Identification of key genes of papillary thyroid carcinoma by integrated bioinformatics analysis

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Abstract

Background:Papillary thyroid carcinoma (PTC) is one of the fastest-growing malignant tumor types of thyroid cancer. Therefore, identifying the interaction of genes in PTC is crucial for elucidating its pathogenesis and finding more specific molecular biomarkers.

Methods:In this study, 4 pairs of PTC tissues and adjacent tissues were sequenced using RNA-Seq, and 3745 differentially expressed genes (DEGs) were screened. The results of GO and KEGG enrichment analysis indicate that the vast majority of DEGs may play a positive role in the development of cancer. Then, the significant modules were analysed using Cytoscape software in the protein-protein interaction (PPI) network. Survival analysis, TNM analysis, and immune infiltration analysis of key genes are all analyzed. And the expression of ADORA1, APOE and LPAR5 genes was verified by qPCR in papillary thyroid carcinoma compared to their matching adjacent tissues.

Results: A total of 25 genes were identified as hub genes with nodes greater than 10. The expression of 25 key genes in PTC were verified by the GEPIA database, and the overall survival and disease free survival analyses of these key genes were conducted with Kaplan–Meier plots. We found that only three genes were confirmed with our validation and were statistically significant in PTC, namely ADORA1, APOE, and LPAR5. Further analysis found that the mRNA levels and methylation degree of these three genes are significantly correlated with the TNM staging of PTC, and these three genes are related to PTC immune infiltration. Verification of the expression of these three genes by RT-qPCR further confirmed the reliability of our results.

Conclusion: Our study identified three genes that may play key regulatory roles in the development, metastasis, and immune infiltration of papillary thyroid carcinoma.

Key words :RNA-Seq, papillary thyroid carcinoma, key gene, bioinformatics

Introduction

Thyroid carcinoma is presently the malignancy with the most rapidly increasing incidence in the world and is the most widely recognized endocrine carcinoma in the Western world(1). Thyroid cancers, derived from follicular thyroid cells, can be sorted into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), and anaplastic thyroid carcinoma (ATC) according to the histological subtype(2). Clinical results vary across these subtypes.

The annual rate of thyroid cancer has more than doubled within the past two decades, with the vast majority of this increase being ascribed to PTC, which accounts for 80–85% of all thyroid carcinomas(1). In addition, patients with PTC suffer from cervical lymph nodes metastasis or remote metastasis, which leads to unfavourable results, and approximately 10–15% of cases may progress to a potentially fatal recurrent ailment(3, 4). Due to these reasons, uncovering the causes of PTC and its fundamental

mechanisms and finding molecular biomarkers for early diagnosis and customized treatment, are significant and important tasks.

With the advancement and continuous improvement of gene sequencing and gene-editing technology, it is now convenient to recognize the hub biomarkers related to neoplasm metastasis and survival status using the large amount of information available by applying bioinformatics(5). Currently, there are no effective sensitive biomarkers for early diagnosis, treatment and prevention of lymph node metastasis of PTC. An examination of differentially expressed genes (DEGs) between tumour and para-carcinoma tissue may help identify critical biomarkers of papillary thyroid carcinoma. As a form of molecular marker, mRNA, containing the most abundant genetic information, is necessary for protein translation, and it is separate from the pathological process of cancer at various stages(6). Some studies utilized public databases such as The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) to identify significant biomarkers of papillary thyroid carcinoma. However, these investigations were just founded on single datasets with constrained sample sizes or just based on online databases used to screen out the DEGs.

In this study, we analysed the DEGs in PTC tissues versus the matched adjacent tissues by RNA-Seq and bioinformatics methods to obtain the DEGs. Then, we screened out the key modules and extracted the key genes in those modules by constructing a DEGs interaction network. Then, the possible role of differentially expressed genes was analysed using GO annotation and KEGG pathway enrichment analysis. The expression validation, survival analysis and functional enrichment analysis of key genes was conducted by using relevant databases. Finally, we found that the three genes ADORA1, APOE and LPAR5 are highly expressed in PTC and are associated with PTC methylation, TNM staging, and immune infiltration.

Materials And Methods

Tissue samples

Thirty pairs of PTC and adjacent tissues were collected from 2019.1 to 2019.7 at the First Affiliated Hospital of Hebei North University. This experiment was approved by the Ethical Committee of the First Affiliated Hospital and all patients provided informed consent. All tissues were frozen in liquid nitrogen after surgical resection.

RNA library construction and sequencing

Total RNA was isolated from 4 adjacent normal and cancerous thyroid samples utilizing TRIzol reagent (Qiagen, Valencia,CA, USA) as indicated by the manufacturer's guidelines. RNAs of PTC tissues and paracancerous tissues (sample numbers: 1C, 1P, 2C, 2P, 3C, 3P, 4C, 4P; the number represents different samples, the “C” indicates a cancer sample, and the “P” represents a matched paracancerous tissue sample) were used. Six libraries were built utilizing an Illumina standard kit as indicated by the manufacturer's protocol All sequencing was carried out on an Illumina Hiseq 4000 (LC Bio, China).

Differentially expressed genes screening

The level of expression of mRNAs was evaluated using StringTie by calculating FPKM(7). The DEGs between PTC and paracancerous tissue were screened with $|\log_2$ (fold change)$|>1$, and $P<0.05$ was regarded as statistically significant; the analyses were conducted using the R package Ballgown(8).

Functional enrichment analysis and pathway analysis

To reveal the functional roles of the DEGs, the Annotation, Visualization and Integrated Discovery function annotation tool (DAVID, http://david.abcc.ncifcrf.gov/home.jsp) was used to perform Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. P values less than 0.05 were considered as cutoff criteria.

PPI network construction and identification of hub genes

PPI networks were constructed successively using STRING database (http://strin g-db.org) (9). The interactions of DEGs with a combined score > 0.7 were set as significant and Cytoscape software (version 3.7.2) was utilized to visualize and analyse the results of the STRING database. To find key (hub) genes in this PPI network, the significant module was analysed by using the plug-in MCODE of Cytoscape software. The criteria for selection were set to the default. The key genes were chosen with degrees ≥10. Subsequently, genes in that module were used to analyse their functional roles with FunRich software.

Data validation and analysis.

To verify the accuracy of our RNA-seq results, we used the Gene Expression Profiling Interactive Analysis database to verify the expression of 25 key genes in PTC and adjacent tissues. The overall survival and disease free survival analyses were performed by Kaplan–Meier plots for these PTC-related hub genes. Genetic alterations of 25 hub genes in PTC and their correlations with other genes were analysed utilizing the cBioPortal for Cancer Genomics. Hub genes related to clinicopathological features were analysed using the online database UALCAN (http://ualcan.path.uab.edu)(10). The correlation of ADORA1, APOE, LPAR5 expression with the immune infiltration level in PTC and the expression of these 3 genes in different kinds of cancers was performed using the Tumor Immune Estimation Resource database(11).

For qRT-PCR analysis, total RNA was isolated from 30 normal and cancerous papillary thyroid samples utilizing TRIzol reagent (Qiagen, Valencia, CA, USA). cDNA was synthesized with RNA reverse transcription kit (TIANGEN BIOTECH., Beijing, China). qRT-PCR was performed with an ABI 7300 Real-Time PCR System (Applied Biosystems Life Technologies, USA). Expression of the genes of interest was normalised to β-actin. The primers for ADORA1, APOE, LPAR5 and β-actin are shown in Table 1.

Statistical analysis

All the data were analyzed by R and SPSS 17.0 (SPSS Inc, USA). Kaplan-Meier method was used to estimate the significant difference in survival between the over-expression group and the low-expression

group of key genes in papillary thyroid carcinoma.The statistical difference was set at $P<0.05$

Results

Differentially expressed genes screening based on RNA-Seq

To screen out the genes or modules that may play a role in promoting cancer in papillary thyroid carcinoma, we performed RNA-Seq experiments on four pairs of thyroid cancer tissues and their matched paracancerous tissues to obtain differentially expressed genes. After RNA-Seq, we acquire 9–11 million reads for each sample. The fold changes between PTC cancer tissues and matched paracancerous samples were calculated. Setting the cut-off criterion as P value <0.05 and a fold change >1, there were 1927 upregulated and 1818 downregulated genes. These 3745 DEGs were considered to be candidate genes for subsequent study. Fig. 1a shows the expression of the top 80 genes in PTC vs matched paracancerous tissues.

Functional enrichment analysis and pathway analysis

To investigate the potential function of DEGs in PTC, genes functional enrichment was conducted by using GO and KEGG pathway analyses. For the biological process category, the DEGs were significantly involved in regulation of extracellular matrix organization, cell adhesion, cell junction and angiogenesis. The cellular component category results showed PTC-related DEGs were enriched in the membrane region, plasma membrane, and integral component of the membrane. DEGs in molecular function are mainly involved in calcium ion binding and axon guidance (Fig. 2a).

As Fig. 2b shows, the KEGG pathway results showed DEGs were enriched in multiple tumourigenesis pathways, including small cell lung cancer, PPAR signalling pathway, p53 signalling pathway, microRNAs in cancer, gastric cancer, cytokine-cytokine receptor interaction, transcriptional misregulation in cancer, colorectal cancer, bladder cancer, cell adhesion molecules and apoptosis.

PPI network construction and module analysis

PPI networks were constructed successively by the database by loading the PTC related DGEs into the STRING database (Fig. 1c). Using Cytoscape, we analysed the most significant module in the PPI network (Fig. 1d). The PPI network consisted of 587 nodes and 1836 edges. Following the use of MCODE in Cytoscape, the significant module was selected. The 25 top hub genes Adenylate Cyclase 8 (ADCY8), Adenosine Receptor A1 (ADORA1), Adrenoceptor Alpha 2C (ADRA2C), Apolipoprotein E (APOE), Complement C5a Receptor 1 (C5AR1), C-C Motif Chemokine Ligand 13 (CCL13), C-C Motif Chemokine Ligand 20 (CCL20), Cadherin 2 (CDH2), Chromogranin B (CHGB), C-X-C Motif Chemokine Ligand 12 (CXCL12), Eva-1 Homolog A, Regulator Of Programmed Cell Death (EVA1A), FAM20A Golgi Associated Secretory Pathway Pseudokinase (FAM20A), Fibronectin 1 (FN1), G Protein Subunit Alpha I1 (GNAI1), Glypican 3 (GPC3), Glutamate Metabotropic Receptor 4 (GRM4), Lysophosphatidic Acid Receptor (LPAR5), Melanotransferrin (MELTF or MFI2), Milk Fat Globule-EGF Factor 8 Protein (MFGE8),

Neuromedin U (NMU), Opioid Receptor Mu 1 (OPRM1), Serpin Family A Member 1 (SERPINA1), Somatostatin Receptor 3 (SSTR3), TIMP Metallopeptidase Inhibitor 1 (TIMP1), and Tenascin C (TNC) were evaluated by degree (>10) in the PPI network (Fig. 1d). The results showed that the functions of the 25 key genes were mainly concentrated in signal transduction, cell communication, G-protein coupled receptor activity, cell adhesion molecule activity and GPCR ligand binding (Fig. 3).

Data analysis and validation

After the key genes were selected, the expression of 25 key genes in PTC and its adjacent tissues were verified by the GEPIA database (Fig. 4). ADORA1, APOE, EVA1A, LPAR5, MFGE8, OPRM1, SERPINA1, SSTR3, and TIMP1 were positively related to the overall survival analysis of PTC patients, while C5AR1 and GNAI1 were negatively related (Fig. 5). ADCY8, ADORA1, CHGB, FN1, LPAR5, NMU, TNC showed positive associations with disease free survival analysis of PTC patients but not APOE (Fig. 6).

Next, we analysed the alterations of the 25 key genes by using the cBioPortal database. The 25 key genes were changed in 224 (56%) of queried samples (Fig. 7b). Figure 7a shows the frequency of alterations of each PTC related key gene. SSTR3, FN1, and ADORA1 were altered the most (8%, 6% and 6%, respectively). Figure 7d shows the network of the 25 genes and their altered neighbouring genes in PTC patients (out of a total of 1278).

Among these genes, only ADORA1, APOE and LPAR5 genes simultaneously showed statistical significance for overall survival analysis and disease free survival analysis of PTC patients. The GEPIA database and qPCR experiments data verified that these three survival-related genes were all overexpressed in PTC (Fig. 8). Then, based on UALCAN, the clinical features and degree of methylation of these 3 genes were analysed. The transcription level of ADORA1, APOE and LPAR5 were significantly higher in PTC patients than normal tissues according to subgroups of sample types, individual stages and nodal metastasis status (Fig. 9). In addition, ADOR1 and LPAR5 exhibited a hypomethylation state in the cancer group, but APOE showed a hypermethylation state in PTC samples (Fig. 10a).

To further clarify the role of these genes, we conducted an analysis of immune infiltration. The ADOR1 expression level was positively corelated with infiltrating levels of B cells (r=0.111, p=1.51e-2), CD8+ T cells (r=0.246, p=3.96e−08), neutrophils (r=0.162, p=3.31e−04) and DCs (r=0.232, p=2.32e−07). The expression of APOE was positively associated with B cells (r=0.228, p=4.39e−07), CD8+ T cells (partial.cor=0.15, p=9.30e−04), neutrophils (r=0.197, p=1.14e−05), and DCs (partial.cor=0.229, p=3.58e−07). LPAR5 expression level was positively related to B cells (r=0.259, p=8.15e−09), CD4+ T cells (r= 0.238, p=1.03e−07), macrophages (r=0.175, p=1.05e−04), neutrophils (r= 0.27, p=1.42e−09) and DCs (r=0.256, p=1.04e−08) and negatively related to Purity (r=−0.099, p=2.94e−02) and CD8+ T cells (r=−0.085, p=6.18e−02) (Figure 10b). These findings strongly suggest that LPAR5, ADOR1 and APOE play specific roles in immune infiltration in PTC, especially those of DCs. Finally, we examined the expression of these three genes in common cancer tissues and adjacent tissues, and we found that these three genes are highly expressed in most cancer tissues (Fig. 11).

Discussion

PTC is a common cancer with great heterogeneity in morphological features and prognosis(12). Although most papillary thyroid carcinomas exhibit low biological activity, there are still a few patients with higher invasive and metastatic clinical features(13). Activation of oncogene expression and loss of function of tumour suppressor genes may lead to the development or progression of PTC. To better clarify the molecular mechanism of PTC occurrence, development and metastasis, we identified 25 key genes related to PTC progression through comprehensive bioinformatics methods, and we screened three of the PTC prognosis-related genes for a comprehensive analysis.

In the present study, we identified 3745 differentially expressed genes by RNA-Seq, with GO enrichment analysis showing that the DEGs were enriched in the regulation of the extracellular matrix, cell adhesion, cell junction, collagen metabolism, and wound healing. KEGG pathway enrichment analyses showed that the DEGs were significantly involved in the pathways associated with cell adhesion molecules (CAMs), apoptosis, endocrine resistance and multiple cancer-related pathways.

To further explore the interrelationship of differentially expressed genes in papillary thyroid carcinoma, we constructed a PPI regulatory network. A total of 25 DEGs with nodes greater than 10 were screened out in the network. The key genes were ADCY8, ADORA1, ADRA2C, APOE, C5AR1, CCL13, CCL20, CDH2, CHGB, CXCL12, EVA1A, FAM20A, FN1, GNAI1, GPC3, GRM4, LPAR5, MELTF, MFGE8, NMU, OPRM1, SERPINA1, SSTR3, TIMP1 and TNC. Biological process and molecular function analyses of these 20 key DEGs indicated that they were significantly involved in cancer regulation processes such as adjustment of cell growth or maintenance, cell immune response, cell adhesion molecular activity, and extracellular matrix structural constituent.

To verify the credibility of the experiments and data, the 25 DEGs screened were verified by the GEPIA database. Among the 25 selected genes, 15 genes showed expression differences consistent with our RNA-Seq data. Among the 15 genes, ADORA1, APOE, CCL13, CDH2, EVA1A, FAM20A, FN1, LPAR5, MFGE8, NMU, SERPINA1, TIMP1 and TNC levels were overexpressed in PTC tissues while GPC3 and GNAI1 were downregulated.

ADORA1 belongs to the G-protein coupled receptor 1 family and protects human tissues and cells under physiological conditions(14). Lin et al. suggested that ADORA1 may promote the proliferation of breast cancer cells by positively regulating oestrogen receptor-alpha in breast cancer cells(15). Similarly, Jayakar indicated that knockdown of APOE expression can reduce the level of MMPs by regulating the AP-1 signalling pathway and thus reduce the invasion and metastasis of oral squamous cell carcinoma(16). Bioinformatics predictions were that APOE mRNA shows a significant increase in PTC(17). CCL13 is a coding gene involved in immune regulation and inflammatory responses, and it has been reported that CCL13 has a role in promoting the proliferation of tumour-forming volume in nude mice(18). CDH2 is overexpressed in various cancers. Some research results indicate that overexpression of CDH2 can increase the invasive ability and induce EMT in lung cancer cells(19). Qiu et al confirmed CDH2 acts as an oncogene in papillary thyroid carcinoma, which is consistent with our findings(20).

EVA1A acts as a regulator of programmed cell death and Shen et al. indicates that EVA1A can inhibit the proliferation of GBM cells by inducing autophagy and apoptosis via inactivating the mTOR/RPS6KB1 signalling pathway(21). FAM20A may play a key role in haematopoiesis. There are few reports on the relationship between FAM20A and cancer, and our experiment found that FAM20A is more highly expressed in papillary thyroid carcinoma than in other cancers. FN1 is involved in regulating cell adhesion, cell movement, wound healing and maintaining cell morphology(22). Some researchers indicated that FN1 participates in regulating many types of cancer progression, such as gastric cancer (23), skin squamous cell carcinoma (24) and papillary thyroid carcinoma (20, 25, 26). It has been shown that LPAR5 is related to the pathogenesis of pancreatic cancer(27). Consistent with our study, Zhang et al. believes that LPAR5 may be involved in the development of papillary thyroid carcinoma(28). According to previous reports, MFG-E8 is involved in the progression of various malignancies, such as breast cancer, melanoma, bladder tumours and ovarian cancer(29–32). MFGE8 is considered to be a potential therapeutic target for ovarian cancer owing to its carcinogenic effect(32). Consistent with our data, Zhang et al. indicate that NMU is one of the DEGs of papillary thyroid carcinoma(28). Recently, a researcher has shown that abnormal expression of NMU is associated with a variety of cancers(33). For SERPINA1, there are currently 6 articles pointing out that SERPINA1 may be a key gene for PTC, consistent with our results(28, 34–38). Clinical studies have shown that high expression of TIMP1 is positively correlated with a poor prognosis of colon, brain, prostate, breast, lung, and several other cancers(39). TNC is a component of the extracellular matrix (ECM) and is closely related to the malignant biological behaviour of cancer. In particular, TNC overexpression is positively associated with liver cancer, oral squamous cell carcinoma, and lymph node metastasis of breast cancer (40, 41). GPC3 belongs to the glypicans family. It has been reported that overexpression of GPC3 can promote the metastasis of hepatocellular carcinoma (42), but we found that it is expressed at low levels in PTC. Similar to GPC3, some scholars believe that GNAI1 is a tumour-promoting gene and reported upregulated GNAI1 mRNA in human glioma, which is inconsistent with our data(43).

Only the ADORA1, APOE and LPAR5 genes simultaneously showed statistical significance for overall survival and disease free survival of PTC patients. Considering that the occurrence and metastasis of cancer is a complex and multi-regulated process, we further analysed the regulatory mechanisms of these three genes. We found that the mRNA and methylation levels of these three genes were significantly correlated with TNM staging. In addition, ADORA1, APOE, and LPAR5 are all related to immune infiltration, especially to dendritic cells. Finally, we found that these three genes are more highly expressed in cancer tissues than matched adjacent tissues.

However, our research has certain limitations. First, only 4 pairs of cancer and adjacent tissues were analysed using RNA-seq in this experiment, so further research requires a larger sample size. Second, further experiments are needed to validate the specific mechanisms of these key genes.

Declarations

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Xu Lin conducted the bioinformatics analysis. Xu Lin and Gang Xue contributed as first authors. Xu Lin wrote the manuscript. Jing -Fang Wu and Gang Xue critically revised the article. Gang Xue and Da Pei obtained clinical specimens, and the others contributed to verification of the RNA-seq results.

DATA AVAILABILITY

The data used to support the findings of this study are available from

the corresponding author upon request.

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References

1. Kitahara CM, Sosa JA: The changing incidence of thyroid cancer. *Nature reviews Endocrinology* 2016; 12(11):646-653.
2. Aschebrook-Kilfoy B, Ward MH, Sabra MM, Devesa SS: Thyroid cancer incidence patterns in the United States by histologic type, 1992-2006. *Thyroid : official journal of the American Thyroid Association* 2011; 21(2):125-134.
3. Pourseirafi S, Shishehgar M, Ashraf MJ, Faramarzi M: Papillary Carcinoma of Thyroid with Nasal Cavity Metastases: A Case Report. *Iranian journal of medical sciences* 2018; 43(1):90-93.
4. Ullmann TM, Gray KD, Moore MD, Zarnegar R, Fahey TJ, 3rd: Current controversies and future directions in the diagnosis and management of differentiated thyroid cancers. *Gland surgery* 2018; 7(5):473-486.
5. Jin X, Deng B, Ye K, Ye D, Huang Y, Chen X, Yang Z, Chen Y: Comprehensive expression profiles and bioinformatics analysis reveal special circular RNA expression and potential predictability in the peripheral blood of humans with idiopathic membranous nephropathy. *Molecular medicine reports* 2019.

6. Rapisuwon S, Vietsch EE, Wellstein A: Circulating biomarkers to monitor cancer progression and treatment. *Computational and structural biotechnology journal* 2016; 14:211-222.
7. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL: StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nature biotechnology* 2015; 33(3):290-295.
8. Frazee AC, Pertea G, Jaffe AE, Langmead B, Salzberg SL, Leek JT: Ballgown bridges the gap between transcriptome assembly and expression analysis. *Nature biotechnology* 2015; 33(3):243-246.
9. von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B: STRING: a database of predicted functional associations between proteins. *Nucleic acids research* 2003; 31(1):258-261.
10. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi B, Varambally S: UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia* 2017; 19(8):649-658.
11. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, Li B, Liu XS: TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells. *Cancer research* 2017; 77(21):e108-e110.
12. Nikiforov YE, Nikiforova MN: Molecular genetics and diagnosis of thyroid cancer. *Nature reviews Endocrinology* 2011; 7(10):569-580.
13. Pusztaszeri M, Auger M: Update on the cytologic features of papillary thyroid carcinoma variants. *Diagnostic cytopathology* 2017; 45(8):714-730.
14. Borea PA, Gessi S, Merighi S, Varani K: Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? *Trends in pharmacological sciences* 2016; 37(6):419-434.
15. Lin Z, Yin P, Reierstad S, O'Halloran M, Coon VJ, Pearson EK, Mutlu GM, Bulun SE: Adenosine A1 receptor, a target and regulator of estrogen receptoralpha action, mediates the proliferative effects of estradiol in breast cancer. *Oncogene* 2010; 29(8):1114-1122.
16. Jayakar SK, Loudig O, Brandwein-Gensler M, Kim RS, Ow TJ, Ustun B, Harris TM, Prystowsky MB, Childs G, Segall JE *et al*: Apolipoprotein E Promotes Invasion in Oral Squamous Cell Carcinoma. *The American journal of pathology* 2017; 187(10):2259-2272.
17. Tan J, Qian X, Song B, An X, Cai T, Zuo Z, Ding D, Lu Y, Li H: Integrated bioinformatics analysis reveals that the expression of cathepsin S is associated with lymph node metastasis and poor prognosis in papillary thyroid cancer. *Oncology reports* 2018; 40(1):111-122.
18. Kuo CY, Wang JC, Hsu SL, Hwang GY: Functional characterization of hepatitis B virus X protein based on the inhibition of tumorigenesis in nude mice injected with CCL13-HBx cells. *Intervirology* 2008; 51(4):253-260.
19. Yamauchi M, Yoshino I, Yamaguchi R, Shimamura T, Nagasaki M, Imoto S, Niida A, Koizumi F, Kohno T, Yokota J *et al*: N-cadherin expression is a potential survival mechanism of gefitinib-resistant lung cancer cells. *American journal of cancer research* 2011; 1(7):823-833.
20. Qiu J, Zhang W, Zang C, Liu X, Liu F, Ge R, Sun Y, Xia Q: Identification of key genes and miRNAs markers of papillary thyroid cancer. *Biological research* 2018; 51(1):45.

21. Shen X, Kan S, Liu Z, Lu G, Zhang X, Chen Y, Bai Y: EVA1A inhibits GBM cell proliferation by inducing autophagy and apoptosis. *Experimental cell research* 2017; 352(1):130-138.
22. Liao YX, Zhang ZP, Zhao J, Liu JP: Effects of Fibronectin 1 on Cell Proliferation, Senescence and Apoptosis of Human Glioma Cells Through the PI3K/AKT Signaling Pathway. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2018; 48(3):1382-1396.
23. Zhang H, Sun Z, Li Y, Fan D, Jiang H: MicroRNA-200c binding to FN1 suppresses the proliferation, migration and invasion of gastric cancer cells. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2017; 88:285-292.
24. Dooley TP, Reddy SP, Wilborn TW, Davis RL: Biomarkers of human cutaneous squamous cell carcinoma from tissues and cell lines identified by DNA microarrays and qRT-PCR. *Biochemical and biophysical research communications* 2003; 306(4):1026-1036.
25. Chakladar J, Li WT, Bouvet M, Chang EY, Wang-Rodriguez J, Ongkeko WM: Papillary Thyroid Carcinoma Variants are Characterized by Co-dysregulation of Immune and Cancer Associated Genes. *Cancers* 2019; 11(8).
26. Li S, Yin Y, Yu H: Genetic expression profile-based screening of genes and pathways associated with papillary thyroid carcinoma. *Oncology letters* 2018; 16(5):5723-5732.
27. Ishii S, Hirane M, Fukushima K, Tomimatsu A, Fukushima N, Tsujiuchi T: Diverse effects of LPA4, LPA5 and LPA6 on the activation of tumor progression in pancreatic cancer cells. *Biochemical and biophysical research communications* 2015; 461(1):59-64.
28. Zhang S, Wang Q, Han Q, Han H, Lu P: Identification and analysis of genes associated with papillary thyroid carcinoma by bioinformatics methods. *Bioscience reports* 2019; 39(4).
29. Jinushi M, Nakazaki Y, Carrasco DR, Draganov D, Souders N, Johnson M, Mihm MC, Dranoff G: Milk fat globule EGF-8 promotes melanoma progression through coordinated Akt and twist signaling in the tumor microenvironment. *Cancer research* 2008; 68(21):8889-8898.
30. Yang C, Hayashida T, Forster N, Li C, Shen D, Maheswaran S, Chen L, Anderson KS, Ellisen LW, Sgroi D *et al*: The integrin alpha(v)beta(3-5) ligand MFG-E8 is a p63/p73 target gene in triple-negative breast cancers but exhibits suppressive functions in ER(+) and erbB2(+) breast cancers. *Cancer research* 2011; 71(3):937-945.
31. Faes S, Dormond O: PI3K and AKT: Unfaithful Partners in Cancer. *International journal of molecular sciences* 2015; 16(9):21138-21152.
32. Tibaldi L, Leyman S, Nicolas A, Notebaert S, Dewulf M, Ngo TH, Zuany-Amorim C, Amzallag N, Bernard-Pierrot I, Sastre-Garau X *et al*: New blocking antibodies impede adhesion, migration and survival of ovarian cancer cells, highlighting MFGE8 as a potential therapeutic target of human ovarian carcinoma. *PloS one* 2013; 8(8):e72708.
33. Przygodzka P, Soboska K, Sochacka E, Boncela J: Neuromedin U: A Small Peptide in the Big World of Cancer. *Cancers* 2019; 11(9).

34. Choi D, Ramu S, Park E, Jung E, Yang S, Jung W, Choi I, Lee S, Kim KE, Seong YJ *et al*: Aberrant Activation of Notch Signaling Inhibits PROX1 Activity to Enhance the Malignant Behavior of Thyroid Cancer Cells. *Cancer research* 2016; 76(3):582-593.
35. Jarzab B, Wiench M, Fujarewicz K, Simek K, Jarzab M, Oczko-Wojciechowska M, Wloch J, Czarniecka A, Chmielik E, Lange D *et al*: Gene expression profile of papillary thyroid cancer: sources of variability and diagnostic implications. *Cancer research* 2005; 65(4):1587-1597.
36. Qu T, Li YP, Li XH, Chen Y: Identification of potential biomarkers and drugs for papillary thyroid cancer based on gene expression profile analysis. *Molecular medicine reports* 2016; 14(6):5041-5048.
37. Teng H, Mao F, Liang J, Xue M, Wei W, Li X, Zhang K, Feng D, Liu B, Sun Z: Transcriptomic signature associated with carcinogenesis and aggressiveness of papillary thyroid carcinoma. *Theranostics* 2018; 8(16):4345-4358.
38. Vierlinger K, Mansfeld MH, Koperek O, Nohammer C, Kaserer K, Leisch F: Identification of SERPINA1 as single marker for papillary thyroid carcinoma through microarray meta analysis and quantification of its discriminatory power in independent validation. *BMC medical genomics* 2011; 4:30.
39. Jackson HW, Defamie V, Waterhouse P, Khokha R: TIMPs: versatile extracellular regulators in cancer. *Nature reviews Cancer* 2017; 17(1):38-53.
40. Chiquet-Ehrismann R, Chiquet M: Tenascins: regulation and putative functions during pathological stress. *The Journal of pathology* 2003; 200(4):488-499.
41. Orend G, Chiquet-Ehrismann R: Tenascin-C induced signaling in cancer. *Cancer letters* 2006; 244(2):143-163.
42. Sun B, Huang Z, Wang B, Yu Y, Lin S, Luo L, Wang Y, Huang Z: Significance of Glypican-3 (GPC3) Expression in Hepatocellular Cancer Diagnosis. *Medical science monitor : international medical journal of experimental and clinical research* 2017; 23:850-855.
43. Liu YY, Chen MB, Cheng L, Zhang ZQ, Yu ZQ, Jiang Q, Chen G, Cao C: microRNA-200a downregulation in human glioma leads to Galphai1 over-expression, Akt activation, and cell proliferation. *Oncogene* 2018; 37(21):2890-2902.

Tables

Table 1 PCR primers

Gene symbol	Primer sequence
ADORA1	F:5′-CCACAGACCTACTTCCACACC-3′
	R:5′-TACCGGAGAGGGATCTTGACC-3′
β-actin	F:5'-CACTCTTCCAGCCTTCCTTCC-3′
	R:5'-AGGTCTTTGCGGATGTCCAC-3′
APOE	F:5′- GTTGCTGGTCACATTCCTGG -3′
	R:5′- GCAGGTAATCCCAAAAGCGAC′
LPAR5	F:5′- CACTTGGTGGTCTACAGCTTG-3′
	R:5′- GCGTAGTAGGAGAGACGAACG-3′

F,forward primer;R,reverse primer;bp,base pair.

Figures

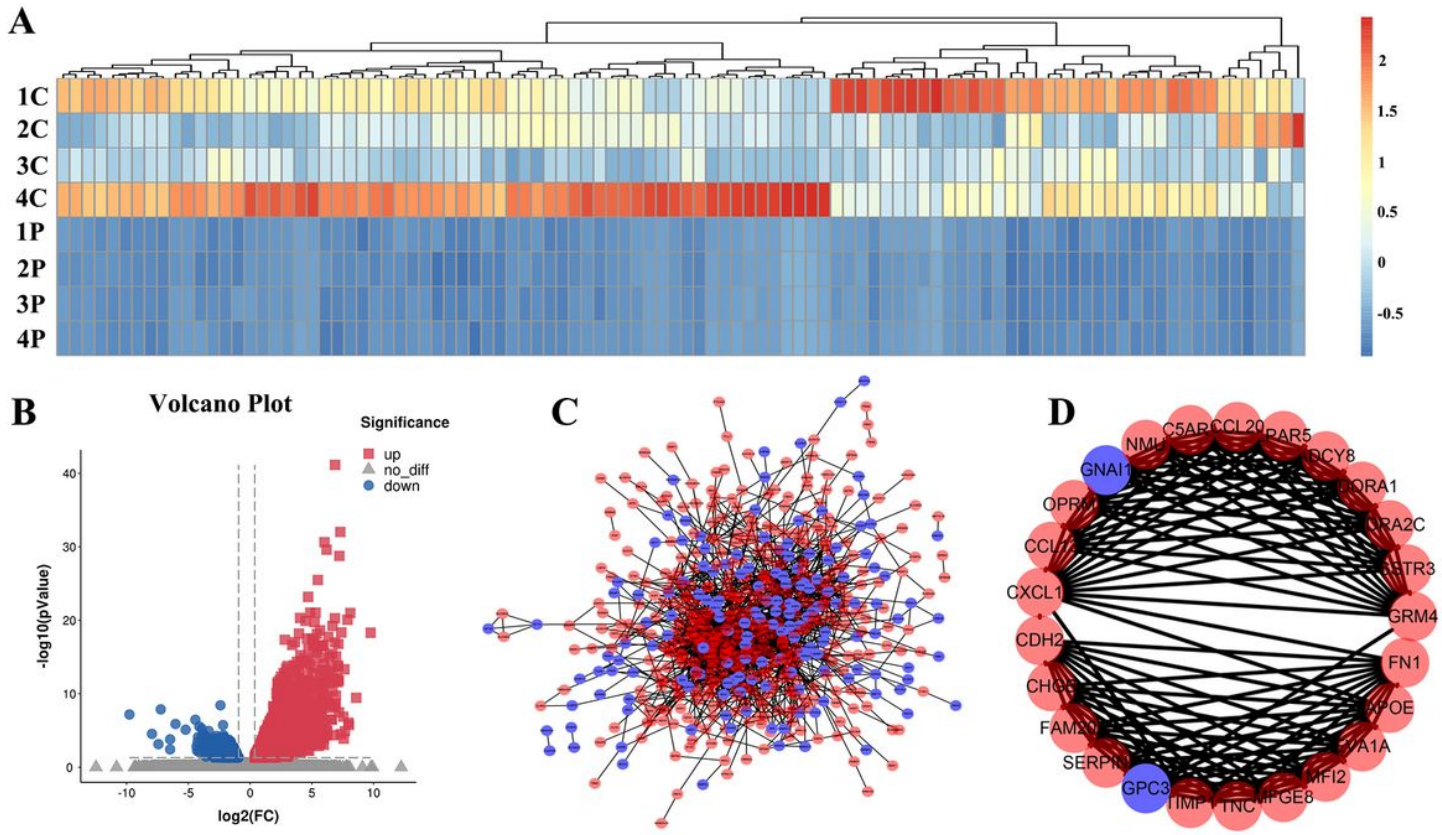

Figure 1

Identification of DEGs by RNA-seq. The heat map (A) and volcano plots of the DEGs (B). (C) The PPI network of the DEGs was conducted by Cytoscape. (D) The most significant module was selected by

MCODE in Cytoscape. Red represents the upregulated genes, and blue represents the downregulated genes.

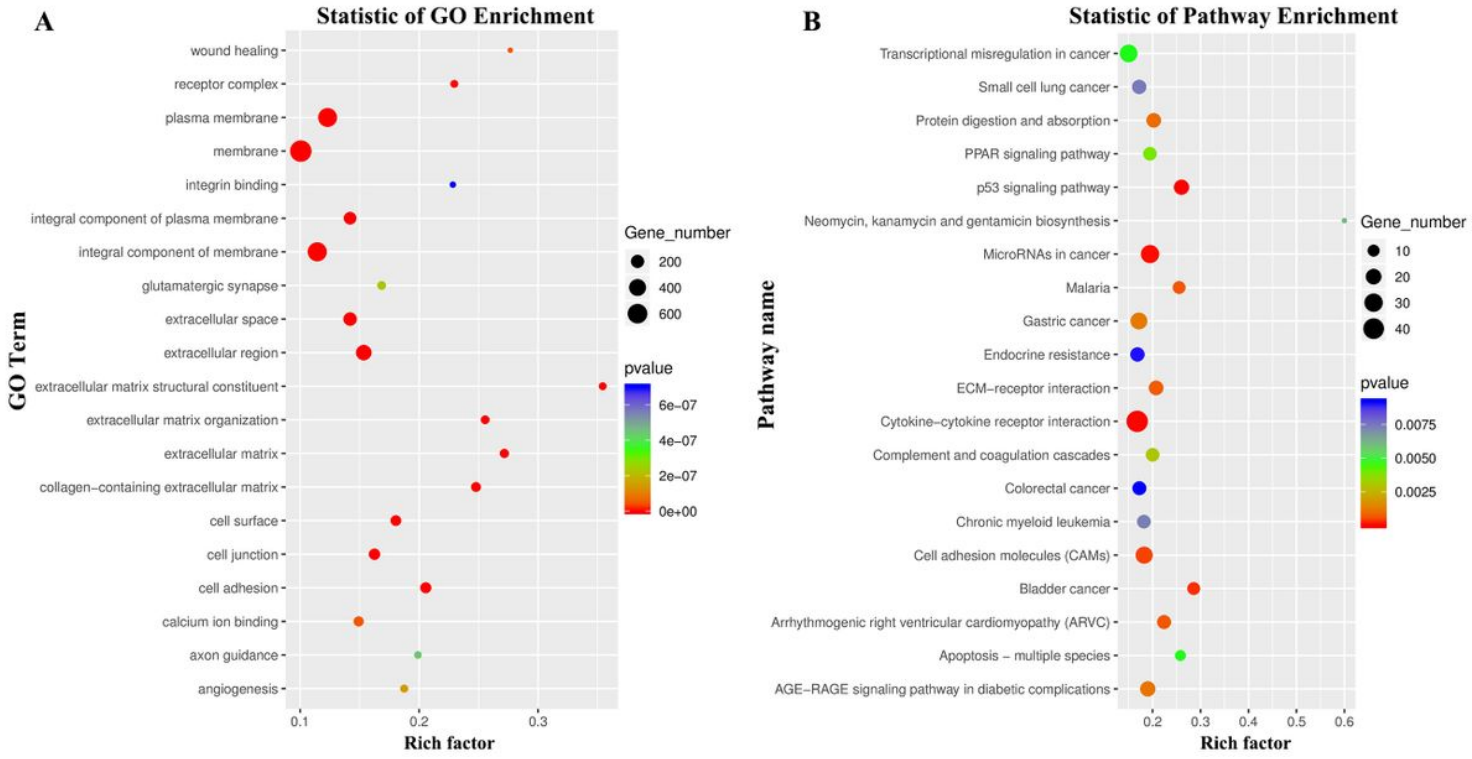

Figure 2

GO and KEGG pathway enrichment analysis of 3745 DEGs through RNA-Seq. (A) Bubble plot of Gene Ontology enrichment analysis of DEGs. (B) Bubble plot of Kyoto Encyclopaedia of Genes and Genomes pathway enrichment analysis of DEGs.

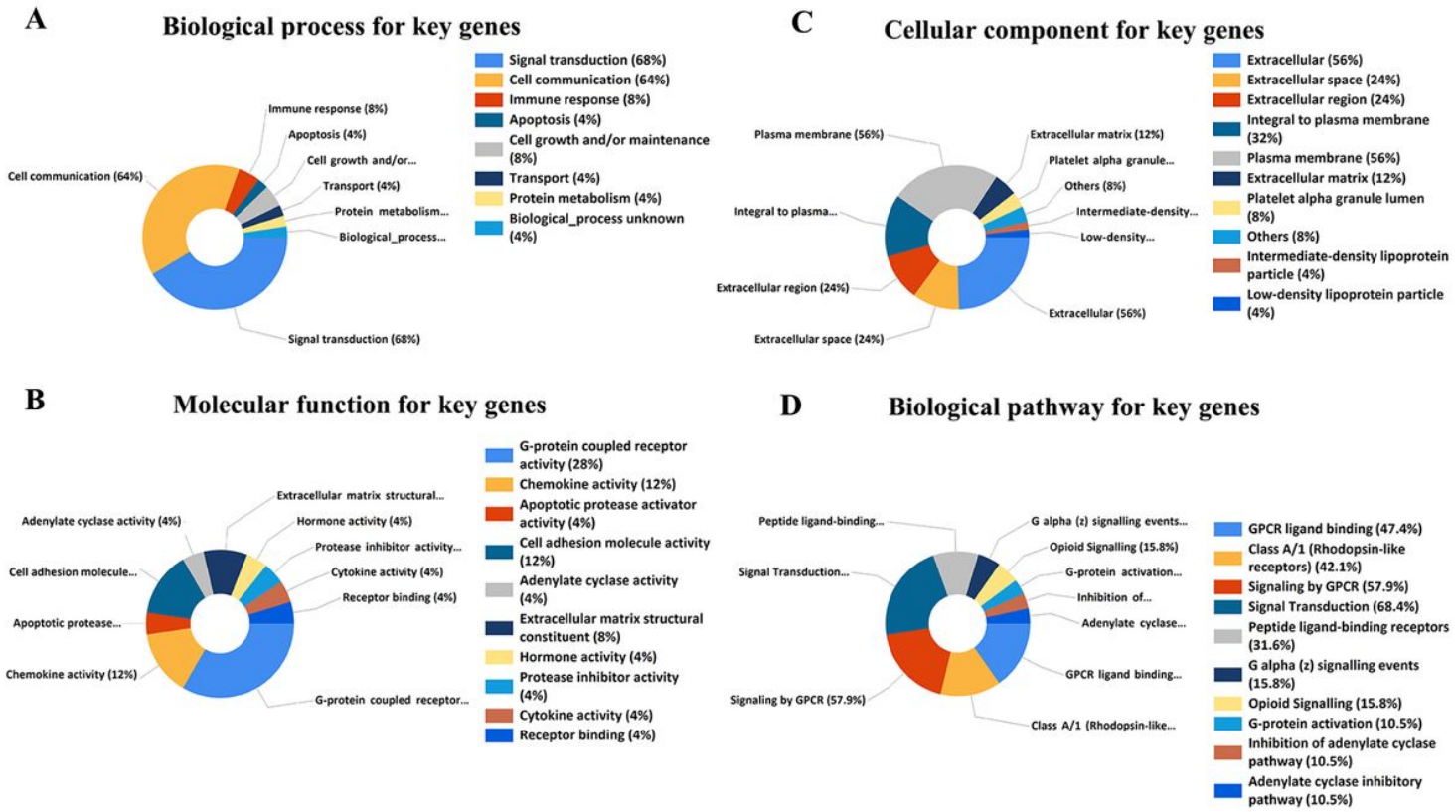

Figure 3

GO enrichment analysis and KEGG analysis for the key genes. (A) Top 10 elements involved in biological processes. (B) Top 10 elements involved in molecular function. (C) Top 10 elements involved in cellular components. (D) Top 10 pathways related to the 25 key genes through KEGG analysis.

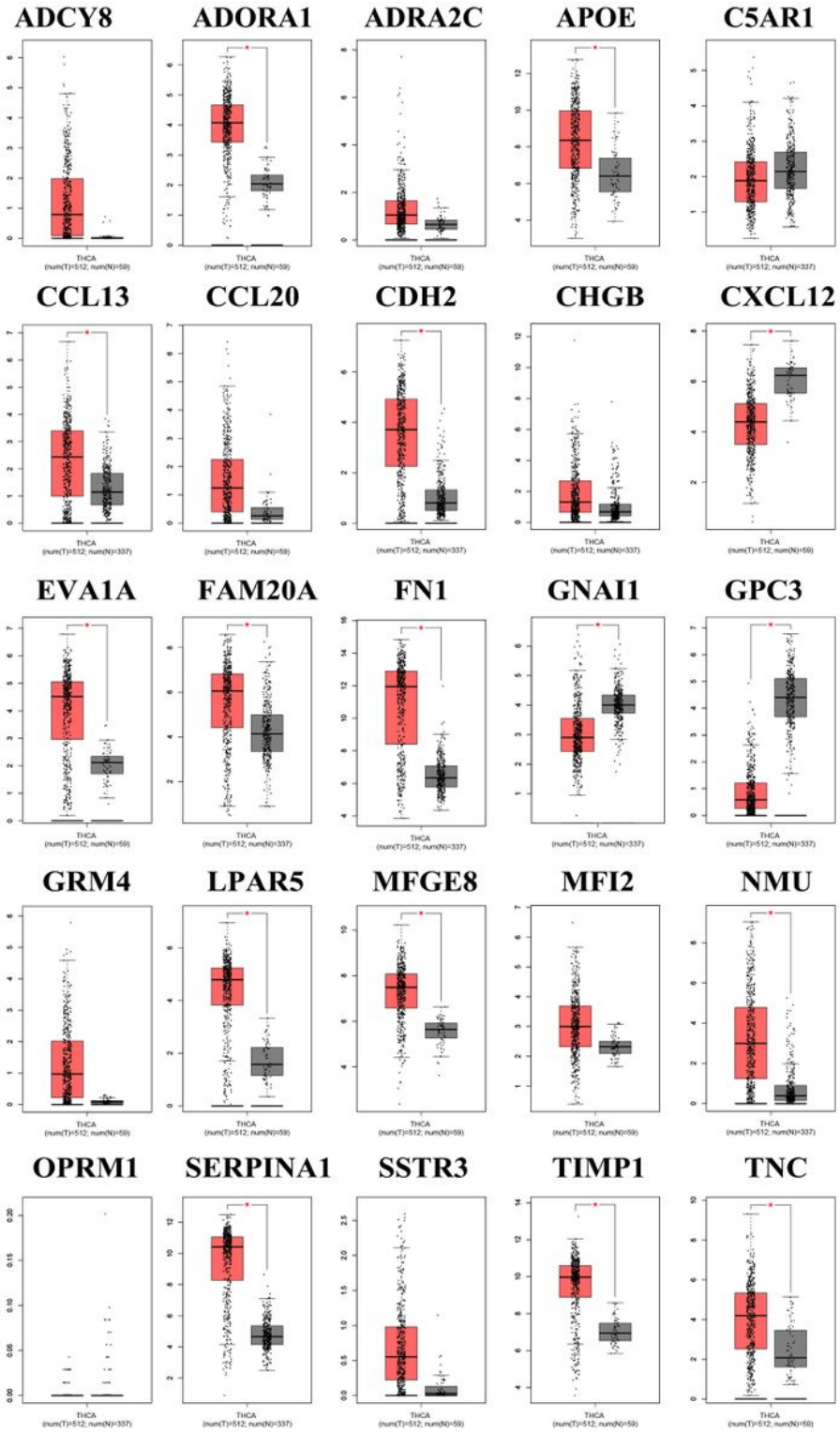

Figure 4

Validation of the 25 key DEGs in the GEPIA database. ADORA1, APOE, CCL13, CDH2, CXCL12, EVA1A, FAM20A, FN1, GNAI1, LPAR5, MFGE8, NMU, SERPINA1, TIMP1 and TNC are overexpressed in PTC tissues compared to paracancerous tissue, while GNAI and GPC3 are downregulated.

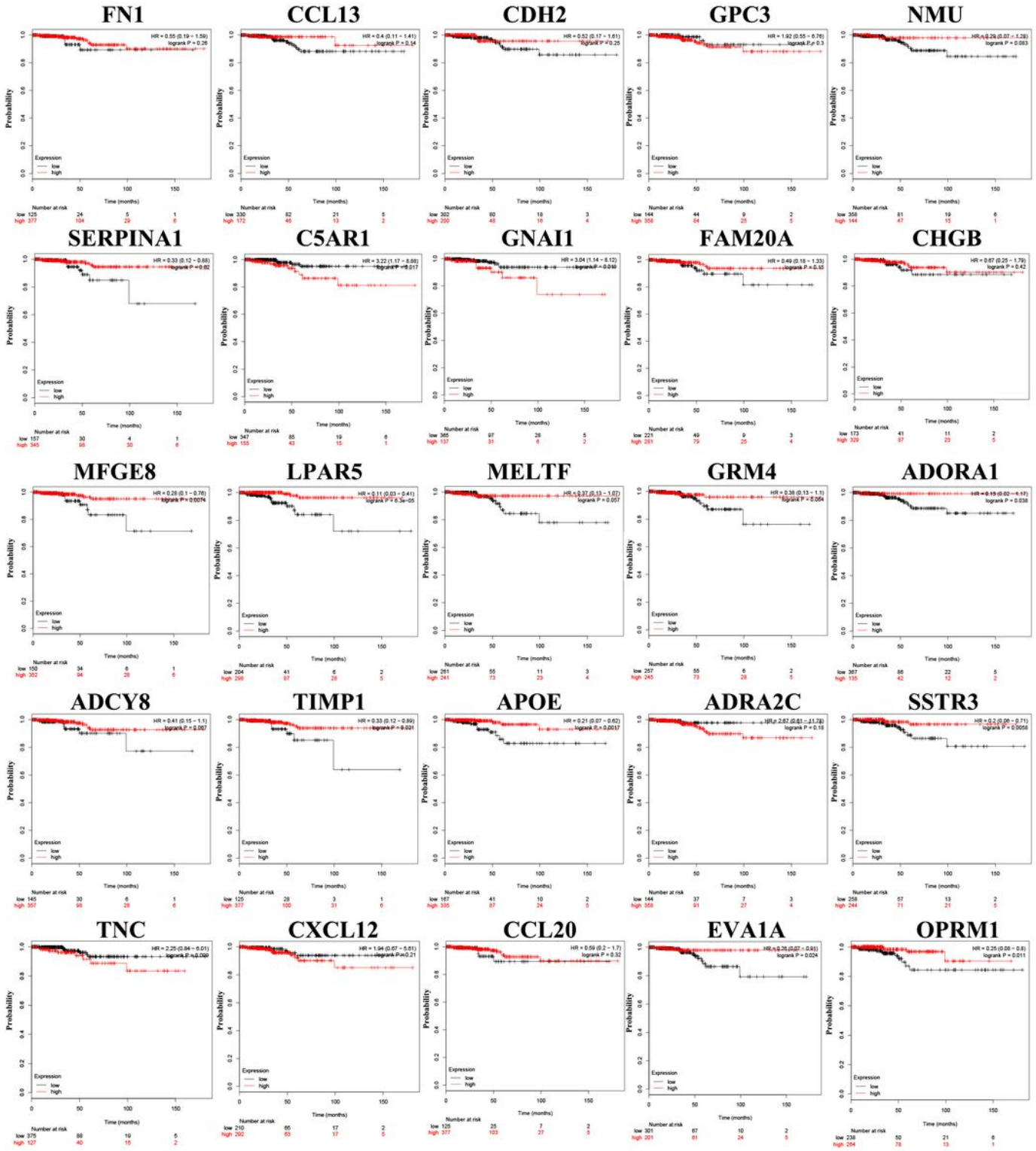

Figure 5

Overall survival analysis of 25 key genes in PTC using Kaplan–Meier plots. Expression levels of ADORA1, APOE, C5AR1, EVA1A, FAM20A, GNAI1, LPAR5, MFGE8, OPRM1, SERPINA1, SSTR3 and TIMP1 are related to the overall survival of patients with PTC.

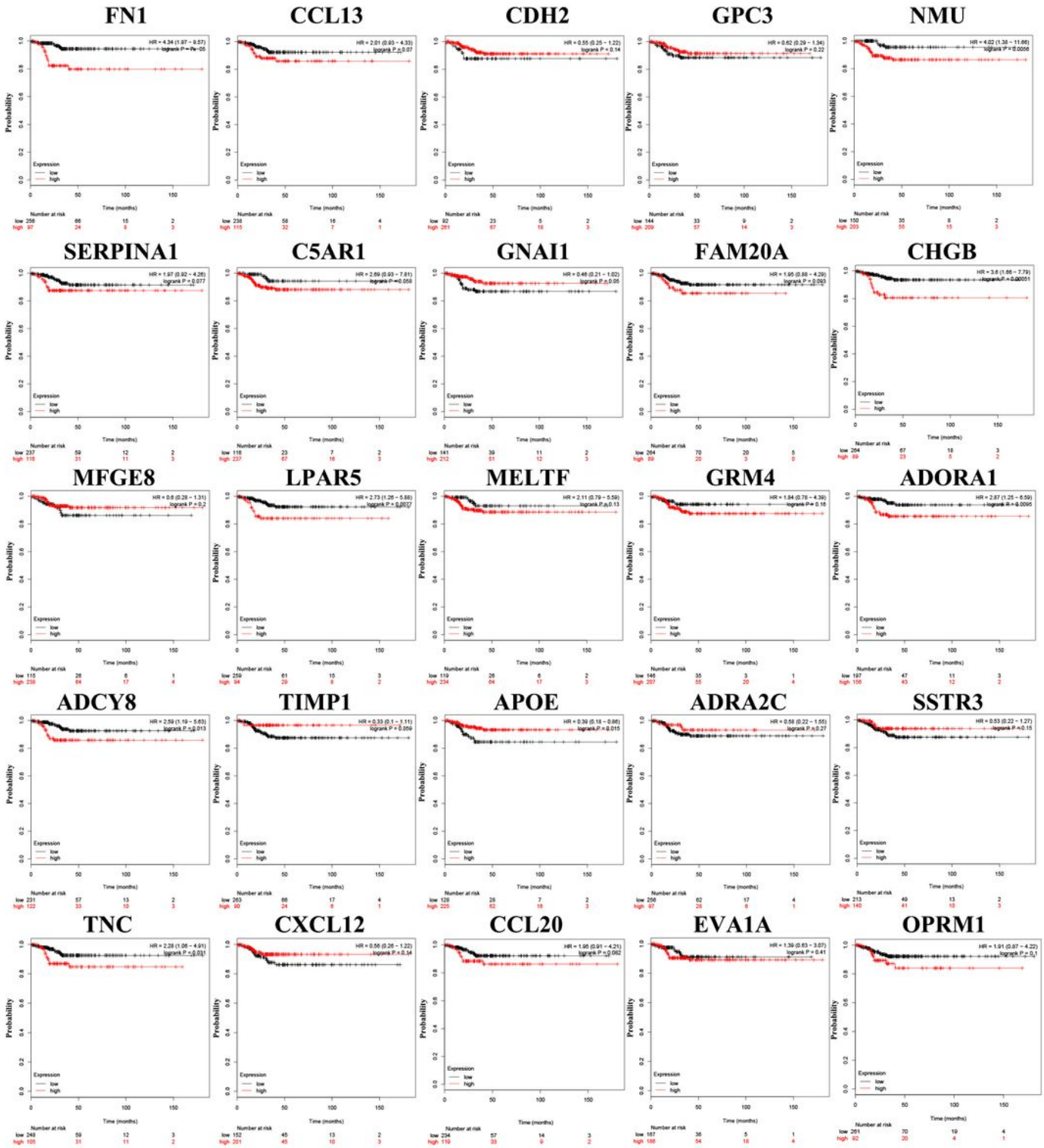

Figure 6

Disease free survival analysis of 25 key genes in PTC using Kaplan–Meier plots. Expression levels of ADCY8, ADORA1, APOE, CHGB, FN1, LPAR5, NMU and TNC are significantly related to the disease free survival of patients with PTC.

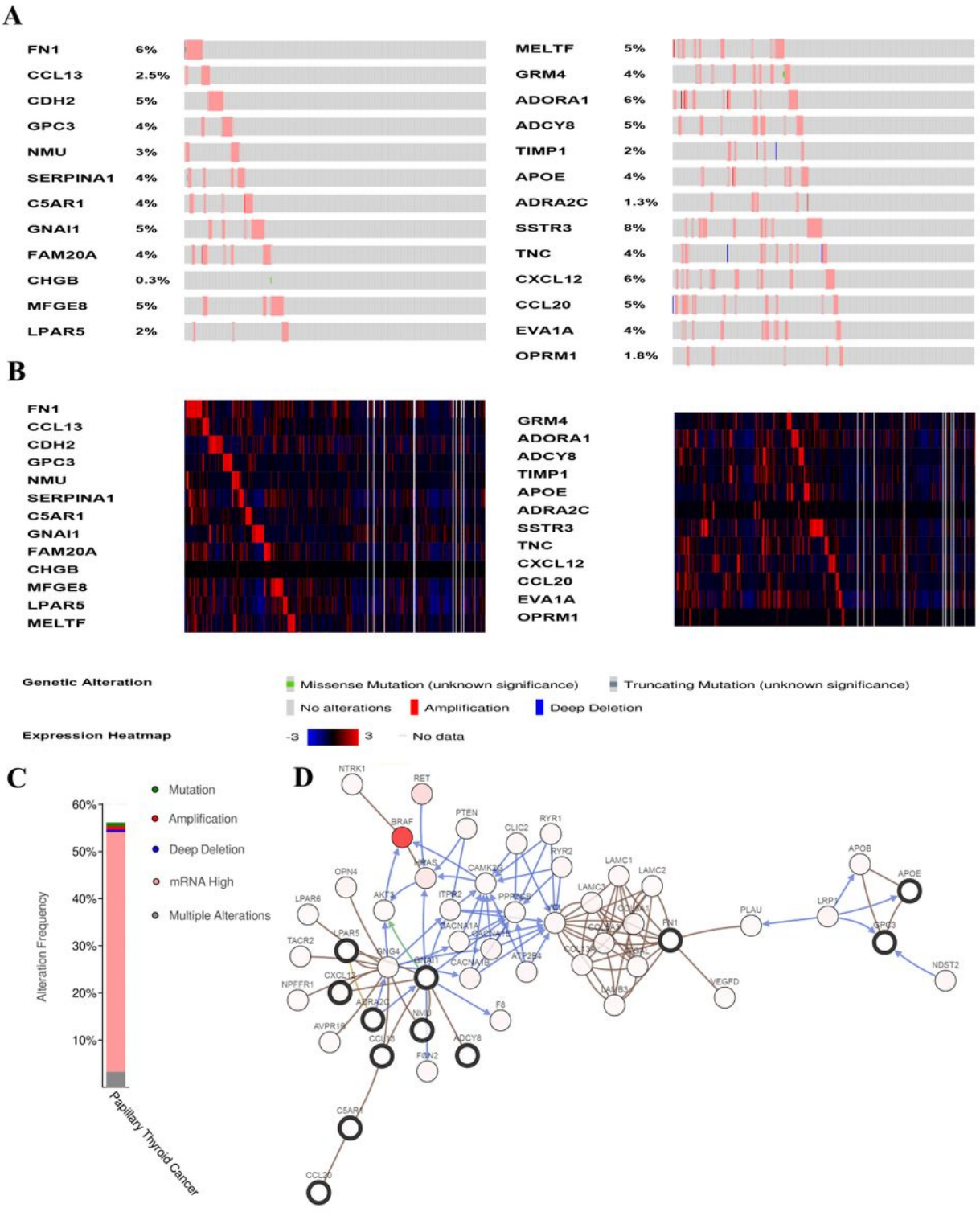

Figure 7

The 25 key genes expression and mutation analysis in PTC by the cBioPortal for Cancer Genomics. (A) The genetic alterations of 25 key genes of 399 PTC samples. Queried genes are altered in 224 (56%) of queried patients/samples. (B) The expression heatmap of 25 key genes. (C) The alteration frequency of 25 key genes in PTC. (D) Network of 25 key genes mutations and their 50 frequently altered neighbouring genes in PTC.

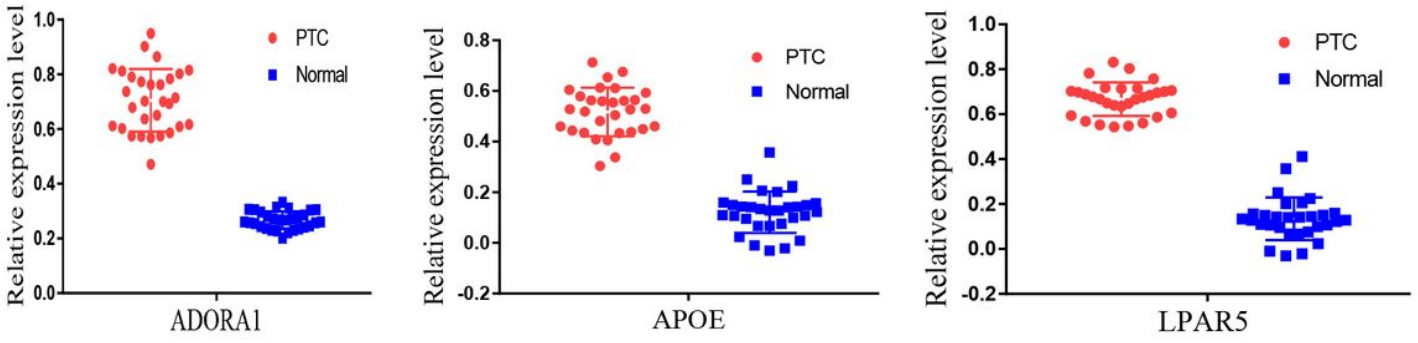

Figure 8

Validation of expression levels of ADORA1, APOE and LPAR5 by RT-qPCR in 30 cases of PTC and matched adjacent tissues.

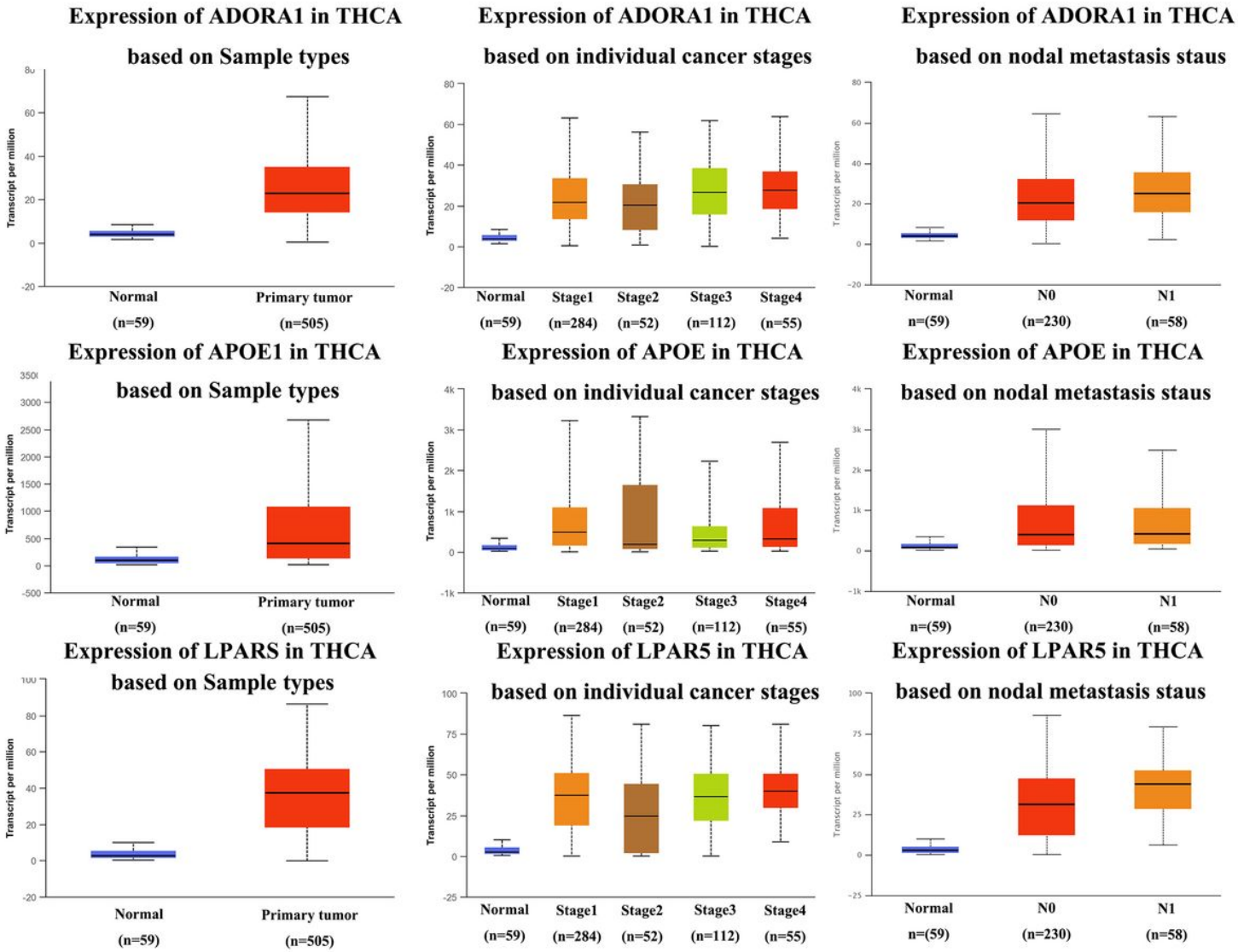

Figure 9

Relative expression of ADORA1, APOE and LPAR5 in normal thyroid tissues and PTC tissues, individual cancer stages and nodal metastasis status, respectively (UALCAN).

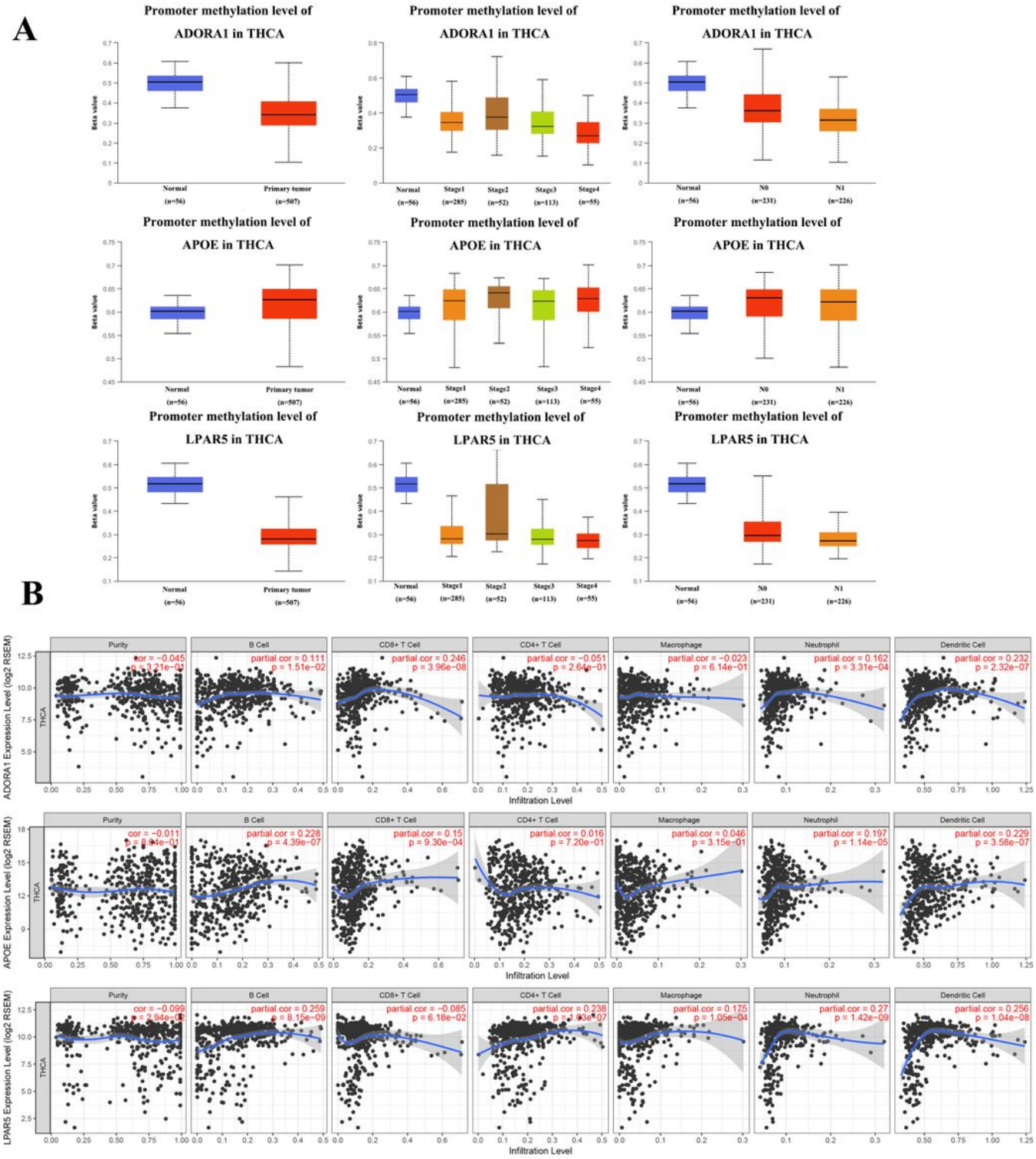

Figure 10

Methylation level and immune infiltration level of ADORA1, APOE and LPAR5. (A) Relative methylation level of ADORA1, APOE and LPAR5 based on normal thyroid tissues and PTC tissues, individual cancer

stages and nodal metastasis status, respectively (UALCAN). (B) The correlation between the 3 genes and TIICs (TIMER). TIICs, tumour infiltrating immune cells.

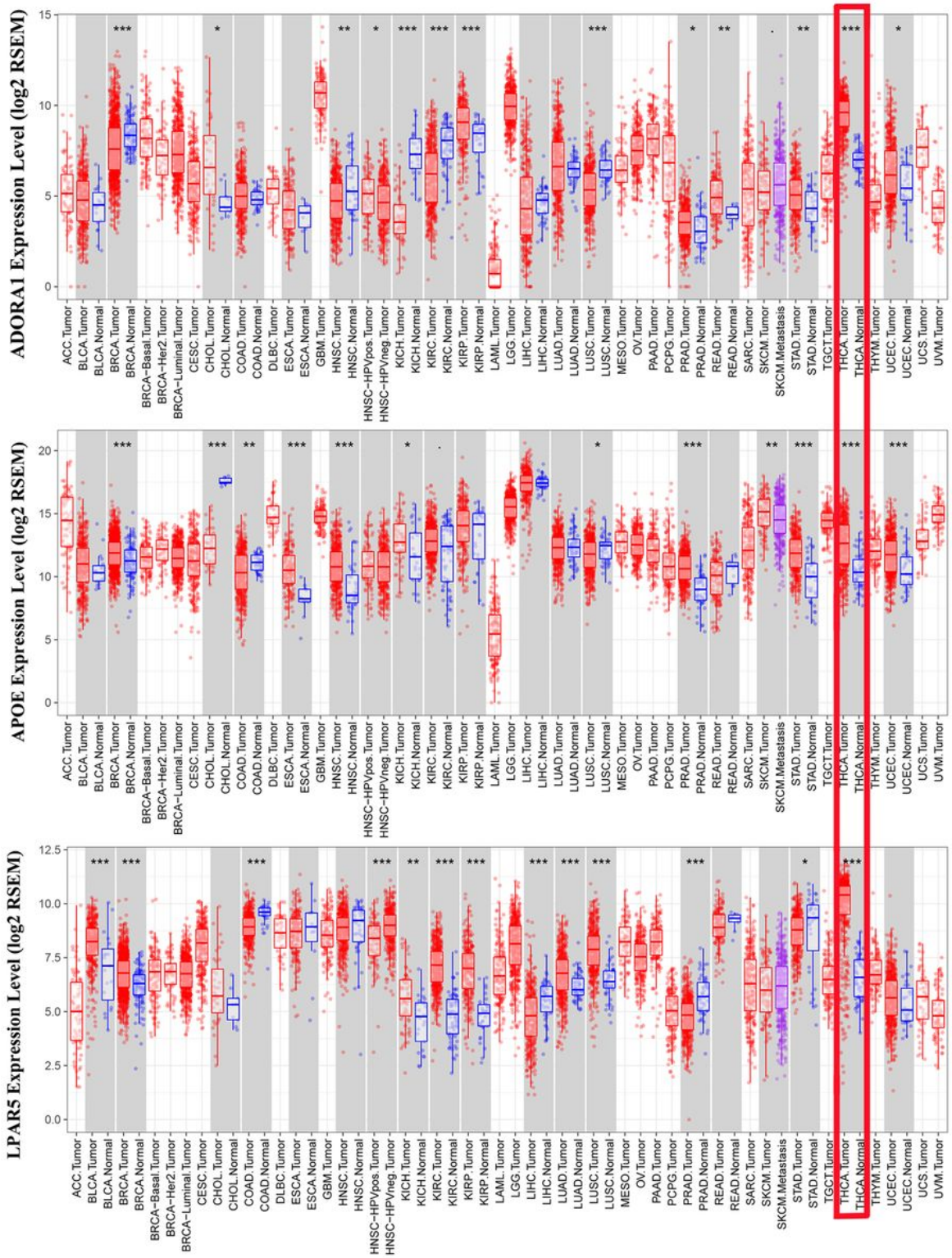

Figure 11

The expression of ADORA1, APOE and LPAR5 in thyroid cancer tissues and normal thyroid tissues. The 3 genes expression were analyzed in different kind of cancer tissues and normal tissues via the TIMER database. *p < 0.05, **p < 0.01, ***p < 0.01