy and chemotherapy^[17]. Studies have pointed out that TIMP-1 can activate YAP/TAZ (Yes-related protein/transcriptional co-activator with PDZ binding motif) as a new downstream target to promote cell proliferation^[18]. Furthermore, TIMP1 can promote cell proliferation in ovarian cancer, revealing TIMP1 as a potential therapeutic target for ovarian cancer^[19]. Our results suggest that TIMP1 is highly expressed in thyroid cancer tissues and is related to the DFS of thyroid cancer, suggesting that it is related to the prognosis of thyroid cancer.

MMP-9 (matrix metalloproteinase 9) is a zinc-containing protease, which is abnormally expressed in a variety of tumors. Studies have found that overexpression of MMP-9 contributes to the aggressiveness of the prostate cancer cell line LNCaP^[20]. In addition, active MMP-9 is significantly related to extrathyroid

invasion, lymph node metastasis, and tumor invasion^[21]. In vitro studies of MMP-9 overexpression showed that Notch-1 activation was increased, while β -catenin was correspondingly decreased, indicating that MMP-9 plays a tumor suppressor role in inflammatory bowel cancer, leading to p21 (WAF1/Cip1) activation and β -catenin inhibition^[22]. Researchers found that the expression level of MMP-9 in thyroid cancer cells was higher than that of normal human thyroid cells, and by observing the changes in the biological behavior of thyroid cancer cell lines after interfering with MMP-9 expression, they found that MMP-9 can promote TGF- β 1 induction. The EMT process of thyroid cancer affects the migration and invasion ability of cells^[23]. Our results also suggest that MMP-9 is highly expressed in thyroid cancer tissues and is related to disease-free survival and clinical stage.

Topoisomerase II α (TOP2A) is involved in DNA replication, transcription, recombination, and chromatin reconstruction. The enzyme maintains the topological state of chromosomes during DNA replication and transcription, catalyzes the instantaneous break and reconnection of two double-helical DNA strands, thereby changing DNA and topological structure, and controlling cell cycle progression. TOP2A is abnormally expressed in various tumors^[24-26] and is used in cancer treatment^[27-30]. Double-strand break (DSB) is the most common type of genetic toxicity of DNA damage, and a single unrepaired DSB can trigger cell apoptosis. TOP2A can cause spontaneous DSB of genomic DNA^[31]. The accumulation of DSB can kill tumor cells, but it may also cause genome instability. Studies have screened TOP2A as the hub gene of thyroid cancer through bioinformatics^[32-34]. Our bioinformatics analysis results also indicate that TOP2A is highly expressed in thyroid cancer and is related to the prognosis, but the histological verification has not obtained the same results. To ensure the reproducibility of the results, a large number of tissue samples and a series of Cell experiments to verify this conclusion.

In summary, our research has identified 20 hub genes that may be related to the occurrence and development of thyroid cancer through multiple gene expression profile data sets and a series of comprehensive bioinformatics analyses. Further database and tissue validation analysis revealed that only 2 hub genes may be considered as potential prognostic biomarkers, including FN1 and TIMP1. In addition, these two hub genes are involved in the cell cycle, suggesting that they may play a role in the occurrence and development of thyroid cancer. However, in the future, a series of molecular biology experiments and a large number of clinical specimens are needed to verify these hub genes to provide a basis for the treatment and prognosis of thyroid cancer.

Abbreviations

GEO: Gene Expression Omnibus; DEGs: differentially expressed genes; PPI: protein-protein interaction; THCA: thyroid cancer; TOP2A: DNA topoisomerase II alpha; FN1: fibronectin 1; TIMP1: Tissue inhibitor matrix metalloproteinase 1; ATC: anaplastic thyroid carcinomas; PTC: papillary thyroid carcinomas; GO: Gene Ontology; MF: molecular function; CC: cell composition; BP: biological process; DFS: Disease Free Survival; MMP9: matrix metalloproteinase 9.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Fudan University Minhang Hospital.

Consent for publication

All recruited patients provided informed consent.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Further details are available from the corresponding author upon request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions XLX and HLY performed the experiments and wrote the manuscript supervised by YLP, GXL, CYY and XS who edited the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Venn plots of overlapping DEGs.

Tumoral vs Normal
Cutoff for logFC is 1
The number of up gene is 159
The number of down gene is 251

Figure 2

Volcano plots of gene expression profile data in GSE33630, GSE65144 and GSE29265.

Figure 3

GO analysis of the upregulated DEGs.

Figure 4

GO analysis of the downregulated DEGs.

Figure 5

KEGG analysis of the upregulated DEGs

Figure 6

KEGG analysis of the downregulated DEGs

Figure 8

The hub genes formed a network of interactions.

Figure 9

The expression level of hub genes in thyroid cancer tissue through GEPIA.

Figure 10

Correlation between hub genes.

Figure 11

The relationship between hub gene and DFS.

Figure 12

The relationship between hub gene and clinical stage.

Figure 13

FN1, TOP2A and MMP9 enriched the "cell cycle".

Figure 14

The expression level of hub gene in thyroid cancer tissue.