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Expression profile of long noncoding RNAs in human papillary thyroid carcinoma

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Long non-coding RNAs (lncRNAs) are strongly associated with cancer biology. The objective of this study is to investigate the expression profile of lncRNAs in human papillary thyroid carcinoma (PTC), and to understand the biological role of lncRNAs and their involvement in PTC oncogenesis. The lncRNAs and messenger RNAs (mRNAs) expression in human PTC and paired adjacent non-cancerous thyroid (NCT) tissues were studied by micro-array, and quantitative real-time polymerase chain reaction (qRT-PCR) validated five differentially expressed lncRNAs. We identified 2,925 significantly differentially expressed lncRNAs with potential roles in PTC, (absolute fold change >2.0; $p<0.05$). Of these, 1,922 were up-regulated and 933 down-regulated and the qRT-PCR results agreed with micro-array results. Gene ontology (GO) enrichment and pathway analysis then investigated gene function and identified significantly enriched pathways in differentially expressed mRNA's. Many of these pathways were related to cancer, including 60 genes associated with "pathways in cancer" and 34 linked to "proteoglycans in cancer". Co-expression network and target prediction analysis of lncRNAs revealed that TCONS_00020457 can have important roles in PTC. In conclusion, the results of this study indicate that lncRNAs can be important regulators in PTC tumorigenesis and provide understanding of the function and mechanism of lncRNAs related to human papillary thyroid carcinoma.

Key words: Papillary thyroid carcinoma, long noncoding RNAs, mRNA, micro-array

Thyroid cancer (TC) is the most frequent endocrine cancer and papillary thyroid carcinoma (PTC) is the prevalent histotype. It accounts for 85–95% of all thyroid malignancies and its incidence has steadily increased over the past decades [1–3]. Despite advanced surgical techniques, almost 5% of PTC patients develop recurrence within five years of initial treatment [1]. Understanding the proliferation, invasion and apoptotic molecular mechanisms in PTC development is paramount in developing therapeutic strategies against PTC recurrence.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that are not translated into protein [4] . LncRNAs have negative or positive roles in regulating gene expression in developmental and differentiation processes. In contrast to microRNAs (miRNAs), lncRNAs can provide greater potential and versatility for recognition of both proteins and targets [5]. They regulate many biological processes, including epigenetic regulation, genomic imprinting, chromosome inactivation and cell differentiation at transcriptional, post-transcriptional and translational levels [6, 7]. Deregulation of lncRNAs in tumors has been suggested in several studies [8, 9], and others have recently recorded lncRNA expression profiles in specific cancers [10–13].

However, studies on lncRNA expression related to PTC development mechanisms are rare, and this study therefore examines the biological role of lncRNAs and their involvement in the oncogenesis of PTC by micro-array analysis of differential lncRNA and mRNA expression in human PTC tissues and paired adjacent non-cancerous thyroid tissue.

Patients and methods

Patients and tissue samples. Samples of human papillary thyroid carcinoma (PTC) and corresponding non-cancerous thyroid tissue (NCT) were obtained from 15 patients at Xiangya Hospital of the Central South University and Hunan Province Tumor Hospital (Changsha, Hunan, China)

between August 2014 and March 2015, and immediately snap-frozen in liquid nitrogen following tissue resection. Five pairs of these samples were used to perform lncRNA micro-array analysis and the remainder provided additional analysis. Table 1 presents the patient characteristics for collected tissues. The ethical approval for our protocol was granted by the Ethics Committee of Xiangya School of Medicine, Central South University (registration number: CTXY-110008-3) and written informed consent was obtained from all patients.

RNA extraction. Total RNA was extracted as in Wang et al. [14] using Trizol reagent (Invitrogen; Thermo Ficher Scientific, Inc., Waltham, MA, USA). The quantity of total RNA was measured by NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the standard used was A_{260}/A_{280} ranging from 1.8 to 2.0. Agarose gel electrophoresis then detected extracted DNA quality.

Micro-array analysis. lncRNA+mRNA micro-array analysis was performed according to Wang et al. [14]. Briefly, double-stranded complementary DNA (cDNA) was synthesized, labeled and hybridized to lncRNA+mRNA Human Gene Expression Micro-array V4.0 (CapitalBio Corp, Beijing, China). GeneSpring software V11.5 (Agilent) was used to summarize, normalize and control the quality of the lncRNA+mRNA micro-array data. Differentially expressed genes were selected based on threshold values (>2.0 or <−2.0 folds) and Benjamini-Hochberg corrected p-values (<0.05). The data was then normalized and hierarchically clustered with CLUSTER 3.0 software. Finally, the Java TreeView software achieved tree visualization (Stanford University School of Medicine, Stanford, CA, USA).

Gene ontology (GO) and pathway analysis. GO and pathway analysis were performed as in Wang et al. [14]. GO analysis was obtained from Gene Ontology (www.geneontology.org) which includes the following three domains: a cellular component, biological process and molecular function. The significance of the GO term enrichment in the differentially expressed mRNA list was defined at $p<0.05$. The latest Kyoto Encyclopedia of Genes and Genomes (KEGG) software (www.genome.jp/kegg/) then determined the biological pathways with significant enrichment of differentially expressed mRNAs ($p<0.05$).

Construction of gene co-expression network. The gene co-expression network followed Wang et al. based on correlation analysis of differentially expressed lncRNAs and mRNAs. LncRNAs and mRNAs with Pearson correlation coefficients greater than 0.99 visualized the molecular interaction network [14].

Reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR). Micro-array data results were validated by qRT-PCR by SYBR Green qRT-PCR Kit according to the manufacturer's recommendations of ABI Prism 7000 Sequence Detection System (Applied BiosystemsInc, Foster City, CA, USA). The total RNA extraction method was as previously described, and then reversed to cDNA following qRT-PCR Kit procedures (Applied Biosystems). Primer sequences used for qRT-PCR are listed in Table 2. The protocol used for the PCR reactions was as follows: holding at 95 °C for 10 min followed by 40 cycles of preliminary denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 15 s. All the reactions were conducted in triplicate for each sample. The qRT-PCR reaction was assessed by melting curve analysis and agarose gel electrophoresis, and calculations for determining relative gene expression levels were according to Meiliu Yang et al. [3].

Statistical analysis. A minimum of three replicates were performed for all assays, with quantitative data presented as the mean ± standard deviation (SD). Gene expression levels were compared between PTC and NCT tissue samples using the Student's t-test, with $p<0.05$ which represented a statistically significant difference.

Table 1. Clinical and phenotypical characteristics of 5 PTC patients analyzed in lncRNA microarray.

Patient No	Sex	Age at diagnosis	Tumor size (cm)	Lymph node involvement
1	Female	44	2.5	Yes
2	Female	49	2.3	Yes
3	Female	42	2	Yes
4	Male	37	3	Yes
5	Male	36	2	Yes

Table 2. The sequences of forward and reverse primers for qRT-PCR.

Gene name	Primer sequence 5'–3'
p2196 - Forward	TGCAGCAAGATAGCTCCAGG
p2196 - Reverse	CCTGGCGTCTGTGTAGTGTT
p4438 - Forward	GCCTGTGAGTGAAGACTGTTG
p4438 - Reverse	GTTCCCTCTCAGTGCAGTCT
p10201 - Forward	CCGATCCCCCTTAAGAAGCG
p10201 - Reverse	AGCTTTTCGCAGTCTCTGCT
p2131 - Forward	GCACTGTCTACACCTGGGAG
p2131 - Reverse	GAGCTCAGAGGCAATCGTCA
p17007 - Forward	TTCAGGAGGGGAAATTGAGCC
p17007 - Reverse	GGTCTTACTCCCAAAACATGTCA
GAPDH - Forward	TGTTGCCATCAATGACCCCTT
GAPDH - Reverse	CTCCACGACGTACTCAGCG

Results

Identification of distinctive lncRNA expressions between PTC and matched NCT tissues. The locus-by-locus lncRNA probes of PTC vs NCT tissues were analyzed. Based on the criteria of corrected p-value <0.05 and absolute fold change (FC)>2, we identified 1,922 up-regulated and 933 down-regulated lncRNAs significantly and differentially expressed in PTC tissues compared to NCT tissues (Figure 1a). The top 10 up-regulated and down-regulated lncRNAs are listed in Table 3. The differentially expressed lncRNAs were then

grouped on their expression levels using hierarchical clustering analysis (Figure 1b). When the cut-off was set at 5-fold, 367 lncRNAs were up-regulated and 175 were down-regulated ($p<0.05$). ENSG00000244125.1 (FC=187.12) was the most up-regulated lncRNA and ENSG00000230257.1 (FC=117.88) the most down-regulated.

GO and pathway analysis of differentially expressed genes. Micro-array analysis revealed that 2,745 mRNAs were significantly differentially expressed in PTC samples compared to NCT samples ($FC \geq 2.0$; $p<0.05$). The highest enriched GOs were; extracellular matrix (cellular component), extracellular matrix organization (biological process) and oxygen transporter activity (molecular function) (Figure 2a). KEGG pathway analysis revealed the most significant pathways comprised "cytokine-cytokine receptor interaction," "cell adhesion molecules," "pathways in cancer" and "proteoglycans in cancer" (Figure 2b).

Validation of microarray data by qRT-PCR. Micro-array results were validated by qRT-PCR with a random selection of five differentially expressed lncRNAs. Figure 3 shows the qRT-PCR-determined quantitative estimates of the expression of the five lncRNAs were close to the mRNA micro-array analysis.

LncRNA classification. The association between lncRNAs and mRNAs was analyzed to identify putative functional relationships. The relationship between lncRNAs and mRNAs was categorized as antisense, sense, divergent, intronic or intergenic. In differentially expressed lncRNAs, there were 463 antisense lncRNAs, 556 sense, 207 divergent, 297 intronic and 1,332 intergenic.

Construction of the lncRNA-mRNA co-expression network. The lncRNA-mRNA co-expression network established correlation between differentially expressed lncRNA and mRNA. The results showed that one lncRNA is correlated with one-to-tens of lncRNAs and mRNAs,

Table 3. Top 10 differentially expressed lncRNAs in PTC compared to NCT.

Gene name	Fold change	p-value
Up-regulatedlncRNAs		
ENSG00000244125.1	187.12	0.00014
XLOC_006163	133.68	0.0044
XLOC_001313	95.71	0.0000068
ENSG00000257973.1	76.86	0.00016
ENSG00000261185.1	74.79	0.00059
ENSG00000223838.1	67.54	0.0029
ENSG00000235070.3	55.08	0.00055
ENSG00000226005.2	47.01	0.0011
LOC646736	44.73	0.00078
XLOC_012852	44.12	0.0039
Down-regulatedlncRNAs		
ENSG00000230257.1	117.89	0.000069
HIX0036730	47.89	0.00072
ENSG00000237463.1	47.28	0.0095
ENSG00000250748.2	48.19	0.00011
ENSG00000259264.1	32.58	0.00092
ENSG00000256268.1	32.57	0.00036
ENSG00000188424.5	31.12	0.0025
LOC100507175	29.87	0.0013
ENSG00000223914.1	27.58	0.0011
ENSG00000215498.4	26.25	0.0087

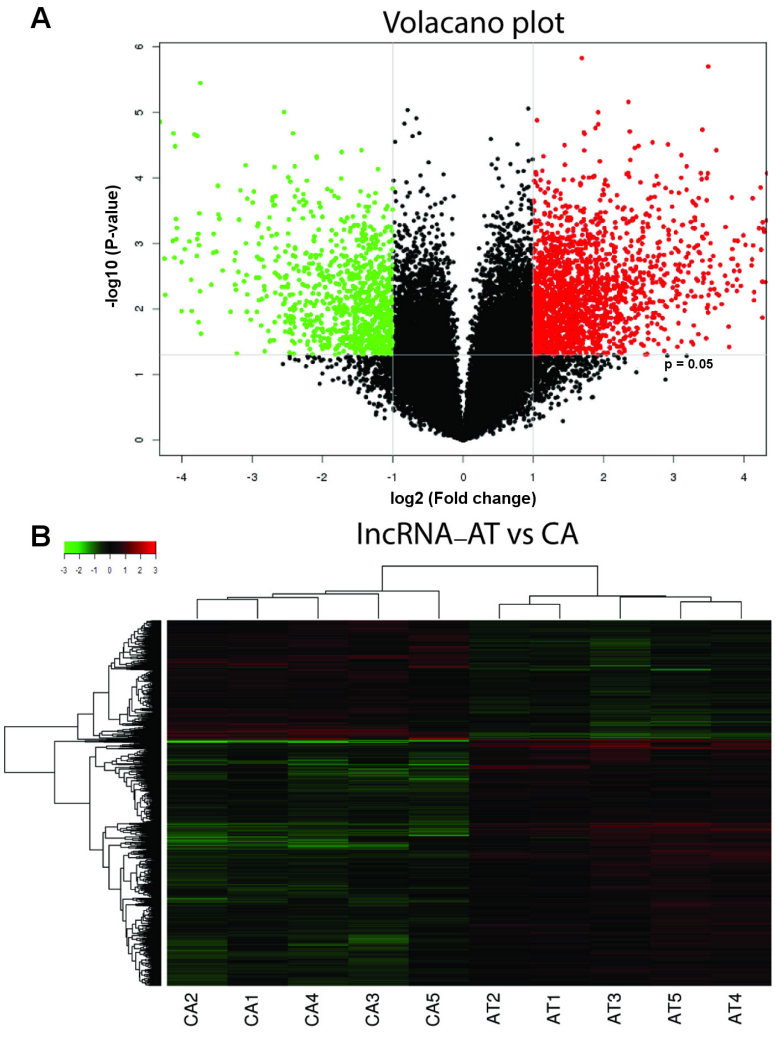

Figure 1. Differentially expressed genes compared PTC with NCT. A) volcano plot of differentially expressed lncRNAs between PTC and NCT. The vertical lines correspond to 2.0-fold up and down and the horizontal line represents a P value of 0.05. B) hierarchical clustering analysis of differentially expressed lncRNAs. Red and green colors indicate high and low expression, respectively. In the heat map, columns represent samples and rows represent each lncRNA.

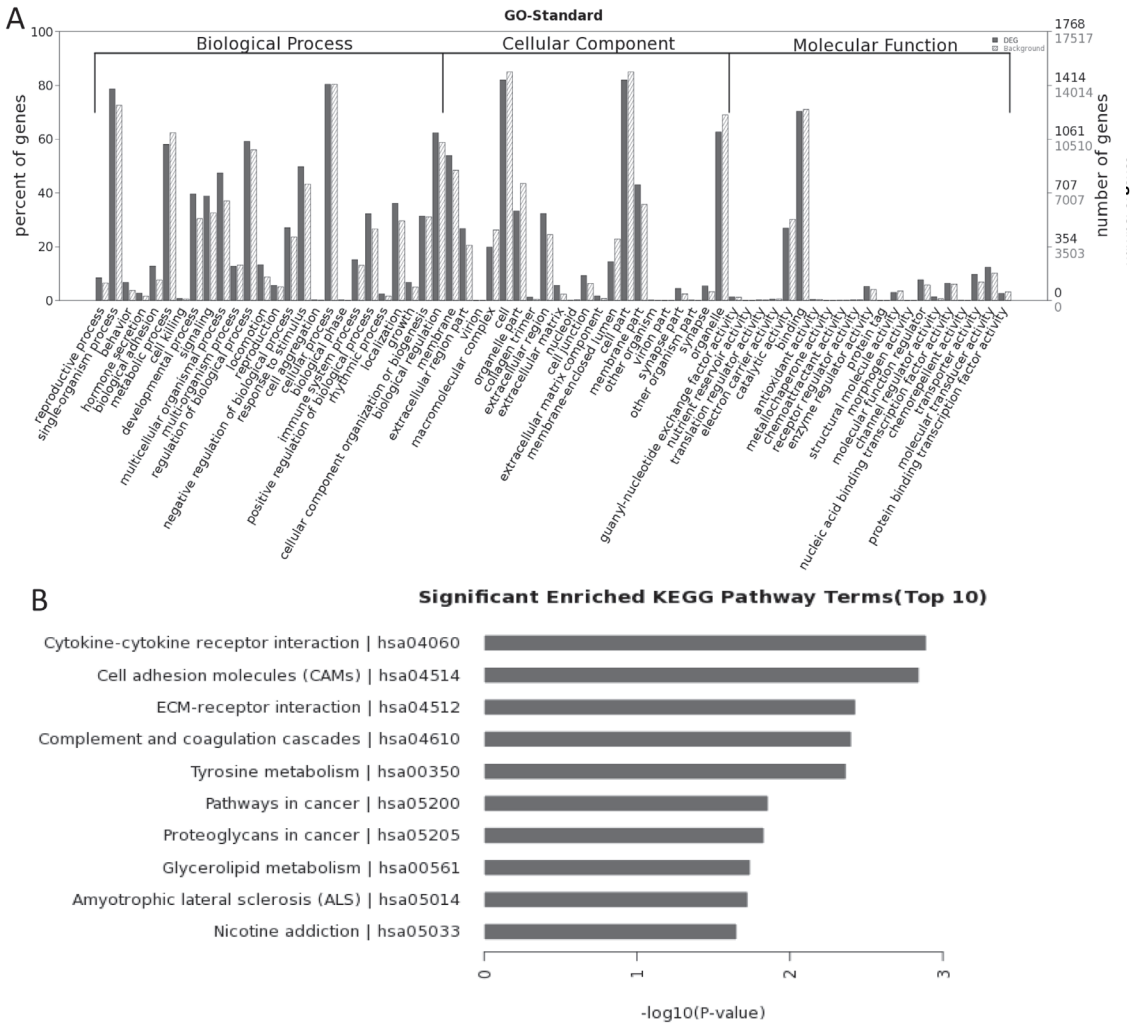

Figure 2. GO and pathway analysis of mRNAs differentially expressed between PTC and NCT. A) GO analysis covers the three domains of biological process, cellular component and molecular function. The abscissa – classification of the database. The ordinates – the gene number and percentage B) the vertical axis represents the pathway category and the horizontal axis represents the enrichment score (–log10 (p-value)) of the pathway.

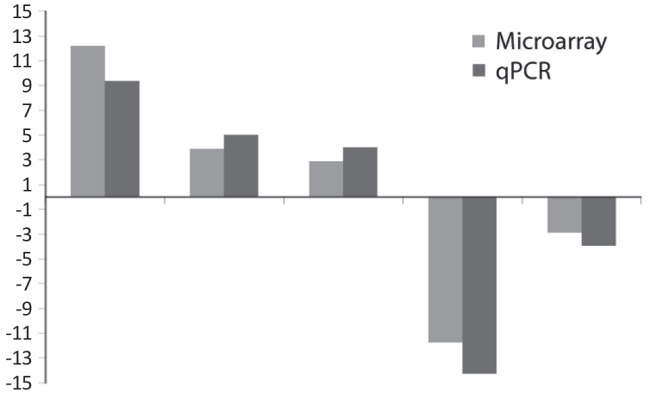

Figure 3. qRT-PCR test for the differentially expressed genes. qRT-PCR was used to confirm the mRNA levels of five selected genes detected as differentially expressed via RNA microarray analysis. The GAPDH gene was used as an internal control.

thus suggesting that significant correlation exists between the expression profile of lncRNAs and mRNAs. A dynamic expression trend of one lncRNA (ENSG00000242808.2) and one mRNA (SLC5A8) was identified between PCT and NCT tissue samples (Figure 4).

Discussion

Papillary thyroid carcinoma (PTC) is the most common thyroid cancer, constituting over 70% of thyroid malignancies [10]. However, the current primary focus centers on PTC protein-coding gene mutations. For example, the three genetic alterations in PTC that are best known are activating mutations in RAS, ligand independent activation of the chimeric RET/PTC genes and mutations in BRAF [15–17]. Recent advances in molecular techniques have resulted in identifying novel lncRNA gene regulators. LncRNAs interact

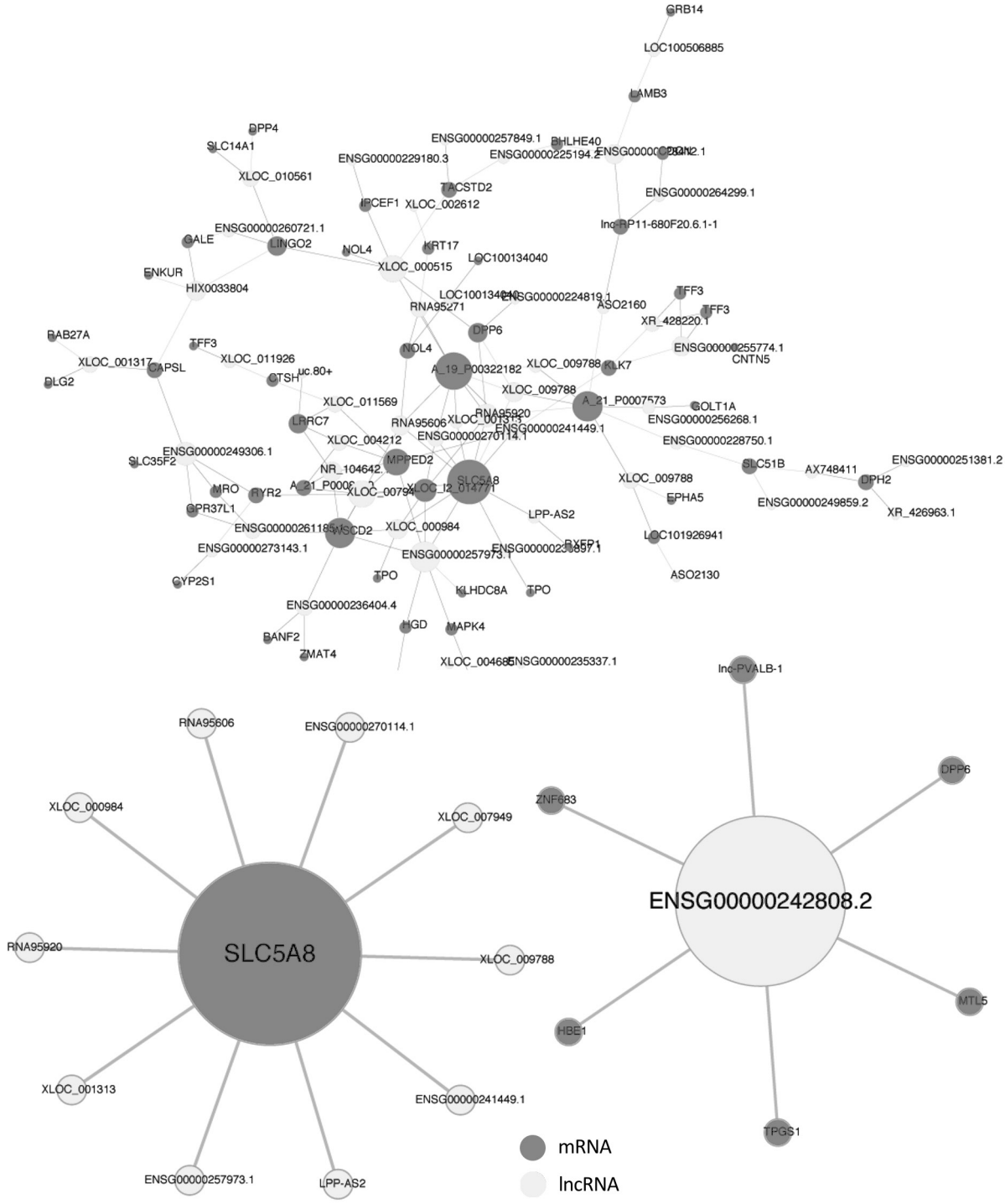

Figure 4. Co-expression network for differentially expressed lncRNAs and mRNAs.

with miRNA or proteins and can potentially regulate other genes [18, 19]. Despite not being as well-characterized as miRNAs, lncRNAs demonstrate a vital role in regulating diverse cellular processes such as cell growth, development and apoptosis [18].

Herein, we demonstrate global expression profiles of lncRNA and mRNA between human PTC and NCT tissue samples using micro-array. We identified that many lncRNAs and mRNAs are significantly differentially expressed in PTC and NCT tissues. Subsequently, we validated the micro-array

results by qRT-PCR. Previous study also demonstrated that 675 lncRNAs were abnormally expressed in the three PTC samples compared to adjacent non-cancerous samples, thus implying that lncRNAs have a crucial role in PTC and could be new molecular targets in its treatment [3].

lncRNAs associated with PTC [20–22] include oncogenic papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3) [22, 23]. The qRT-PCR experiments show that PTCSC3 expression is strongly down-regulated in PTC tissues, and the restoration of PTCSC3 expression in PTC cells inhibits cell growth and affects the expression of numerous genes [23]. The expression level of PTCSC3 in our micro-array study is consistent with the literature [20], with a FC of 3.87 (up-regulated) in NCT compared to PTC tissue samples.

In our study, we implemented bioinformatic analysis to further investigate these differentially expressed lncRNAs and mRNAs. GO and KEGG pathway analyses determined that these genes are involved in a variety of diverse biological processes, cellular complexes and molecular functions. KEGG analysis identified numerous pathways related to TC cancer, and "Pathways in cancer" and "proteoglycans in cancer" were significantly enriched in the top 10 pathways. These are related to 60 and 34 differentially expressed target genes, respectively. While Lan et al's genome-wide screening and lncRNA expression profile analysis recorded that thousands of lncRNAs and mRNAs are differentially expressed in sixty-two PTC tissues compared to adjacent non-cancerous thyroid tissue, the interaction network between mRNA and lncRNA requires further study [24]. Herein, we identified 2,925 significantly differentially expressed PCT lncRNAs, and our differentially expressed lncRNA and mRNA co-expression network analysis revealed a dynamic expression trend of one mRNA (SLC5A8) and one lncRNA (ENSG00000242808.2) in PCT and NCT tissue samples.

The SLC5A8 sodium transporter is reported as a tumor suppressor gene in many cancers; including papillary thyroid carcinomas and colon and breast cancer [25–28]. Porra et al. reported that PTC SLC5A8 expression was selectively down-regulated (40-fold), and low SLC5A8 expression level was significantly associated with BRAF T1796A mutation [28]. Our results support the expression level of SLC5A8 in previous literature [28], with a FC of 89.49 (down-regulated) in PTC compared to NCT tissue samples.

LncRNAs may also be functionally related to neighboring cis-regulating coding genes [29]. For example; (1) XISTfunctions in a *cis* manner to regulate immediate genomic neighbors which recruit chromatin modifying complexes to silence their adjacent sites [30]; (2) co-expression network analysis indicates that TCONS_00020457 is the lncRNA dynamic expression trend with SLC5A8 and (3) HMGA2 is the predicted TCONS_00020457 *cis* target gene.

Moreover, heightened expression of HMGA2 has been identified in a variety of human cancers [31, 32], and our results confirm that the expression level of HMGA2 was significantly up-regulated (26.31-fold) in PTC compared to NCT tissues. This indicates that HMGA2 could well be the target gene of TCONS_00020457 in PTC. While the functions of the differentially expressed lncRNAs and their potential regulatory relationship with PTC cis-genes require confirmation in additional investigations, our prediction of lncRNA targets provides essential information, robust potential functional lncRNA candidates and PTC target genes for future investigations.

In conclusion, we identified the inclusive differential expression profiles of lncRNAs in human PTC. Thousands of lncRNAs and mRNAs were found to be differentially expressed in PTC compared to NCT tissues. In addition, our bioinformatics prediction identified target genes which correlate with the candidate PTC lncRNAs. This enables future studies of these lncRNAs' functional analysis which can explain the regulatory mechanisms of lncRNAs in PTC. Finally, our combined results establish that lncRNAs could be critical regulators in PTC development, and our data will lay the foundation for future studies aimed to investigate the functional and mechanistic roles of lncRNAs associated with human PTC.

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