Integrated bioinformatics analysis reveals that the expression of cathepsin S is associated with lymph node metastasis and poor prognosis in papillary thyroid cancer

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Abstract. The prognosis of the majority of patients with papillary thyroid cancer (PTC) is excellent, although there are patients who experience disease recurrence and progression. The aim of the present study was to identify potential prognostic risk markers in PTC. Differentially expressed genes (DEGs), identified from four Genome Expression Omnibus cohorts were subjected to functional enrichment analyses with Gene Ontology terms and the Kyoto Encyclopedia of Genes and Genome pathways. Hub genes, filtered from cytoHubba, were validated using the The Cancer Genome Atlas (TCGA) cohort, and their associations with clinicopathological features and prognosis were analyzed. A total of 277 DEGs were identified following data preprocessing. DEGs were primarily enriched in 'small cell lung cancer', 'ECM-receptor interaction', 'pathways in cancer'and 'tyrosine metabolism'. Hub genes [*APOE*, cathepsin S (*CTSS*), insulin receptor substrate 1 *(IRS1), KIT, LGALS3, RUNX2* and *TGFBR1*] were extracted from cytoHubba. Their expression in the TCGA cohort was consistent with that in the GEO cohorts. *CTSS* (P=0.006) and *IRS1* (P=0.005) were associated with disease-free survival, as determined using the Kaplan-Meier analysis. *CTSS* was an independent risk factor for poor disease-free survival (HR, 2.649; 95% CI, 1.095-6.409; P=0.031). Patients with high expression of *CTSS* exhibited different histological types (increased tall-cell subtype and reduced follicular subtype; P<0.001), more frequent lymph node metastasis (P<0.001) and advanced tumor-node-metastasis stages (P=0.049) compared with the low-expression group. High expression of *CTSS* was independently associated with lymph node metastasis (OR, 2.015; 95% CI, 1.225-3.315; P=0.006). Therefore, *CTSS* may serve as a predictive risk marker for the progression and prognosis of PTC.

Introduction

Thyroid cancer is the most common endocrine malignancy worldwide, with the most rapidly increasing incidence (>5% per year) (1). It is the fifth most common type of cancer in women in the United States, with ~56,870 cases being newly diagnosed in 2017 and ~2,010 mortalities resulting from thyroid cancer (2). Papillary thyroid cancer (PTC) accounts for ~80% of all thyroid cancer cases (3). The majority of patients with this indolent tumor have an excellent prognosis, however >25% of patients experience disease recurrence during long-term follow-up (4). Therefore, it is important to identify the biomarkers associated with the progression and prognosis of PTC.

BRAF and *RAS* mutations and fusions involving the *RET* and *NTRK1* tyrosine kinases accelerate PTC tumorigenesis and progression by aberrantly activating the mitogen-activated protein kinase (MAPK) signaling pathway (5-8). Mutations in members of the phosphoinositide 3-kinase (PI3K) pathway, including *PTEN, PIK3CA* and *AKT1*, play a fundamental role in thyroid tumorigenesis (9,10), and the NF-κB pathway controls proliferative and anti-apoptotic signaling pathways in

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Abbreviations: CTSS, cathepsin S; PTC, papillary thyroid cancer; DEGs, differentially expressed genes; DFS, disease-free survival; LNM, lymph node metastasis; ETE, extrathyroidal extension; TNM, tumor-node-metastasis; CI, confidence interval; HR, hazard ratio; OR, odds ratio

Key words: CTSS, papillary thyroid cancer, lymph node metastasis, prognosis, bioinformatics analysis, differentially-expressed genes

thyroid cancer cells (11,12). Furthermore, the WNT-β-catenin pathway may play a particularly important role in the aggressiveness of thyroid cancer (10,13). In addition, the overexpression of microRNA-21 is associated with aggressive tall-cell histology and may play a crucial role in the pathogenesis of PTC. In addition, *TERT* promoter mutations identify a subset of aggressive and poorly differentiated PTCs (14). Despite significant research progress, the mechanisms underlying PTC tumorigenesis remain elusive. Therefore, a systemic approach is required to identify predictive biomarkers for disease progression and prognosis.

In the present study, a comprehensive bioinformatics analysis was used to identify key genes associated with PTC. A comparison of the expression profiles of PTC and normal tissues uncovered differentially expressed genes (DEGs). The key genes were extracted using cytoHubba (15) and their expression in PTC and normal tissues was compared using The Cancer Genome Atlas (TCGA) database. To determine whether their expression was correlated with a poor prognosis, a series of survival analyses were conducted. The relationships between key genes that were aberrantly expressed and clinicopathological factors, as well as tumor progression in patients with PTC, were also analyzed.

Materials and methods

Microarray data. Four gene expression profiles, GSE3467, GSE29265, GSE33630 and GSE50901, were acquired from the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). GSE3467 included 9 PTC tissue samples and 9 normal samples. GSE29265 consisted of 20 PTC tissue samples and 20 normal samples. GSE33630 comprised 49 PTC tissue samples and 45 normal samples. GSE50901 included 61 PTC tissue samples and 4 normal samples.

Screening DEGs. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) is available to analyze almost any GEO series between two sample groups under the same experimental conditions (16). This tool uses established Bioconductor R packages to analyze GEO data. In the present study, GEO2R was applied to screen for DEGs between PTC and normal tissue samples. An adjusted P-value (adj. P) <0.01 and |logFC| >1 were set as the cut-off criteria. Common DEGs from the four datasets were selected for further analyses.

Identification of key genes and predictions of function. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) online tool, with an enrichment threshold of P<0.05. GO analysis comprises biological process (BP), cellular component (CC) and molecular function (MF) terms. A DEG-associated protein-protein interaction (PPI) network was constructed using the STRING database (https://string-db.org/) (17) and was subsequently visualized using Cytoscape (18). Hub genes were filtered from the intersection of the top 55 genes of 12 topological analysis methods using the Cytoscape plugin cytoHubba.

Patients and clinicopathological data. Normalized RNA sequencing data (Illumina HiSeq) and the corresponding clinicopathological data for PTCs from the TCGA dataset were downloaded from Firebrowse (http://firebrowse.org/) and cBioPortal for Cancer Genomics (http://www.cbioportal.org/). Normalized expression levels of mRNAs were log2-transformed and used for further analyses. The expression of key genes was validated using RNA sequencing data from 501 PTC and 59 normal thyroid samples. The clinicopathological data included the following variables: sex, age at diagnosis, maximum tumor size, multifocality, extrathyroidal extension (ETE), histological subtypes, lymph node metastasis (LNM), and tumor-node-metastasis (TNM) stage.

Statistical analysis. All statistical analyses were performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 software (GraphPad, Inc., San Diego, CA, USA). An independent-sample Student's t-test was applied to compare the differential expression of DEGs between two groups. Disease-free survival (DFS), which was defined as the time from registration to detection of tumor recurrence or progression or until the last follow-up date, was calculated using Kaplan-Meier analysis followed by the log-rank test. Non-parametric receiver operating characteristic (ROC) analyses were performed to calculate the best cut-off value for hub gene expression levels predictive of LNM and DFS. The multivariable Cox proportional hazards regression model was used to determine independent predictive factors for DFS. Initial candidate variables with P<0.5 in the univariate analyses were included in further multiple logistic regression. To analyze the association between key genes and clinicopathological parameters, patients were divided into low-expression and high-expression groups according to the median expression levels of the key genes. The associations between the expression of key genes and clinicopathological characteristics were evaluated using the χ^2 test. Multivariable logistic regression was performed to identify independent predictors of LNM. Multivariate survival analysis was performed on all parameters that were revealed to be significant on univariate analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DEGs. In total, 615, 752, 1,189 and 1,025 DEGs were identified in the GSE3467, GSE29265, GSE33630 and GSE50901 gene expression profile datasets, respectively. Among them, 277 genes showed identical expression trends in the four datasets, including 160 upregulated genes (Fig. 1A) and 117 downregulated genes (Fig. 1B) in PTC tissues compared with normal tissues. A heat map demonstrated the significant differential distribution of the DEGs using data profile GSE3467 as a reference (Fig. 2).

Identification of key genes and prediction of function. A total of 140 DEGs (89 upregulated and 51 downregulated genes) were filtered into the PPI network, which comprised 140 nodes and 255 edges, using the STRING online database and were visualized using Cytoscape software (Fig. 3). Seven key

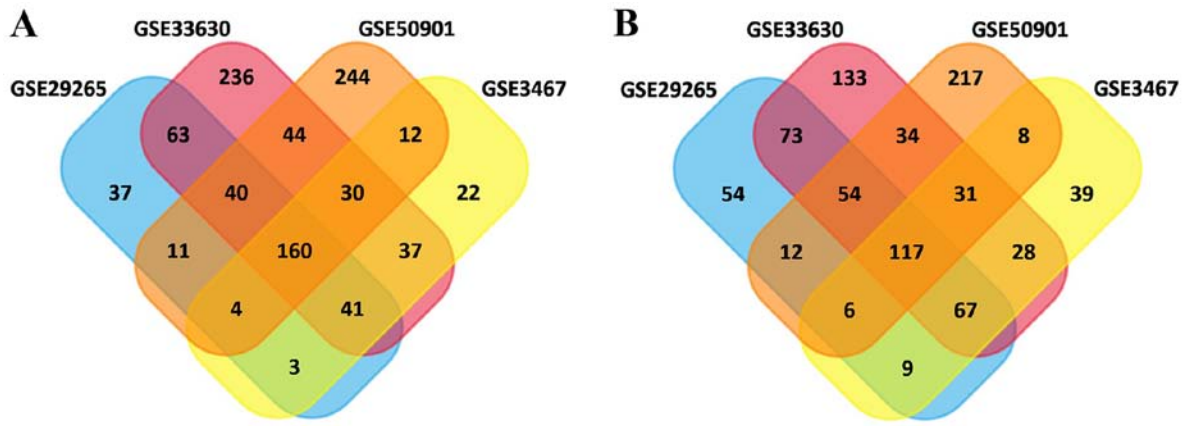

Figure 1. Identification of DEGs in mRNA expression profiling datasets GSE3467, GSE29265, GSE33630 and GSE50901. (A) The crossed areas indicated the commonly upregulated DEGs. (B) The crossed areas indicated the commonly downregulated DEGs. DEGs, differentially expressed genes.

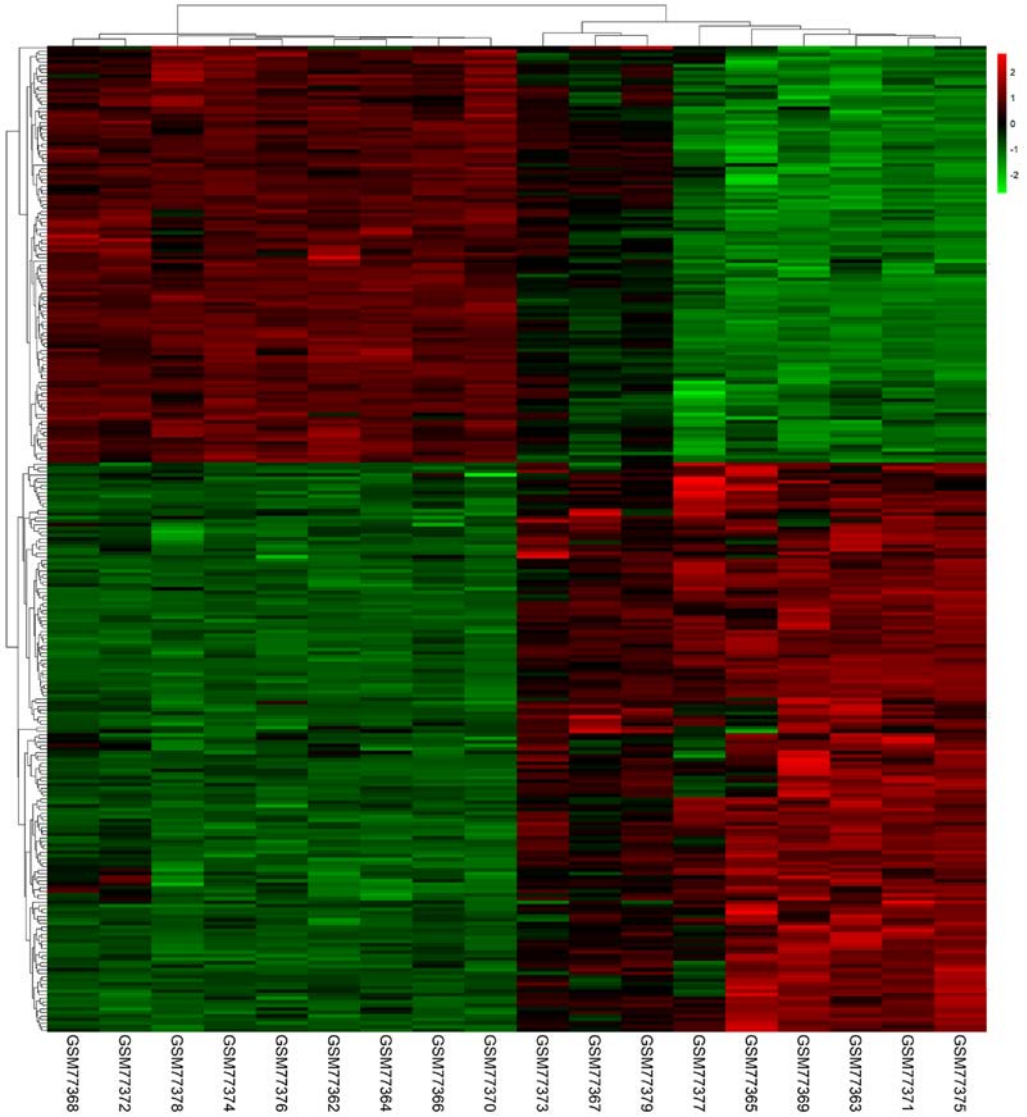

Figure 2. Heatmap plot of the 277 overlapped genes between PTC and normal samples in GSE3467 dataset. Red represents higher expression and green represents lower expression. PTC, papillary thyroid cancer; DEGs, differentially expressed genes.

genes were selected from the intersection of the top 55 genes from 12 algorithms by cytoHubba, including apolipoprotein E (*APOE*), *CTSS*, *IRS1*, KIT proto-oncogene receptor tyrosine kinase *(KIT)*, *LGALS3*, *RUNX2* and transforming growth factor-β receptor type 1 (*TGFBR1*). DEGs were subjected to functional enrichment analyses, which indicated

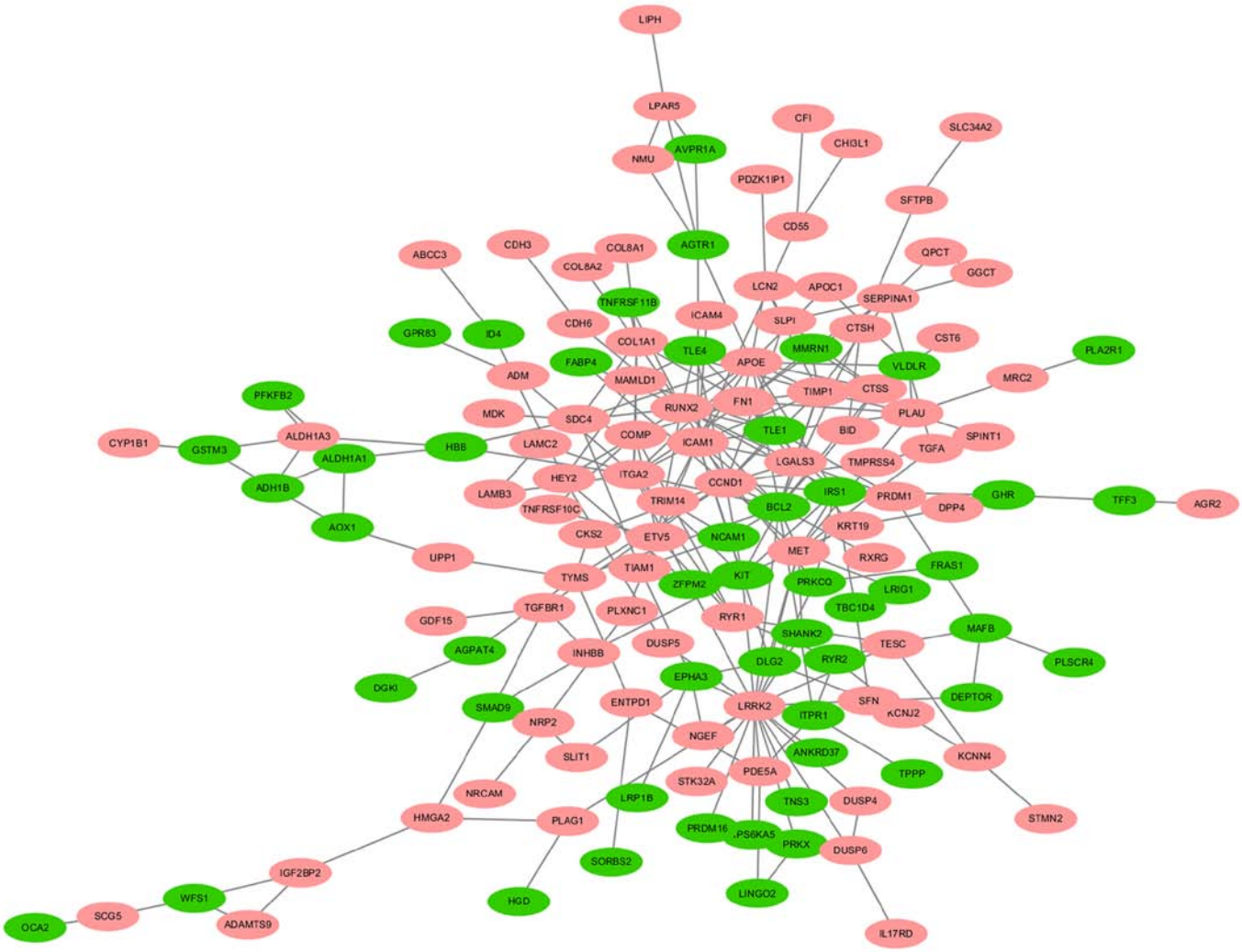

Figure 3. A protein-protein interaction (PPI) network of DEGs. Red nodes stand for upregulated genes, while green nodes stand for downregulated genes. DEGs, differentially expressed genes.

that the upregulated genes were mainly enriched in the terms 'cell adhesion', 'protease binding', 'small cell lung cancer', 'ECM-receptor interaction' and 'pathways in cancer', while the downregulated genes were mainly enriched in the terms 'signal transduction', 'protein binding' and 'tyrosine metabolism' (Fig. 4). Therefore, these DEGs may be associated with thyroid tumorigenesis.

Expression of key genes in the TCGA cohort. To determine the reliability of the identified DEGs from the four cohorts, their expression levels in 501 PTC samples and 59 normal thyroid tissues included in the TCGA database were evaluated. Consistent with the GEO analysis, 5 upregulated and 2 downregulated genes were significantly overexpressed and underexpressed respectively, in the PTC samples compared with the normal tissue samples (P<0.0001; Fig. 5).

Key genes associated with DFS. To investigate the association between the expression of key genes and DFS in patients with PTC, Kaplan-Meier survival analyses comparing the expression of the seven key genes and the DFS in the TCGA cohort were performed. From the Kaplan-Meier survival curves, *CTSS* (Fig. 6A; P=0.006) and *IRS1* (Fig. 6B; P=0.005) were observed to exhibit significant correlations with DFS. A PPI network analysis revealed that there was no direct association between IRS1 and CTSS (Fig. 7A). A significant positive correlation was observed between *CTSS* and *IRS1* mRNAs, both in PTC tissues and normal thyroid tissues (Pearson's correlation, in PTC tissues, r=-0.26, P<0.001, Fig. 7B; in normal thyroid tissues, r=-0.29, P<0.05, Fig. 7C).

Key genes independently predicting DFS. The Cox proportional hazards regression model was used to determine whether aberrant expression levels of CTSS and IRS1 may be independent risk factors for DFS in PTC. Univariate analysis revealed that the significant variables associated with DFS were increased LNM (HR, 3.242; 95% CI, 1.300-8.083; P=0.012), advanced TNM stage (HR, 2.323; 95% CI, 1.102-4.895; P=0.027), higher expression of *CTSS* (HR, 2.705; 95% CI, 1.287-5.688; P=0.009), and lower expression of IRS1 (HR, 0.360, 95% CI, 0.171-0.756; P=0.007). Multivariate analysis adjusted for sex, histological type, T stage, LNM, TNM stage, as well as the expression of *CTSS* and *IRS1* indicated that only the increased expression of *CTSS* was an independent predictive factor for DFS (HR, 2.649; 95% CI, 1.095-6.409; P=0.031). Therefore,

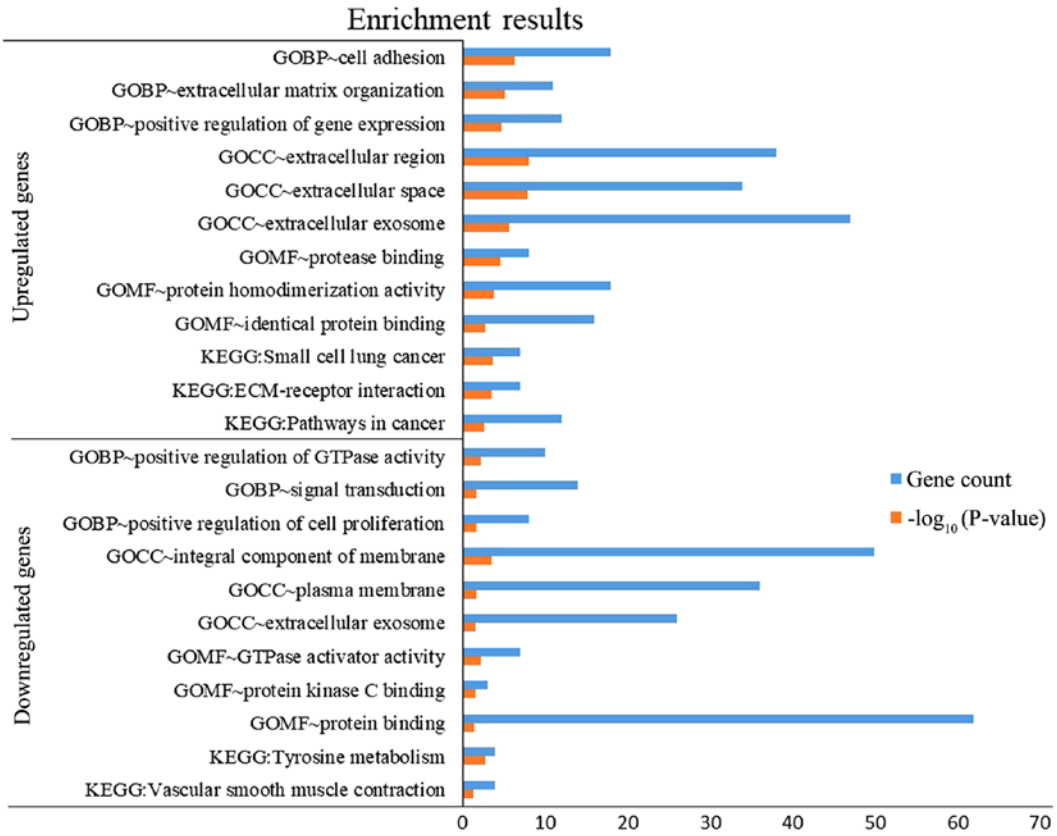

Figure 4. The cellular component (CC), biological process (BP) and molecular function (MF) terms, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched by DEGs. DEGs, differentially expressed genes.

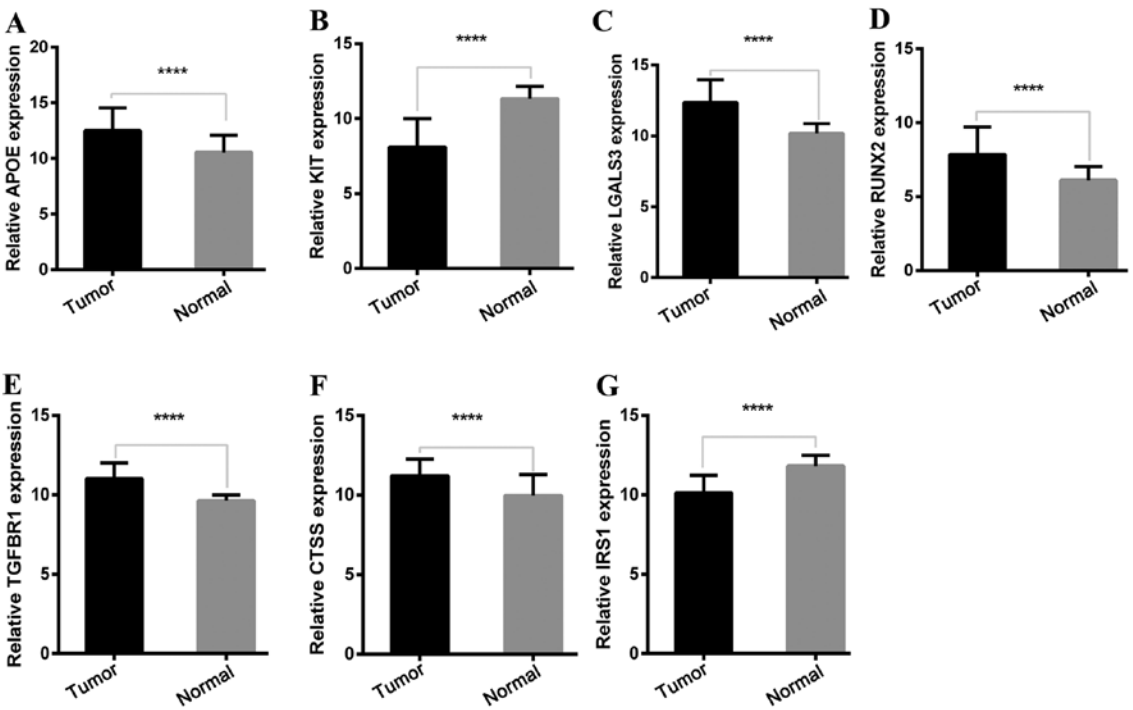

Figure 5. mRNA expression level of key genes in TCGA cohort. (A) APOE was significantly upregulated in 501 PTC samples compared with 59 normal thyroid samples. (B) KIT was significantly downregulated in 501 PTC samples compared with 59 normal thyroid samples. (C) LGALS3 was significantly upregulated in 501 PTC samples compared with 59 normal thyroid samples. (D) RUNX2 was significantly upregulated in 501 PTC samples compared with 59 normal thyroid samples. (E) TGFBR1 was significantly upregulated in 501 PTC samples compared with 59 normal thyroid samples. (F) CTSS was significantly upregulated in 501 PTC samples compared with 59 normal thyroid samples. (G) IRS1 was significantly downregulated in 501 PTC samples compared with 59 normal thyroid samples. Statistical differences were analyzed using independent t-test. ****P<0.0001. APOE, apolipoprotein E; KIT, KIT proto-oncogene receptor tyrosine kinase; LGALS3, galectin-3; RUNX2, runt-related transcription factor 2; TGFBR1, transforming growth factor-β receptor type 1; CTSS, cathepsin S; IRS1, insulin receptor substrate 1.

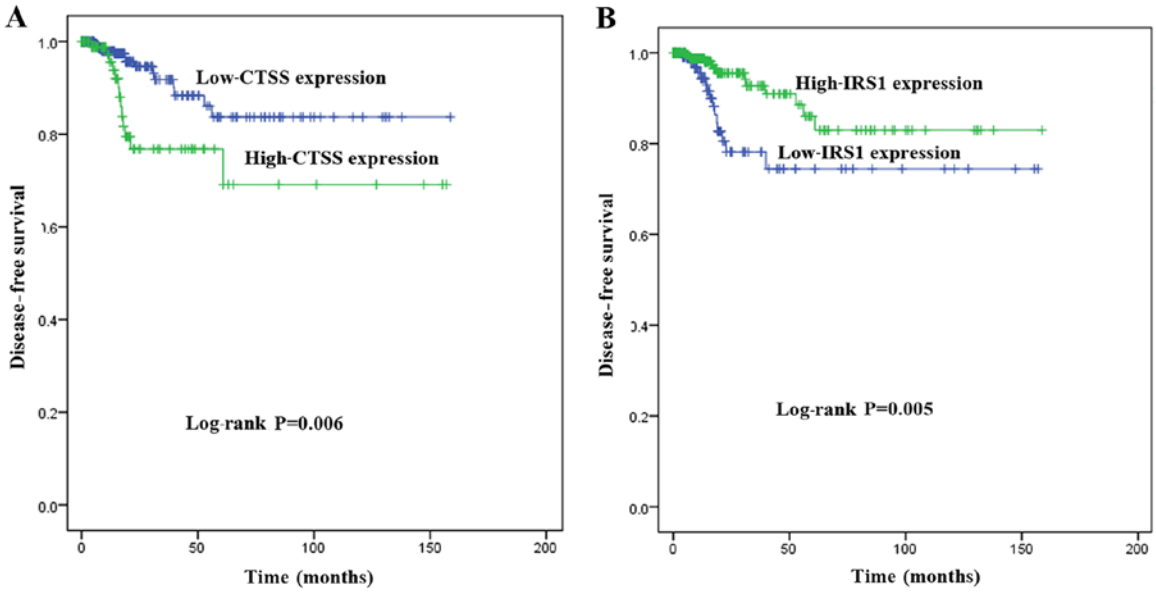

Figure 6. Kaplan-Meier survival analysis of the association between key gene expression level and DFS. (A) Patients with high expression of CTSS demonstrated significantly shorter DFS than those with low level of CTSS expression (P=0.006). (B) Patients with low expression of IRS1 demonstrated significantly shorter DFS than those with high level of IRS1 expression (P=0.005). Survival curves were compared using log-rank test. DFS, disease-free survival.

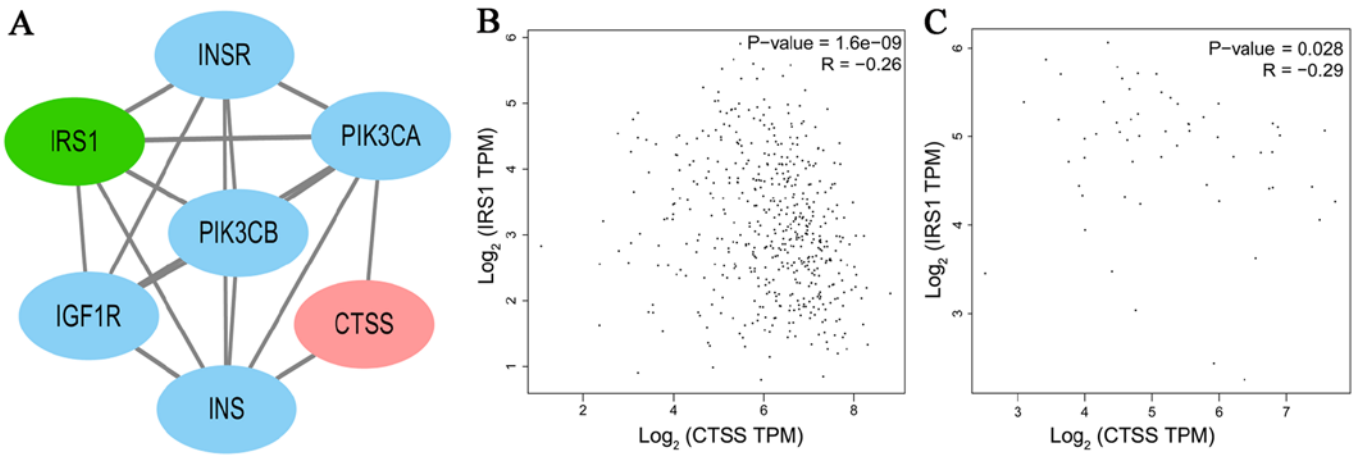

Figure 7. Correlation between the expression of CTSS and IRS1 in the protein-protein interaction network (PPI) and the Gene Expression Profiling Interactive Analysis (GEPIA) database(http://gepia.cancer-pku.cn/). (A) A PPI of IRS1 and CTSS. (B) The expression of *CTSS* was negatively correlated with *IRS1* mRNA expression in PTC tissues (r=-0.26, P<0.001). (C) The expression of *CTSS* was negatively correlated with *IRS1* mRNA expression in normal thyroid tissues (r=-0.29, P<0.05).

the increased expression of *CTSS* was considered to have a significant negative impact on the DFS of patients with PTC (Table I).

Expression of CTSS and clinicopathological factors. To further analyze the association between the expression of *CTSS* and clinicopathological characteristics in patients with PTC, patients were divided into high-expression and low-expression groups based on *CTSS* levels. It was revealed that high expression of *CTSS* was significantly associated with different histological subtypes (increased tall-cell subtype and reduced follicular subtype; P<0.001), more frequent LNM (P<0.001) and advanced TNM stage (P=0.049). However, there was no significant association between the expression of *CTSS* and any other clinicopathological characteristic, including age, sex, maximum size of tumor, multifocality and T stage (P>0.05; Table II).

Correlation between the expression of CTSS and LNM. Binary logistic regression analysis was performed to determine whether the overexpression of CTSS may be an independent risk factor for LNM in PTC. As listed in Table III, univariate analysis revealed that LNM was associated with older age (OR, 0.610; 95% CI, 0.420-0.887; P=0.010), larger tumor size (OR, 1.884; 95% CI, 1.203-2.951; P=0.006), ETE (OR, 2.751; 95% CI, 1.809-4.183; P<0.001), advanced T stage (OR, 2.646; 95% CI, 1.792-3.908; P<0.001), histological subtype (OR, 0.172; 95% CI, 0.091-0.326; P<0.001), overexpression of IRS1 (OR, 0.335; 95% CI, 0.228-0.492; P<0.001) and overexpression of CTSS (OR, 2.688; 95% CI, 1.780-4.059; P<0.001). After

Table I. Univariate and multivariate Cox regression analysis of factors associated with disease-free survival (DFS).

	Univariate analysis			Multivariate analysis		
All variables	P-value	HR	95% CI	P-value	HR	95% CI
Age (years)						
<45		1				
≥45	0.939	1.030	0.488-2.172	nd	nd	nd
Sex						
Male		1			1	
Female	0.273[b]	0.641	0.290-1.418	0.152	0.530	0.222-1.265
Maximum tumor size (cm)						
<4		1				
≥4	0.619	1.229	0.545-2.771	nd	nd	nd
Multifocality						
Unifocal		1				
Multifocal	0.794	1.108	0.515-2.383	nd	nd	nd
ETE						
No		1				
Yes	0.952	1.024	0.470-2.230	nd	nd	nd
LNM						
N0		1			1	
N1	0.012[c]	3.242	1.300-8.083	0.112	2.400	0.816-7.056
T stage						
T1-T2		1	1			
T3-T4	0.326[b]	1.451	0.690-3.052	0.611	1.239	0.542-2.835
TNM stage						
I-II		1			1	
III-IV	0.027[c]	2.323	1.102-4.895	0.100	2.117	0.867-5.173
Histological type						
Classical PTC		1			1	
Follicular PTC	0.369[b]	0.575	0.172-1.925	0.672	1.312	0.373-4.610
Tall-cell PTC	0.557	0.548	0.073-4.086	0.190	0.251	0.032-1.980
CTSS						
≥ cut-off[a]	0.009[d]	2.705	1.287-5.688	0.031[c]	2.649	1.095-6.409
< cut-off[a]		1			1	
IRS1						
≥ cut-off[a]	0.007[d]	0.360	0.171-0.756	0.208	0.551	0.218-1.392
< cut-off[a]		1			1	

[a]Cut-off was calculated by non-parametric receiver operating characteristic analyses to predict disease-free survival; PTC, papillary thyroid cancer; ETE, extrathyroidal extension; LNM, lymph node metastasis; TNM, tumor-node-metastases; HR, hazard ratio; CI, confidence interval; nd, not done; [b]P<0.5; [c]P<0.05; [d]P<0.01.

adjusting for these variables, multivariate analysis indicated that overexpression of *CTSS* (OR, 2.015; 95% CI, 1.225-3.315; P=0.006) was an independent risk factor for LNM, whereas age ≥45 years was a significant protective predictive factor (OR, 0.527; 95% CI, 0.331-0.840; P=0.007), as was the overexpression of IRS1 (OR, 0.579; 95% CI, 0.362-0.926; P=0.022) and follicular-variant histology was significantly associated with a lower incidence of LNM compared with classical PTC (OR, 0.205; 95% CI, 0.102-0.414; P<0.001). No significant associations were observed between LNM and other factors, including tumor size, ETE and T stage (P>0.05; Table III).

Discussion

The present study identified 277 DEGs in tumor samples from four GEO cohorts. Enrichment analyses indicated that the majority of the DEGs could be associated with thyroid tumorigenesis. The top DEGs [*APOE, CTSS, IRS1, KIT*, galectin-3

Table II. Correlation between the expression of CTSS and patient clinicopathological characteristics.

Variables	CTSS subgroup[a]		P-value[b]
	Low (%)	High (%)	
No. of cases	251	250	
Age (years)			0.140
<45	106 (46.5)	122 (53.5)	
≥45	145 (53.1)	128 (46.9)	
Sex			0.244
Male	62 (45.9)	73 (54.1)	
Female	187 (51.8)	174 (48.2)	
Maximum tumor size (cm)			0.529
<4	177 (49.6)	180 (50.4)	
≥4	64 (52.9)	57 (47.1)	
Multifocality			0.495
Unifocal	129 (48.7)	136 (51.3)	
Multifocal	117 (51.8)	109 (48.2)	
ETE			0.230
No	183 (55.1)	149 (44.9)	
Yes	59 (48.8)	62 (51.2)	
T stage			0.133
T1-T2	162 (52.8)	145 (47.2)	
T3-T4	89 (45.9)	105 (54.1)	
LNM			<0.001[d]
N0	129 (57.1)	97 (42.9)	
N1	87 (38.7)	138 (61.3)	
TNM stage			0.049[c]
I-II	177 (53.0)	157 (47.0)	
III-IV	72 (43.6)	93 (56.4)	
Histological type			<0.001[d]
Classical PTC	144 (44.6)	179 (55.4)	
Follicular PTC	77 (77.8)	22 (22.2)	
Tall-cell PTC	8 (23.5)	26 (76.5)	

[a]Median expression level was used as a cut-off to divide the 501 patients into low-CTSS group (n=251) and high-CTSS group (n=250); [b]Chi-square test; ETE, extrathyroidal extension; LNM, lymph node metastasis; TNM, tumor-node- metastasis; [c]P<0.05. [d]P<0.001.

(*LGALS3*), runt-related transcription factor 2 (*RUNX2*) and *TGFBR1*] were extracted as hub genes, and their aberrant expression in the TCGA cohort was consistent with the GEO analysis, supporting the validity of the results.

Apolipoprotein E (APOE) is a 299-amino acid glycoprotein that is known to play a role in cholesterol transport and metabolism. *APOE* can play diverse roles in a number of biological processes, including cell growth, differentiation, immune stress and survival (19). *APOE* has been identified to be overexpressed in lung (20) and ovarian cancer (21), however, the data available regarding *APOE* in PTC are limited. The present study revealed that *APOE* was significantly upregulated in PTC tissues. Therefore, *APOE* may be a typical biological characteristic of PTC.

KIT proto-oncogene receptor tyrosine kinase (*KIT*), a type-III receptor tyrosine kinase (RTK), plays a crucial role in the occurrence of cancer. Dysregulation of *KIT* (including overexpression and gain-of-function, loss-of-function and point mutations) has been detected in several types of human cancer (22-25). In thyroid tumors, the expression levels of *KIT* were significantly lower in malignant than in benign tumors, which was considered a very strong predictive indicator for discriminating malignant from benign tumors (26,27). Consistent with the results of previous studies, the present study revealed that *KIT* was significantly downregulated in PTC tissues. Therefore, *KIT* may be a useful diagnostic marker in PTC.

LGALS3, a member of a β-galactoside-binding protein family, is involved in normal growth development, and cancer progression and metastasis, as well as implicated in cell growth, differentiation, adhesion and malignant transformation. Previous studies have demonstrated that *LGALS3* may serve as a diagnostic marker for thyroid cancer (28,29). The present study demonstrated that the expression levels of *LGALS3* were significantly higher in PTC tissues than in normal tissues, which supported the hypothesis that *LGALS3* is a valuable diagnostic marker for thyroid cancer.

Runt-related transcription factor 2 (*RUNX2*) is a transcription factor required for bone development. The involvement of *RUNX2* in tumor progression and metastasis has been increasingly recognized in different types of tumor cells (30-35). The present study revealed that *RUNX2* was upregulated in PTC tissues. Additionally, *RUNX2* was enriched in the GO BP terms 'positive regulation of cell proliferation' and in the KEGG pathway 'transcriptional misregulation in cancer'. In agreement with this, previous studies have identified an association between the expression of *RUNX2* and thyroid cancer progression (36-39). Therefore, *RUNX2* may play an important role in thyroid tumorigenesis.

Transforming growth factor-β (*TGF-β*) is a member of the disulfide-bonded cytokine family and plays an important role in cell growth, differentiation, apoptosis and survival. *TGFBR1* is a receptor for *TGF-β* ligands. Following stimulation with *TGF-β* ligands, *TGFBR1* activates several different signaling pathways, including the Smad, MAPK, PI3K and serine/threonine protein kinase (Akt) pathways, and contributes to the invasion, metastasis and progression of cancer (40-42). However, the oncogenic role of *TGFBR1* in thyroid cancer has not been adequately investigated. The present study revealed that *TGFBR1* was associated with the GO BP terms 'positive regulation of apoptotic signaling pathway', 'positive regulation of cell proliferation', 'positive regulation of cell migration' and the KEGG term 'pathways in cancer'. Furthermore, the present study demonstrated that *TGFBR1* was highly expressed in PTC tissues, indicating a potential role in thyroid tumorigenesis.

In the present study, aberrant expression of *CTSS* and *IRS1* was revealed to be associated with DFS. To the best of our knowledge, there have been no previous studies on the relationship between IRS1 and CTSS. Although there was no direct association between *IRS1* and *CTSS*, a significant positive correlation was observed between CTSS and IRS1 mRNAs, both in PTC and normal thyroid tissues. The mechanisms underlying

Table III. Univariate and multivariate logistic regression analysis of the factors associated with lymph node metastasis (LNM).

	Univariate analysis			Multivariate analysis		
Variables	P-value	OR	95% CI	P-value	OR	95% CI
Age (years)						
<45		1			1	
≥45	0.010[b]	0.610	0.420-0.887	0.007[c]	0.527	0.331-0.840
Sex						
Male		1				
Female	0.051	0.659	0.434-1.001	nd	nd	nd
Maximum tumor size (cm)						
<4		1			1	
≥4	0.006[c]	1.884	1.203-2.951	0.246	1.443	0.776-2.682
Multifocality						
Unifocal		1				
Multifocal	0.088	1.386	0.953-2.017	nd	nd	nd
ETE						
No		1			1	
Yes	<0.001[d]	2.751	1.809-4.183	0.392	1.430	0.630-3.246
T stage						
T1-T2		1			1	
T3-T4	<0.001[d]	2.646	1.792-3.908	0.346	1.495	0.648-3.448
Histological type						
Classical PTC		1			1	
Follicular PTC	<0.001[d]	0.172	0.091-0.326	<0.001[d]	0.205	0.102-0.414
Tall-cell PTC	0.185	1.671	0.783-3.567	0.932	1.038	0.439-2.458
CTSS						
≤ cut-off[a]		1			1	
> cut-off[a]	<0.001[d]	2.688	1.780-4.059	0.006[c]	2.015	1.225-3.315
IRS1						
≤ cut-off[a]		1			1	
> cut-off[a]	<0.001[d]	0.335	0.228-0.492	0.022[b]	0.579	0.362-0.926

[a]Cut-off was calculated by non-parametric receiver operating characteristic analyses to predict lymph node metastasis. ETE, extrathyroidal extension; PTC, papillary thyroid cancer; OR, odds ratio; CI, confidence interval; nd, not done; [b]P<0.05; [c]P<0.01; [d]P<0.001.

the relationship between *CTSS* and *IRS1* must be explored in future studies. Insulin receptor substrate 1 (*IRS1*) is an adaptor protein that functions as a downstream messenger from activated cell surface receptors to numerous signaling pathway cascades. Early studies on *IRS1* introduced its role in metabolism, but a great deal of recent research has focused on its role in cancer progression and metastasis. The PI3K/Akt pathway is one of the main downstream signaling pathways activated by phosphorylated *IRS1* (43). However, the regulatory roles of *IRS1* in tumorigenesis are controversial. *IRS1* has been shown to promote the proliferation and metastasis of pancreatic (44) and colon cancer (45). However, in other studies, suppression of *IRS1* promoted the metastasis of head and neck squamous cell carcinoma (46), neutrophil elastase-mediated degradation of *IRS1* induced lung tumor cell proliferation (47). Previous studies have also demonstrated that *IRS1* may be both a tumor promoter and suppressor in breast cancer (48,49). The present study revealed that *IRS1* was a protective factor for LNM, and the low-*IRS1* expression group exhibited a shorter DFS time compared with the high-*IRS1* expression group, indicating it as a negative regulator in PTC. This finding was consistent with studies that have indicated that IRS1 was a metastatic suppressor (50). Therefore, the role of IRS1 may depend on the type of cancer.

In the present study, the overexpression of *CTSS* was the only independent predictive risk factor for DFS. The high-*CTSS* expression group exhibited different histological types, advanced TNM stage and increased LNM compared with the low-*CTSS* expression group. Furthermore, *CTSS* was identified as an independent risk factor for LNM in PTC. The results of the present study indicated that *CTSS* may play an important role in tumorigenesis, and that high *CTSS* expression may be associated with disease recurrence and progression in PTC.

Cysteine cathepsin proteases, which are endo-lysosomal proteases, contribute to diseases such as cancer, osteoporosis and arthritis (51). The initial attention of researchers in cathepsins in cancer is attributed to the overexpression of cathepsin in tumors in comparison with normal tissues. Previous studies spanning decades have suggested that cathepsins make crucial contributions to tumor progression of numerous cancers by diverse mechanisms. For example, cysteine cathepsins are associated with protein catabolism and autophagy, degradation of the extracellular matrix (ECM) and activation and degradation of growth factors, cytokines and chemokines (52-55).

The human family of cysteine cathepsins comprises 11 members that are involved in diverse physiological processes, including bone protein degradation, autophagy, antigen presentation, growth factor receptor recycling, cellular stress signaling and lysosome-mediated cell death (56). *CTSS* is a lysosomal cysteine protease of the papain superfamily of cysteine proteases, which plays an important role in cancer by contributing to apoptosis, autophagy, angiogenesis, invasion, migration and clinical prognosis (57-60). *CTSS* has characteristics distinct from numerous other family members. Firstly, it remains active at neutral and acidic pH (61), whereas many other cysteine cathepsins are active at an acidic pH. Secondly, the expression of *CTSS* is unique among the cysteine cathepsin family, as it is specifically expressed by antigen-presenting cells (APCs) (62). Thirdly, *CTSS* exerts more physiological effects than other cathepsins in the processing of proteins in the extracellular microenvironment (63).

Previous studies have indicated that *CTSS* was frequently overexpressed in colorectal (58,64), lung, gastric (65) and prostate cancer (66), as well as in hepatocellular carcinoma (67), glioma (68) and breast cancer (69). Although the results of these recent *in vitro* and *in vivo* studies demonstrated that *CTSS* played an important role in tumorigenesis, no data are currently available regarding the abnormal expression of *CTSS* or its association with the clinical outcomes of patients with PTC. The present study revealed that *CTSS* was frequently upregulated in PTC samples compared with normal tissues. Furthermore, it was revealed that high *CTSS* expression was significantly associated with more frequent LNM and a poorer DFS. However, the exact mechanisms underlying how the overexpression of *CTSS* influenced LNM remained unclear. It has been reported that the expression of *CTSS* was regulated by the PI3K/Akt and Ras/Raf/MAPK signaling pathways through the activation of NF-κB (69), which has been well established to contribute to thyroid tumorigenesis (70). Therefore, all these results indicated that the expression of CTSS plays a key role in thyroid tumorigenesis and its overexpression may be associated with aggressiveness and progression in thyroid cancer.

Accumulating evidence has demonstrated that the expression of *CTSS* is an independent predictor of a poor prognosis in some types of human cancer, including gastric (65) and colorectal cancer (58), as well as glioma (71) and lung cancer (72). The present study extended this observation to PTC. A shorter DFS time was observed in patients with high expression of *CTSS* compared with those with low expression of *CTSS*. These results indicated that the overexpression of *CTSS* may provide guidance for identifying postoperative patients at high risk of disease recurrence or progression who may benefit from effective adjuvant therapy.

To the best of our knowledge, the present study was the first to demonstrate that *CTSS* was significantly upregulated in PTC samples compared with normal tissues. Furthermore, a high expression of *CTSS* was significantly associated with LNM and disease recurrence or progression in patients with PTC. Further studies are required to confirm the results of the present study and to identify the mechanism underlying the association between *CTSS* expression and the pathogenesis of PTC. Confirmation of the results of the present study may establish the expression of *CTSS* as a diagnostic and prognostic marker of PTC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JT and YL conceived and designed the study. XA, TC, BS and HL analyzed the data. JT and HL wrote the paper. XQ, DD and ZZ were also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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