European Review for Medical and Pharmacological Sciences 2020; 24: 10003-10014

Comprehensive analysis of lncRNA-mediated ceRNA network in papillary thyroid cancer

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Abstract. – OBJECTIVE: Papillary thyroid cancer (PTC) is one type of thyroid cancer. Although it has a good prognosis, the recurrence and metastasis rates remain high.

MATERIALS AND METHODS: The microarray dataset GSE66783 was downloaded from the Gene Expression Omnibus (GEO). With the R package, the differentially expressed genes (DEGs) and lncRNAs between normal adjacent tissues and cancer tissues of PTC were identified. The miRNAs that were targeted by DElncRNAs and the mRNAs that were targeted by miRNAs were discovered through miRcode and through miRTarBase, TargetScan, and miRDB, respectively. Furthermore, the ceRNA network was constructed. GO and KEGG enrichment analyses were performed on the DEGs. The PPI network of the DEGs was obtained from the STRING database, and the top 5 hub genes that had a tight correlation with the disease were obtained by using Cytoscape. Finally, the study used the Kaplan-Meier method to analyze PTC patient survival time, and the Human Protein Atlas database was used to retrieve the expression of the hub genes in normal and PTC patient tissues.

RESULTS: Five hub genes showed significant differences in expression in the PPI network, and 12 lncRNA-miRNA-mRNA pathways might participate in the potential pathophysiological process of PTC.

CONCLUSIONS: The study indicated that these ceRNAs might contribute to future therapies for PTC.

Key Words:
PTC, LncRNA, CeRNA, MiRNA.

Introduction

Thyroid cancer (TC) is the most common malignant tumor of the endocrine system and is also the major cause of death in endocrine tumors[1]. In the past decades, with the continuous improvement of disease diagnosis technology, the incidence of TC has been increasing worldwide[2,3]. Based on a 2018 GLOBOCAN report, nearly 567,000 patients around the world suffer from TC, which ranked 9th in incidence among diseases[4]. Among the four known pathological types of TC, papillary thyroid cancer (PTC) is the most frequent subtype, accounting for more than 80% of all TC cases[5,6]. Even though most PTCs have a relatively favorable prognosis, there are still some patients with lymph node or distant metastasis or with the invasion of the tumor into the surrounding tissues, which may cause recurrence of the disease and death of the patients[5,7,8]. Therefore, it is also necessary to identify the potential molecular mechanisms of PTC and clarify the pathogenesis of PTC to find novel molecular targets to achieve a quick diagnosis and treatment.

In the human genome, 98% of the genes are not translated, and only approximately 20,000 genes can encode proteins[9]. These non-translated RNAs are called non-coding RNAs (ncRNAs). Among these, the RNAs longer than 200 nucleotides are called long non-coding RNA (lncRNAs)[10]. They play an important role in biological processes, such as interfering with the shearing of mRNAs, directly binding to proteins to regulate their activity or change protein localization, and participating in recombinant gene expression as a competitive endogenous RNA (ceRNA)[11]. The functions of lncRNAs in the gene expression regulatory processes of transcription, posttranscriptional regulation and translation have also been verified[12]. Moreover, multiple pieces of evidence[13,14] have indicated that lncRNAs may take part in carcinogenesis and cancer metastasis. With the deepening of ceRNA knowledge, the supposition that lncRNA

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and mRNA could restore natural miRNA sponges and inhibit miRNA function by binding to shared 3'-UTR has been gradually accepted[15]. Therefore, the ceRNA network has been recognized in various diseases, including cancers[16]. However, studies on the relationship between lncRNAs and PTC are not sufficient, and further research needs to be undertaken.

In the present study, through the search of a microarray dataset from the Gene Expression Omnibus (GEO) database, which contained lncRNA expression information for PTC tissue and noncancerous tissues, differentially expressed lncRNAs (DElncRNAs) were obtained, after which we used online tools to predict potential miRNAs and mRNAs. After filtering the differentially expressed miRNAs (DEmiRNAs) and mRNAs (DEmRNAs), the ceRNA networks of PTC that were mediated by lncRNA were constructed, and the top 5 differentially expressed genes (DEGs) were selected for further analysis. Finally, overall survival (OS) analyses were performed on these hub genes that were all contained in the ceRNA network, and information was collected from the Human Protein Atlas database to verify expression in the PTC cases. These meaningful targets may provide new biomarkers for PTC diagnosis and therapy.

Materials and Methods

Data Collection

The gene expression profile of PTC was downloaded from the GEO (https://www.ncbi.nlm.nih.gov/geo/) database. The GSE66783 dataset was based on GPL19850 (Agilent-060228 Human LncRNA v5 4X180K), which contained 5 PTC and 5 adjacent noncancerous thyroid tissues. The raw data of GSE66783 was normalized and annotated by gene biotype.

Identification of DElncRNAs and DEmRNAs

To find the DElncRNAs and DEmRNAs from the PTC and adjacent noncancerous thyroid tissues, the limma package of R (https://www.r-project.org/) was utilized, and the screening criteria were set as an adjusted p-value<0.05 and a |log fold change (logFC)| ≥1. Next, the heatmaps of the DElncRNAs and DEmRNAs that were recovered, as outlined above, were drawn by the pheatmap package of the R software.

Construction of the ceRNA Network

The online miRNA reference database miRcode (http://www.mircode.org/), which contains a very "highly conservative microRNA family" file, was used to detect the potential interaction of the lncRNAs and miRNAs[17]. In addition, the target mRNAs of the miRNAs were obtained from the three well-known online prediction websites, miRTarBase (http://mirtarbase.mbc.nctu.edu.tw)[18], TargetScan (http://www.targetscan.org)[19] and the miRDB databases (http://www.mirdb.org/)[20]. Notably, only mRNAs with the same result in the three databases could be exported, and these mRNAs were then compared with the different mRNAs obtained previously; the duplicated mRNAs were then used in the construction of the lncRNA-miRNA-mRNA network. Finally, the ceRNA network was constructed by Cytoscape (version 3.7.0)[21].

Functional Enrichment Analysis of the DEmRNAs

Gene Ontology (GO) is an important tool of bioinformatics. It can simply annotate genes and analyze the function of DEmRNAs based on three aspects: molecular function, biological process and cell composition[22]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database that closely links genome information with biological systems. The main biochemical metabolic pathways and signal transduction pathways that the DEmRNAs were most involved in can be determined by this database[23]. To forecast the functions of the DEmRNAs, GO and KEGG pathways enrichment analyses were performed through the clusterProfiler package of R software with a p-value<0.05[24].

Construction of the Protein-Protein Interaction (PPI) Network and Identification of the Hub Genes

Through the Search Tool for the Retrieval of Interacting Genes (STRING, Version 11.0) (https://string-db.org/)[25], the PPI network was constructed and visualized by the Cytoscape software. The gene screening conditions for constructing the network were set to have a comprehensive score greater than 0.7. In the network, the nodes represent the proteins encoded by the corresponding DEmRNAs, and the degree of nodes was considered as the number of interactions with other DEmRNAs. The higher degree nodes acted as the central genes in the PPI networks. After that, through the application

cytoHubba from Cytoscape, the top 5 hub genes with the highest degree of connection to the others were considered as significant and marked with a different color.

Reconstruction of the SubceRNA Network

To confirm the ceRNA network of the hub genes, the corresponding miRNA and lncRNA were also retrieved from the original ceRNA network and were used to reconstruct a subsequent subceRNA network.

Prognostic Analyses of the Hub Genes

The Kaplan-Meier Plotter is a publicly available database containing different types of tumor patients' survival information to evaluate the prognosis among patients with PTC. Therefore, the overall survival curves with a log-rank test and the Kaplan-Meier method were retrieved for the hub genes. A p-value <0.05 was considered statistically significant. Moreover, from the perspective of histopathology, the expression of hub genes in PTC patients was recovered from the Human Protein Atlas database (HPA, Version 19.1) (https://www.proteinatlas.org/), which is dedicated to providing tissue and cell distribution information for all 24000 human proteins with a free public inquiry.

Results

Identification of the DElncRNAs and DEmRNAs

There were 5 PTC patient tissues and 5 normal control samples collected from the GSE66783 dataset. With the limma package in R, 37 lncRNAs (8 upregulated and 29 downregulated) (Figure 1A) and 2009 mRNAs (945 upregulated and 106 downregulated) (Figure 1B) were identified based on the standard of the adjusted p-value<0.05 and a |log fold change (logFC)| ≥1.

CeRNA Network Construction

According to the online miRNA reference database, the potential miRNAs that had a relationship with the lncRNAs were obtained, as shown in the Supplementary Table I. Based on the mRNA prediction databases, the target genes of the miRNA were also identified. In addition, after crosschecking with the DEmiRNAs and DEmRNAs, we selected those that overlapped with those that were identified. Finally, there were 26 lncRNAs, 34 miRNAs and 132 mRNAs involved in the ceRNA network represented by the Cytoscape software (Figure 2).

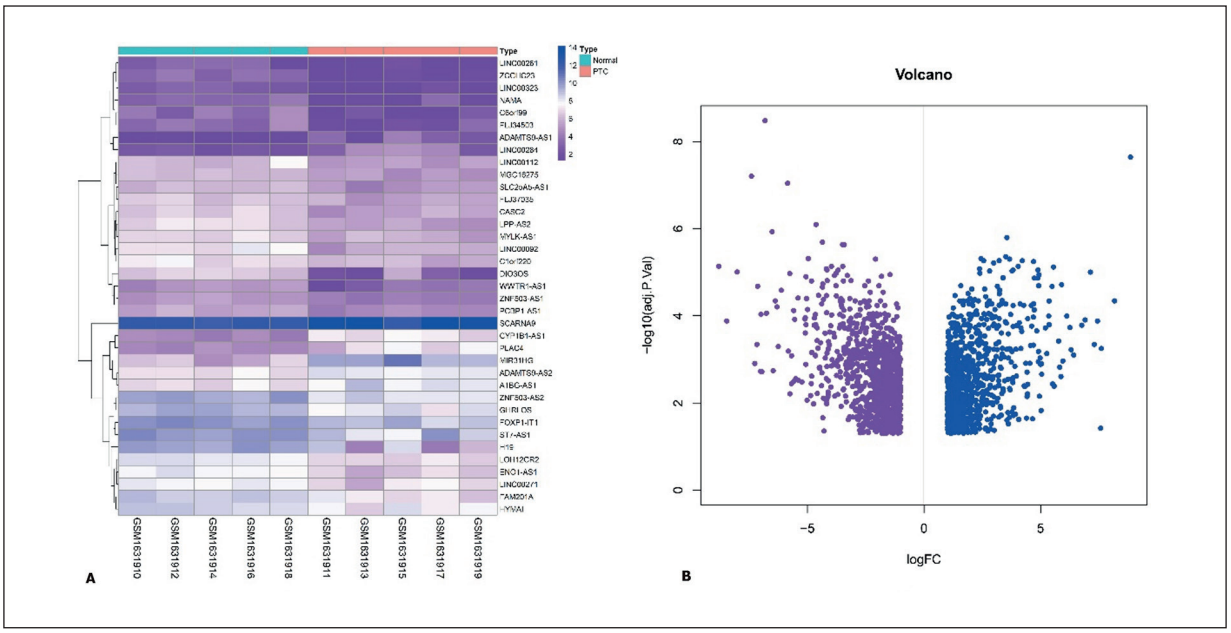

Figure 1. Differentially expressed genes in PTC (|logFC| ≥1.0 and adjusted p-value < 0.05) between 5 cancer tissues and 5 normal tissues. The heatmap plot of DEmiRNAs (**A**). The Volcano map of DEmRNAs (**B**). Blue stands for upregulations, purple stands for downregulations.

Table I. Go analysis of DEmRNAs.

Categories	Terms	Description	Courts	*p*-value
Biological process	GO:0007165	Signal transduction positive	24	1.20E-05
	GO:0045944	regulation of transcription from RNA polymerase II promoter		
	GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	16	2.73E-04
	GO:0043547	Positive regulation of GTPase activity	13	9.71E-04
	GO:0008285	Negative regulation of cell proliferation	12	1.70E-04
	GO:0008284	Positive regulation of cell proliferation	12	6.75E-04
	GO:0045892	Negative regulation of transcription, DNA-templated	12	0.001178
	GO:0045893	Positive regulation of transcription, DNA-templated	12	0.001515
	GO:0010628	Positive regulation of gene expression	11	2.47E-05
	GO:0006366	Transcription from RNA polymerase II promoter	11	0.004733
Cellular component	GO:0005634	Nucleus	54	0.003102
	GO:0005886	Plasma membrane	50	4.48E-05
	GO:0005654	Nucleoplasm	29	0.029165
	GO:0005887	Integral component of plasma membrane	23	3.31E-04
	GO:0009986	Cell surface	13	4.40E-04
	GO:0048471	Perinuclear region of cytoplasm	11	0.012236
	GO:0043235	Receptor complex	9	3.17E-06
	GO:0009897	External side of plasma membrane	8	7.73E-04
	GO:0005925	Focal adhesion	8	0.020536
	GO:0005667	Transcription factor complex	6	0.011738
Molecular function	GO:0005515	Protein binding	91	2.19E-06
	GO:0003700	Transcription factor activity, sequence-specific DNA binding	21	2.24E-05
	GO:0043565	Sequence-specific DNA binding	13	4.33E-04
	GO:0004672	Protein kinase activity	11	3.18E-04
	GO:0042802	Identical protein binding	11	0.049137
	GO:0008134	Transcription factor binding	9	0.001189
	GO:0003682	Chromatin binding	8	0.025377
	GO:0044212	Transcription regulatory region DNA binding	7	0.004894
	GO:0019899	Enzyme binding	7	0.036331
	GO:0000978	RNA polymerase II core promoter proximal region sequence-specific DNA binding	7	0.047081

Functional Enrichment and Pathway Analysis of the DEmRNAs

GO and KEGG pathway enrichment analyses were performed to indicate the functions of the DEmRNAs that were in the ceRNA network. In Table I, the GO results showed that in biological process (BP), the DEmRNAs were mostly enriched in signal transduction, transcription of RNA polymerase II, regulation of cell proliferation, regulation of transcription, DNA-templated, positive regulation of GTPase activity, gene expression, and others (p-value<0.05). In terms of cellular component (CC), the DEmRNAs were significantly enriched in the nucleus, plasma membrane and its surrounding area, the nucleoplasm and its perinuclear region, receptor complex, focal adhesion and transcription factor complex (p-value<0.05). In terms of molecular function (MF), the DEmRNAs were mainly enriched in the binding of protein, transcription factor, chromatin and enzyme, protein kinase activity, transcription regulatory region sequence-specific DNA binding and RNA polymerase II core promoter proximal region sequence (p-value<0.05). Moreover, the KEGG analysis revealed the top 20 pathways that the DEmRNAs were most-

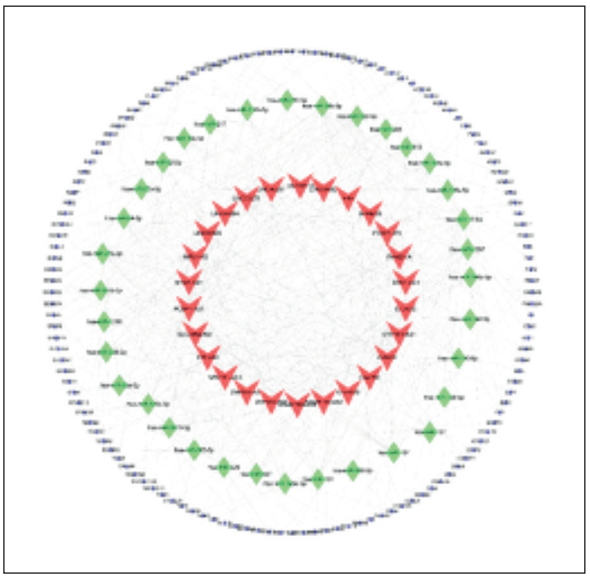

Figure 2. The lncRNA-associated ceRNA network. Red nodes represent DElncRNAs. Green nodes represent predictive miRNAs. Blue nodes represent predictive mRNAs.

ly involved. These pathways were significantly enriched in cancer-related pathways, such as colorectal, gastric, pancreatic, breast, small cell lung, bladder, prostate, melanoma, chronic myeloid leukemia, and hepatocellular cancers, as well as microRNAs, transcriptional misregulation, proteoglycans, human T-cell leukemia virus 1 infection, classical pathways such as MAPK signaling pathway and the p53 signaling pathway, cellular senescence, axon guidance, Cushing syndrome and endocrine resistance (adjusted *p*-value<0.05) (Figure 3).

PPI Network Construction and Hub Genes Identification

To indicate the interrelationships among the 132 DEmRNAs, the STRING database was utilized to construct the PPI network. In this network, the interaction score was set >0.7, and the disconnected nodes in the network were deleted. To identify the top 5 hub genes in the PPI network, cytoHubba was used to evaluate the degree of each gene and to select the top 5: the red color represented the top gene EZH2, followed by the hub genes E2F1 and JUN, both in orange, and PPARG and CCND1, both in yellow (Figure 4A). Figure 4B shows the specific score of the hub genes.

Reconstruction of the LncRNA-miRNA-Hub Genes' Network

Based on the hub genes, the lncRNA-miRNA-hub genes' subnetwork was reconstructed, which contained 12 subnetwork relations (LINC00261-has-miR-139-5p-JUN, LINC00284-has-miR-17-5p-E2F1, LINC00284-has-miR-17-5p-CCND1, DIO3OS-has-miR-139-5p-JUN, DIO3OS-has-miR-217-EZH2, CASC2-has-miR-17-5p- E2F1, CASC2-has-miR-17-5p-CCND1, PCBP1-AS1-has-miR-17-5p- E2F1, PCBP1-AS1-

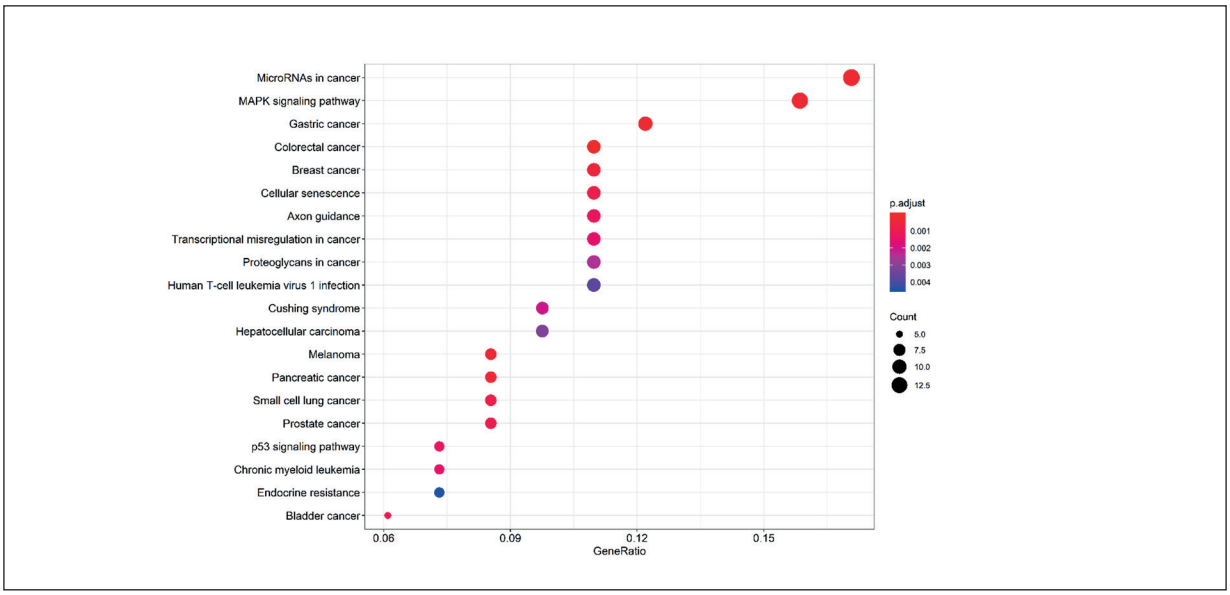

Figure 3. The KEGG pathway analysis of DEmRNAs.

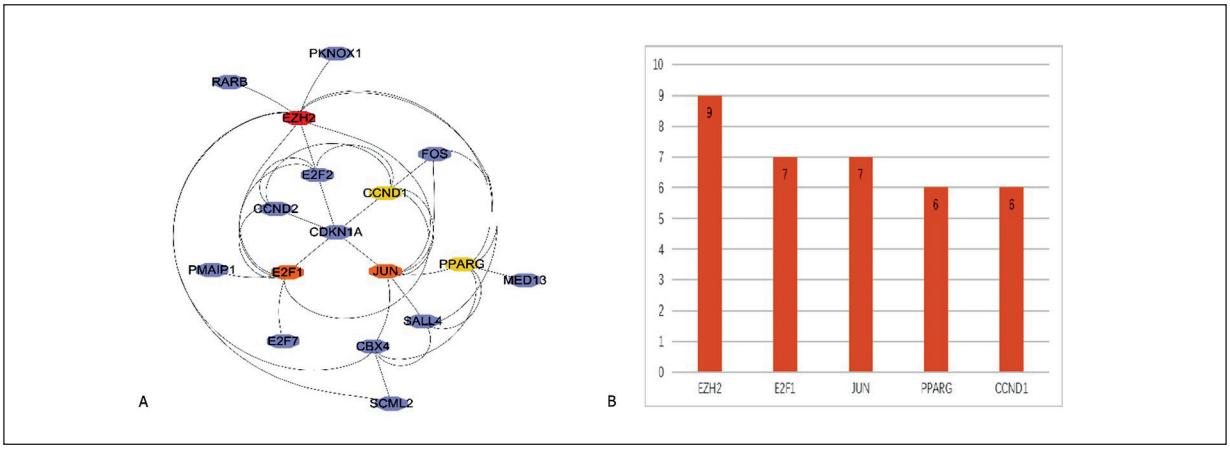

Figure 4. PPI network of DEmRNAs and hub genes. PPI network of DEmRNAs. Blue nodes represent the interaction among DEmRNAs, red, orange and yellow nodes represent the hub genes (**A**). The individual score of 5 hub genes were represented (**B**).

has-miR-17-5p-CCND1, PCBP1-AS1-has-miR-139-5p-JUN, PCBP1-AS1-has-miR-217-EZH2, SLC25A5-AS1-has-miR-139-5p-JUN) (Figure 5).

Identifying Prognostic Hub Genes in Patients

The top 5 hub genes of the lncRNA-miRNA-mRNA network were analyzed by the Kaplan-Meier method. The outcome determined that high expression of PPARG was associated with a poor OS (HR=3.14, 95% CI:1.18-8.39, p=0.0016) (Figure 6A), and the low expression of E2F1 was associated with a poor OS (HR=0.15, 95% CI:0.05-0.47, p=0.00015), as was CCND1 (HR=0.26, 95% CI: 0.1-0.71, p=0.0046) (Figure 6B and Figure 6C). However, no prognostic value for EZH2 and JUN was found in the OS (Figure 6D and Figure 6E). Furthermore, the PTC expression of significant genes (PPARG, E2F1 and CCND1) was also verified through immunohistochemical analysis via the Human Protein Atlas database. EZH2 and CCND1 were not detected in normal thyroid tissue, but EZH2 was expressed at low or moderate levels in PTC tissues, while CCND1 was expressed at low or high levels in PTC tissues. The JUN and E2F1 genes were discovered to be expressed at a higher level in normal tissues, but in PTC tissues, JUN's expression was not detected, either at low or medium levels. E2F1 was expressed at a low level in PTC tissues. PPARG was expressed at a medium level in normal tissues and at a high level in PTC tissues (Figure 7).

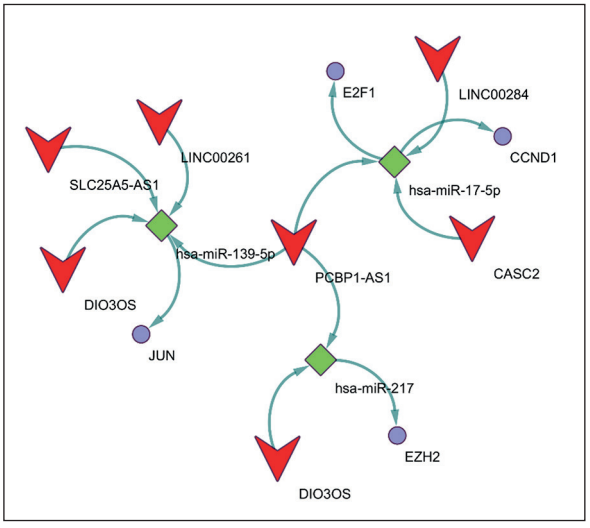

Figure 5. The lncRNA-miRNA-hub gene subceRNA network. Red nodes represent lncRNAs. Green nodes represent miRNAs. Blue nodes represent hub genes

Discussion

LncRNA plays a prominent role in the normal physiological and pathological processes of cells[26]. Up to now, tens of thousands of lncRNAs have been found, which are closely related to the occurrence and development of malignant tumors[27]. Mutations in non-coding portions of the genomes are the main reason for disease, and changes in the expression of some transcripts may lead to significant changes in cell processes, causing disease[28]. At present, it is universally acknowledged that lncRNA is a specific cell-

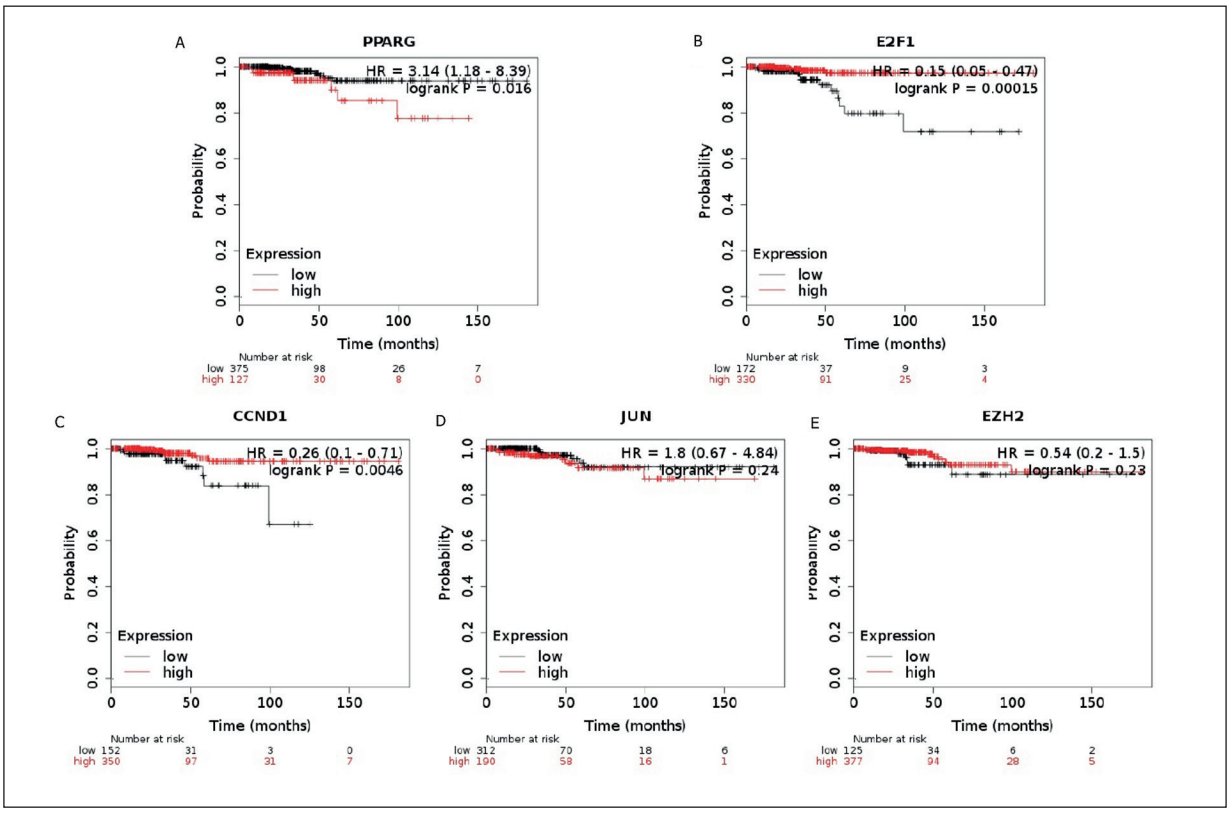

Figure 6. Overall survival analysis of the hub genes. PPARG (**A**). E2F1 (**B**). CCND1 (**C**). JUN (**D**). EZH2 (**E**).

state signal, which is differentially expressed in normal and cancerous tissues through inhibiting growth inhibitory factors and inducing angiogenesis around tumor cells[29,30]. Based on these theoretical principles, the lncRNA-related ceRNA regulation network exerts an important role in the progression of tumors[31,32]. The studies on the lncRNA-related ceRNA network in PTC have not been sufficient to date, and their expression pattern and mechanism in PTC need to be further explored. In this research, we downloaded 5 pairs of PTC tissues from a public database to identify the DElncRNAs, DEmiRNAs and DEmRNAs between tumor tissues and nontumor tissues and

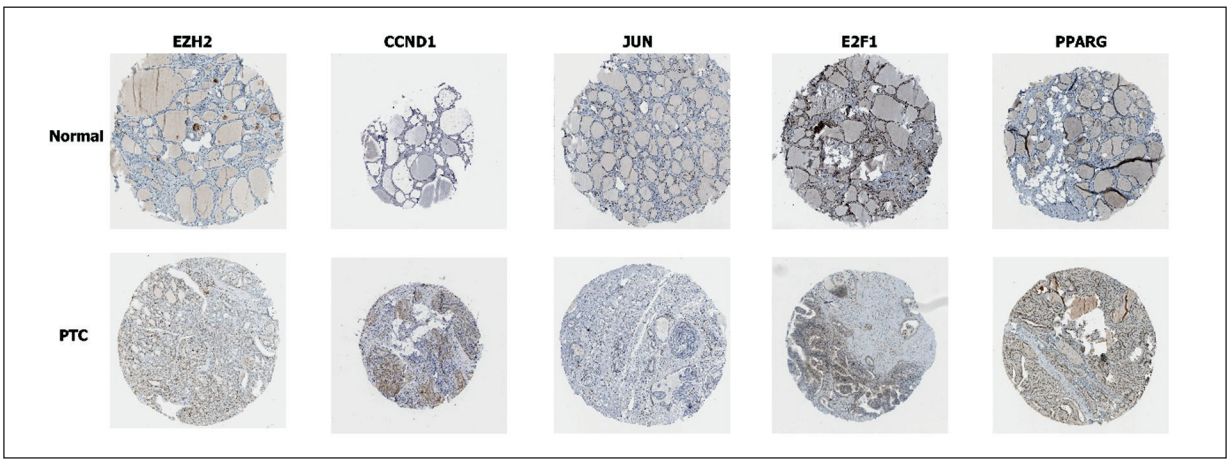

Figure 7. Validation of thyroid expression of hub genes in the human protein atlas database.

construct a lncRNA-miRNA-mRNA regulatory network.

LncRNAs display a dysregulated expression in PTC and participate in the disease pathogenesis and prognosis[33-35]. LINC00284 was highly expressed in gastric cancer and ovarian cancer[36,37]. Moreover, Zhao et al[38] showed that LINC00284 may have a close relationship with miR-205, and its dysregulation was positively correlated with the overall survival of PTC patients. Similarly, our study also found that LINC00284 was a potential biomarker in PTC. Cancer susceptibility candidate 2 (CASC2) is a newly discovered lncRNA that was first discovered in 2004[39]. CASC2 played a crucial role in various tumor tissues such as lung, colon-rectum, kidney, stomach, osteosarcoma, and gliomas[40-44]. Similar to our research, Huang et al[45] determined that CASC2 was one of the regulators of PTC cancer. They discovered that the expression level of CASC2 was significantly decreased in PTC when compared with normal tissues. Furthermore, Zhou et al[46] showed that when the CASC2 was overexpressed, cell proliferation, migration and invasion of PTC would be inhibited. In addition, we found another 4 lncRNAs, LINC00261, DIO3OS, PCBP1-AS1 and SLC25A5-AS1, that are in the ceRNA network; however, LINC00261 was mainly related to colon cancer, lung cancer and endometrial cancer[47-49]. DIO3OS was discovered to interact with miR-122 to promote the proliferation and invasion of pancreatic cancer cells by upregulating ALDOA[50]. In addition, PCBP1-AS1 showed a distinctive differential expression in oral squamous cell carcinoma compared with healthy oral mucosa[51], and Luan et al[52] found that PCBP1-AS1, with prognostic value, was identified in glioma. Finally, SLC25A5-AS1 was considered as a biomarker in clear cell renal cell carcinoma and gastric cancer[53,54]. Although these lncRNAs were first reported in our study to be a potential biomarker in PTC, the relevant evidence is still not sufficient, and in-depth studies need to be carried out in the future.

MicroRNA (microRNA, miRNA) is a single strand of RNA composed of 18-22 nucleotides. It regulates the expression of the target gene by binding with the non-transcript region of the target gene and then affects the biochemical processes and signal transduction pathways of the cell, causing the occurrence and development of tumor[55]. In our work, we found that miR-17-5p, miR-217 and miR-139-5p play significant roles in the regulation of PTC. MiR-17-5p, which belongs to the family of miR-17-92, is located on chromosome 13q31-32 and takes part in cell proliferation, differentiation and disease development[56]. Takakura et al[57] indicated that miR-17-5p was overexpressed in anaplastic thyroid carcinoma compared with normal tissue[57]. In contrast, the Liu et al[58] team supposed that miR-17-5p was notably downregulated in thyroid cancer tissues and cell lines[58]. These different conclusions may result from the dual characteristic of miR-17-5p, but its function in the tumor cannot be ignored. miR-217 is located in the human chromosome 2q16.1 region and is one of the member of the miR-216s cluster. Similar to the function of miR-17-5p, the expression level of miR-217 is different in different types of tumors. It has been stated that miR-217 is at a low level of expression in hepatocellular carcinoma[59], colorectal cancer[60], ovarian cancer[61], osteosarcoma[62], esophageal adenocarcinoma[63] and pancreatic ductal adenocarcinoma[64]; on the other hand, miR-217 was overexpressed in breast cancer[65], cutaneous squamous cell carcinoma[66], and glioblastoma[67]. In the TC field, the role of miR-217 is still being researched. Lin et al[68] demonstrated that miR-217 not only acted as a tumor suppressor, which was downregulated in the tumor tissue, but was associated with the clinical stage and lymph node metastasis. Finally, in our study, we also found miR-139-5p had a tight connection with tumor regulation. MiR1395p is a positive factor in the process of tumorigenesis and has been considered a potential biomarker in various tumor types[69-71]. Furthermore, the Cancer Genome Atlas Research Network[72] reported that miR-139-5p was downregulated in thyroid cancer. Cai et al[73] studied PTC cells and discovered that miR-139-5p was overexpressed when the cells were transfected with a miR-139-5p mimic and was upregulated compared with the control groups.

In the present study, GO and KEGG enrichment function analyses were carried out as well. The results indicated that most of the DEmRNAs' functions were focused on signal transduction and tumor-related signal pathways. Five hub genes were screened, and the PPI network consisting of these genes was constructed. Moreover, in the subceRNA network, 4 hub genes (EZH2, E2F1, JUN and CCND1) were at the center. In past research, enhancer of zeste homolog 2 (EZH2) was related to a poor survival and was expressed in poorly differentiated and anaplastic thyroid cancers[74]. This may because EZH2 is a key methyltransferase

and is expressed abnormally in various cancers, including thyroid cancer[75,76]. E2F1 is one factor in the transcription factor E2F family, which is related to the cell cycle[77]. Zhang et al[78] reported that the transcription level of lncRNA RGMB-AS1 could be enhanced by E2F1 in PTC. Cyclin D1 (CCND1), a recognized proto-oncogene that was discovered early on, is one of the members of the cyclin family. By combining with cyclin dependent kinase, CCND1 can promote the transformation of cells from the G1 phase to the S phase and complete the regulation of cell cycle[79]. Bièche et al[80] revealed that the overexpression of the CCND1 gene is related to thyroid cancers. In the present study, we downloaded the tissue immunohistochemical results of normal and PTC tissues from the HPA database and found that CCND1 was not detected in normal tissue but was expressed abnormally in PTC patients. Li et al[81] indicated that CCND1 was also one of hub genes in the PPI network of PTC, which an RT-PCR experiment has verified. In addition, JUN was also identified as a key gene in the PTC regulation network in their research[81]. Similar to CCND1, JUN is also a proto-oncogene[82] and related to multiple tumors[83-85]. Chen et al[86] reported that JUN could be a potential biomarker in the prognosis of PTC. In addition, JUN was discovered to have a close connection with microRNAs and lncRNAs, but in KM analysis, no prognostic value of JUN was found in OS, and thus further research is needed. With the development of bioinformatics, the study of the ceRNA network in PTC has gained more attention.

In our study, there are still limitations. First, because this study involved data mining, the results were limited by the number of cases and research methods. Second, as there is no experimental verification at this stage, the results can only play a predictive role, but our further studies will continue to identify these results in *in vivo* and *in vitro* experiments.

Conclusions

In summary, through the GEO database, we performed expression profiling of lncRNAs, miRNAs, and mRNAs between normal adjacent tissues and cancer tissues of PTC. In addition, a ceRNA network was constructed to reveal the potential mechanisms of PTC, which could provide novel targets for the treatment of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Data Availability

The dataset used and/or analyzed during the current study is available from GEO database.

Compliance with Ethical Standards

For GEO database is an open public website, additional approval was not required.

Authors' Contribution

Ning Xia conceived and designed the experiments; Yue Sun performed the experiments, wrote the manuscript; Weiran Dai analyzed the data, reviewed drafts of the paper.

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