Value of pyruvate carboxylase in thyroid fine-needle aspiration wash-out fluid for predicting PTC lymph node metastasis

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Research

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Abstract

Background

The incidence of papillary thyroid carcinoma (PTC) is increasing yearly. Knowing whether there is lymph node metastasis is of great value in choosing surgery and surgical methods, but ultrasound cannot qualitatively diagnose and effectively detect lymph node metastases in the central area. Metabolic dysregulation is an important factor associated with malignancy and metastasis of tumors. Pyruvate carboxylase (PC) is a major anaplerotic enzyme that catalyzes the carboxylation of pyruvate to form oxaloacetate (OAA) and is involved in tumorigenesis in several cancers. This study aims to explore the relationship between *PC* mRNA expression in thyroid fine-needle aspiration (FNA) wash-out fluid and lymph node metastasis of papillary thyroid carcinoma (PTC).

Methods

The expression levels of *PC* in PTCs and normal thyroid tissues were first compared based on bioinformatics analysis of public databases, including the Gene Expression Profiling (GEPIA), Oncomine and Gene Expression Omnibus (GEO) databases. Then, the *PC* mRNA levels were measured by RT-PCR in surgical tissues in a total of 42 patients with surgically confirmed PTC and compared to those of patients with and without central lymph nodal metastasis (CLNM). Further, to identify *PC* expression in diagnostic biopsies, a total of 71 thyroid nodule patients with ultrasound-guided fine-needle aspiration wash-out fluid samples and cytological diagnosis were prospectively enrolled in the study.

Results

Through data mining, we found that *PC* is overexpressed in PTC and PTC with lymph node metastasis. In 42 surgically diagnosed PTC patient tumor tissues, *PC* was significantly higher in patients with CLNM (n=15) than in those without (n=27) nodal metastasis (median: 6.490 vs. 2.430, p = 0.014). Furthermore, in thyroid FNA wash-out fluid, *PC* was significantly higher in patients with cytologically diagnosed PTC (n=52) than in those with benign nodules (n =19) (median: 2.456 vs. 0.495, p = 0.005). Thirty-four of the 52 patients with PTC received a total thyroidectomy, and *PC* was also significantly higher in patients with CLNM (n=17) than in those without nodal metastasis (n=17) (median: 3.665 vs. 1.621, p = 0.013). The area under the ROC curve for PC predicting PTC with lymph node metastasis in FNA wash out samples was 0.751 (p = 0.013), and the sensitivity and specificity were 70.6% and 76.5%, respectively.

Conclusions

The findings of this study suggest that PC may predict malignancy in thyroid nodules and lymph nodal metastasis with thyroid FNA wash-out fluid in PTC patients.

Background

Thyroid carcinoma is the most common endocrine tumor in the world, and its worldwide incidence has significantly increased yearly (1;2). Nearly 90% of thyroid cancers are differentiated, including papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) (3). PTC is the most common subtype, accounting for 80%-85% of all malignant thyroid tumors (2). PTCs exhibit an indolent biological behavior (4), and the 5-year survival rate reaches almost 95% after treatment (5), such as surgical operation and radioactive iodine therapy. There is a debate about whether PTC, especially papillary thyroid microcarcinoma (PTMC), is over-diagnosed and overtreated. Ito et al (6) reported that only a small portion of PTMCs showed progression and suggested that subclinical low-risk PTMC should be surveilled instead of receiving immediate surgery. Alternatively, some cases of PTMCs show nodule enlargement and early lymph node metastasis during the surveillance period. It is critical to develop a tool to identify those patients with early nodal metastasis to guide clinical management.

At present, the main clinical diagnostic methods of PTC are ultrasonographic and fine-needle aspiration biopsy (FNAB) (7). It is difficult to identify the aggressiveness of PTC based on these diagnostic methods given their inherent limitations (8). With the development of basic medical research, molecular genetic alteration has been increasingly studied, and substantial tumor biomarkers were found, improving the accuracy of PTC risk categories (4). The most widely studied and clinically used PTC tumor markers are BRAF and TERT promoter mutations (9), though the incidence of the two mutations are 83.7% and 7.5%-27%, respectively (10) (11), and neither has significant prognostic value for predicting the high risk clinicopathological features of PTC (12). Therefore, it is important to explore other tumor markers that affect the aggressive biological behavior of PTC including nodal metastasis.

Cancer cell proliferation requires energy, which is dependent on cellular metabolism (13). In proliferating cancer cells, the replenishment of intermediates for the tricarboxylic acid (TCA) cycle provides biosynthetic precursors to synthesize proteins, nucleic acids and lipids (14;15). The pyruvate carboxylase (PC)-mediated anaplerosis pathway for TCA intermediates plays an important role in improving the PTC cellular TCA cycle (16). By detecting the role of *PC* expression levels in thyroid FNA wash-out fluids from PTC patients with LNM, we explored the feasibility of PC as a novel tumor biomarker for PTC tumor aggressiveness.

Materials And Methods

Gene expression data acquisition and bioinformatic analysis

Gene Expression Profiling Interactive Analysis (GEPIA)-(http://gepia.cancer-pku.cn/) (17) is an online server for profiling and interactive analyses of cancer and normal gene expression. Oncomine (https://www.oncomine.org/) (18;19) is a cancer microarray database and integrated data-mining platform online tool. We searched PC in the two databases to analyze *PC* expression in thyroid cancer and other types of cancer. In addition, we used the expression profiling arrays (GSE60542) from Gene Expression Omnibus databases to analyze the different expression genes between thyroid cancer tissue

with or without lymph metastasis. GSE60542 dataset was analyzed by Affymetrix Human Genome Array with annotation platform GPL570 [HG-U133_plus_2]. The GSE60542 dataset contained 14 PTC samples with no lymph nodes metastasis, 19 samples with lymph node invasion and 10 control samples from normal thyroid. Using R packages, we assessed GSE60542 RAW datasets by background correction, normalization, expression calculation, and probe integration. Robust multi-array average (RMA) and mismatch probe (PM) were created for processing the datasets. *P*-values was adjusted by the Benjamini-Hochberg method and fold-changes (FC) were calculated using the false discovery rate (FDR) procedure. PTC-DEGs with a |log2-fold change| > 1 and $P < 0.05$ were selected. The basic features of the GEO database are shown in Table 1.

Table 1
Basic features of the GSE 60542 database

GEO no.	Subtype
GSM 1481838	Normal thyroid
GSM 1481842	Normal thyroid
GSM 1481852	Normal thyroid
GSM 1481862	Normal thyroid
GSM 1481877	Normal thyroid
GSM 1481891	Normal thyroid
GSM 1481895	Normal thyroid
GSM 1481901	Normal thyroid
GSM 1481907	Normal thyroid
GSM 1481913	Normal thyroid
GSM 1481844	Papillary thyroid carcinoma, N1
GSM 1481890	Papillary thyroid carcinoma, N1
GSM 1481847	Papillary thyroid carcinoma, N1
GSM 1481875	Papillary thyroid carcinoma, N1
GSM 1481929	Papillary thyroid carcinoma, N1
GSM 1481854	Papillary thyroid carcinoma, N1
GSM 1481905	Papillary thyroid carcinoma, N1
GSM 1481861	Papillary thyroid carcinoma, N1
GSM 1481882	Papillary thyroid carcinoma, N1
GSM 1481849	Papillary thyroid carcinoma, N1
GSM 1481865	Papillary thyroid carcinoma, N1
GSM 1481883	Papillary thyroid carcinoma, N1
GSM 1481903	Papillary thyroid carcinoma, N1
GSM 1481915	Papillary thyroid carcinoma, N1
GSM 1481839	Papillary thyroid carcinoma, N1
GSM 1481872	Papillary thyroid carcinoma, N1
GSM 1481926	Papillary thyroid carcinoma, N1

GEO no.	Subtype
GSM 1481889	Papillary thyroid carcinoma, N1
GSM 1481914	Papillary thyroid carcinoma, N1

Surgically-confirmed PTC patient populations

The study enrolled 42 surgically-confirmed PTC patients with surgical tissues from July 2018 to November 2018. Clinical characteristics of the patients are shown in Table 2.

Table 2
Clinicopathological characteristics of 42 surgically confirmed PTC patients

Clinicopathological characteristics		Value
Gender (M/F)		13/29
Age (Y), Mean ± SD		42.3 ± 1.9
≥ 55, n (%)		8 (19.0%)
< 55, n (%)		34 (81.0%)
Cytology diagnosis (71)		
CLNM	Positive	15 (35.7%)
	Negative	27 (64.3%)

Thyroid nodule patient populations

A total of 71 thyroid nodule patients with FNA wash-out fluid samples from November 2019 to January 2020 were also included. Fifty-two of the 71 patients were diagnosed as PTC, and 19 had benign nodules based on cytological findings. Thirty-four of the 52 cytologically diagnosed PTC patients underwent total thyroidectomy and central nodal dissection with final tissue sample confirmation. Clinical characteristics of the patients are shown in Table 3.

Table 3
Clinicopathological characteristics of 71 thyroid nodule patients

Clinicopathological characteristics		Value
Gender (M/F)		28/43
Age (Y), Mean ± SD		44.5 ± 1.5
≥ 55, n (%)		20 (28.3%)
< 55, n (%)		51 (71.8%)
Cytology diagnosis (71)		
PTC		52 (73.2%)
Benign nodule		19 (26.3%)
Surgically diagnosed PTC (34)		
CLNM	Positive	17 (50.0%)
	Negative	17 (50.0%)

Note: PTC, papillary thyroid carcinoma; CLNM, central lymph nodal metastasis; PC, pyruvate carboxylase

Sample preparation

Surgical PTC tissues were preserved in RNA protector and frozen at -80ºC. For ultrasound-guided FNA, 3 passes were performed for each thyroid nodule, and direct smears were prepared from each pass for HE stain after air dry. Thyroid FNA wash-out fluid with RNA protector was collected from needles.

Quantitative real-time reverse transcription PCR

RNA was extracted from PTC tissues using TRIzol (Sangon Biotech, Shanghai, China). RNA of the thyroid nodule FNA wash-out fluid samples was extracted following the RNA Kit Protocol (Qiagen Hilden, Germany). All RNAs were first reverse-transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa Bio Inc., Japan) (37 °C for 15 min, 85 °C for 5 s, and cooled to 4 °C). RT-PCR of *PC* and reference gene *b-actin* was performed following the protocol of TB Green® Premix Ex Taq™ II (TaKaRa Bio Inc., Japan) in an Applied Biosystems 7500 Real-Time PCR System. The temperature cycling protocol consisted of 30 sec denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 sec, and 60 °C for 34 sec. The 40 cycles were followed by 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec. The *PC* primers used in this study were 5'-ATGTTGCCCACAACTTCAGCAAGC-3' (forward primer) and 5'-AGTTGAGGGAGTCAAACACACGGA-3' (reverse primer). The *β-actin* primers were 5'-GCACCACACCTTCTACAATG-3' (forward primer) and 5'-TGCTTGCTGATCCACATCTG-3' (reverse primer). *PC* expression level was normalized to *b-actin* mRNA. The cycle threshold (Ct) values below 35 were used in this study. The $2^{-\Delta Ct}$ of (*PC* mRNA - *β-actin* mRNA) were used to evaluate the expression levels of *PC*.

Statistical analyses

Unless otherwise indicated, data were expressed as median, [interquartile range]. The mRNA expression levels of *PC* in cytologically diagnosed PTC patients were compared to benign nodule patients using the Mann-Whitney *U* test. Univariate analyses for the association between clinicopathologic factors and CLNM of surgically diagnosed PTC patients were assessed using the λ^2 test or Mann-Whitney *U* test. The binary logistic regression test was used for multivariate analysis. All statistical analyses were conducted using SPSS 22.0. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Dataset online analysis of PC expression in thyroid cancer

PC mRNA expression through the GEPIA datasets differs in different types of tumors (Fig. 1A, B) but is significantly overexpressed in thyroid cancer (THCA) (Fig. 1C, D). In addition, expression of *PC* is also significantly higher in PTC than in normal tissues in 2 separate public datasets in Oncomine. In Vasko's dataset, *PC* is overexpressed in thyroid cancer with a fold change of 2.516 (Fig. 2A). In He's dataset, *PC* is also highly expressed with a fold change of 2.061 (Fig. 2B). According to GSE60542, *PC* expression level is significantly higher in PTC with or without lymph metastasis tissues compared to normal thyroid samples, and the fold change is 1.10 and 1.22, respectively (Fig. 2C).

The correlation of PC expression between PTC tumor tissues with or without CLNM

As *PC* is overexpressed in thyroid cancer with lymph metastasis, we used surgical tissues of 42 surgically diagnosed PTC patients to further assess the relationship between PC and CLNM. There were 15 PTC patient cases with negative CLNM and 27 cases with positive CLNM. *PC* expression was higher in CLNM-positive patients compared to CLNM-negative patients: 6.490 [2.351 ~ 10.002] vs. 2.430 [1.466 ~ 4.976] ($p = 0.014$).

PC expression and patient age were independent predictors for PTC with CLNM after FNA

The abovementioned results were measured in surgical tissues. To investigate the role of PC in preoperative diagnostic ultrasound-guided FNA, *PC* expression levels were measured in 71 thyroid nodule FNA wash-out fluid samples. Cytological diagnosis divided the 71 patients into two groups: 52 PTC and 19 benign thyroid diseases. A Mann-Whitney *U* test showed that *PC* expression levels were significantly higher in PTCs compared to benign nodules (2.456, [0.442 ~ 3.779] vs. 0.498, [0.262 ~ 2.010], $p = 0.005$; Fig. 3A), consistent with the public datasets described above. Furthermore, 34 out of the 52 PTC patients underwent total thyroidectomy and nodal dissection, and the *PC* expression level was higher in the CLNM positive group than the CLNM negative group (3.665 [2.378 ~ 6.691] vs. 1.621 [0.228 ~ 3.144], $p = 0.013$; Fig. 3B). Univariable logistic regression analysis revealed that PC expression and ages < 55 were associated with CLNM (all $p < 0.05$). Furthermore, multivariable analysis showed that PC and younger

ages were independent predictors for CLNM (Table 4). To validate the sensitivity and specificity of PC expression as a biomarker for PTC with CLNM, we applied ROC curve analysis. The area under the curve (AUC) was 0.751 (P = 0.013, Fig. 4). The sensitivity and specificity were 70.6% and 76.5%, respectively. The results suggest that PC expression is potentially a novel biomarker for LNM of PTC.

Table 4
Univariable and multivariate analyses of predictive factors for CLNM in patients with PTC after FNA

Variable		**Univariate analysis**			**Multivariate analysis**		
		P	OR	95% CI	*P*	OR	95% CI
Gender	male	0.493	1.607	0.414–6.240			
	female		1				
Ages (years)	< 55	0.034	6.667	1.151–38.598	0.035	9.905	1.177–83.338
	≥ 55		1				
Tumor size (cm^3)	< 5	0.698	1.354	0.293–6.261			
	≥ 5						
PC		0.029	1.472	1.04–2.085	0.024	1.587	1.061–2.373

Note: CLNM, central lymph nodal metastasis; PC, pyruvate carboxylase; CI, confidence interval; OR, odds ratio

Discussion

Although most PTCs tend to have “bioinert” characteristics, others show higher invasiveness and aggressive clinical features. Cervical lymph node metastasis is a common aggressive clinical feature, occurring in nearly half of PTC patients (20) and in 20% – 90% of PTMC patients (21;22).

A review of a surveillance, epidemiology, and end results (SEER) database study found that cervical lymph node metastases represent a high risk of locoregional recurrence and survival (23–25). Bake et al (26) reported that the PTC with LNM group has a shorter disease-free survival period than the LNM-negative group. The ratio of the number of LNMs being greater than 30% of the monitored lymph nodes is a significant independent prognostic factor in PTC (27). Central lymph node metastasis, which is the sentinel lymph node of cervical lymph node metastasis, is also a risk factor of recurrence (28). However, it is sometimes difficult to recognize central lymph nodes with ultrasonography (29).

Molecular tests have improved the accuracy of US-FNA cytology (30–32), but there is no effective molecular marker for predicting lymph node metastasis. Although $BRAF^{V600E}$ and $TERT^{C228T}$ mutations are widely tested in the clinic, 40% of DTCs with distant metastases show negative BRAF or TERT genetic

mutation (33). Liu et al. (34) found that the $TERT^{C228T}$ mutation and the $BRAF^{V600E}$ mutation led to dedifferentiation and aggressive biological behavior of thyroid cancer (35). However, the probability of the $BRAF^{V600E}$ and $TERT^{C228T}$ mutations occurring simultaneously in PTC is 13% (36). In addition, Ren et al (37) reported that coexistence of $BRAF^{V600E}$ and $TERT^{C228T}$ mutations has no obvious correlation with PTC lymph node metastasis. Hence, finding a new biomarker is important in the management of PTC.

Overexpression of PC is found in many human cancers (38–40). Using bioinformatics analysis of online databases, we found that *PC* mRNA expression level was significantly higher in the PTC group than in the normal group. Additionally, PC expression is also higher in the CLNM positive group compared to the negative group. Then, we confirmed the above results in surgical PTC tumor tissues, suggesting that PC plays an important role in predicting PTC tumor aggressiveness, consistent with a previous finding (16). To our knowledge, this study is the first to study the diagnostic role of PC in thyroid nodule patients after FNA. Our results suggested that PC has a certain significance for suggesting malignant thyroid nodules. Additionally, younger age is another independent predictor for CLNM of PTC, consistent with previous findings.

The mechanism of high *PC* expression in PTC with LNM may be related to its involvement in cancer metabolic pathways (41). The aerobic glycolysis response restrains pyruvate oxidation in mitochondria, inducing more TCA cycle intermediates (41;42), and the PC-regulated anaplerotic reaction is important for cancer cells to replenish TCA cycle intermediates, which has been identified in many cancers (43). Christen et al (38) used ^{13}C tracer analysis and found that the *PC* mRNA expression level and PC enzyme activity were increased in breast cancer with lung metastasis compared to primary breast cancer. Lee et al (45) suggested that PC may be associated with tumor cell invasion by activating the Wnt/Snail signaling pathway and EMT (epithelial-mesenchymal transition) in breast cancer cells. However, the role of PC regulating LNM in PTC needs further research.

Because PC may be an independent predictor for CLNM and *PC* mRNA expression in thyroid FNA wash-out fluid can be easily obtained, quantitatively determined, and specifically reflected lymph node metastasis of PTC, surgical treatment should be actively performed on PTC patients whose thyroid FNA wash-out fluid shows high expression of *PC*, which may improve the patient's prognosis and survival rate. Alternatively, active surveillance should be suitable for patients with low or no *PC* expression, which may improve the quality of life of PTC patients.

The specificity of PC for predicting CLNM is higher than the sensitivity, which may be because it is difficult to distinguish lower PC expression of PTC from benign tissue. Although it is not ideal to predict the sensitivity and specificity of CLNM by PC expression level in thyroid FNA wash-out fluid, it is possible to further improve their diagnostic sensitivity and specificity by combining other molecular markers, such as BRAF gene detection.

Conclusion

Our study is the first to report that PC is an independent predictor for CLNM and reflects malignant thyroid nodules after FNA treatment. These results may provide clinical guidance for active surgical treatment of thyroid nodules with high *PC* expression and present a good application prospect.

Abbreviations

p>papillary thyroid carcinoma (PTC); pyruvate carboxylase (PC); oxaloacetate (OAA); thyroid fine-needle aspiration (FNA); Gene Expression Profiling (GEPIA); Gene Expression Omnibus (GEO); central lymph nodal metastasis (CLNM); follicular thyroid carcinoma (FTC); papillary thyroid microcarcinoma (PTMC); fine-needle aspiration biopsy (FNAB); tricarboxylic acid (TCA); Robust multi-array average (RMA); mismatch probe (PM); fold-changes (FC); false discovery rate (FDR); epithelial-mesenchymal transition (EMT); Area under the receiver operating characteristic curve (AUC); confidence interval (CI).

Declarations

Ethics approval and consent to participate

The study design was approved by the Ethical Review Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. All patients had signed informed consents.

Consent for publication

Not applicable.

Availability of data and material

The authors declare that data supporting the findings of this study are available within the article.

Competing interests

The authors declare that they have no competing interests.

Note

CLNM, central lymph nodal metastasis; PC, pyruvate carboxylase; CI, confidence interval; OR, odds ratio

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Authors' contributions

Chang Liu and Lu Zhang contributed equally to the article. Chang Liu contributed to the collection of the thyroid FNA wash-out fluid samples, RT-PCR, data analysis for the work, and drafting the manuscript. Lu Zhang contributed to the operation of ultrasound-guided fine-needle aspiration and data analysis for the work. Yang Liu and Qingqing Zhao contributed to the collection of the surgical tissues. Yu Pan and Yifan Zhang contributed to the conception and design of the work and critically revised the manuscript.

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Figures

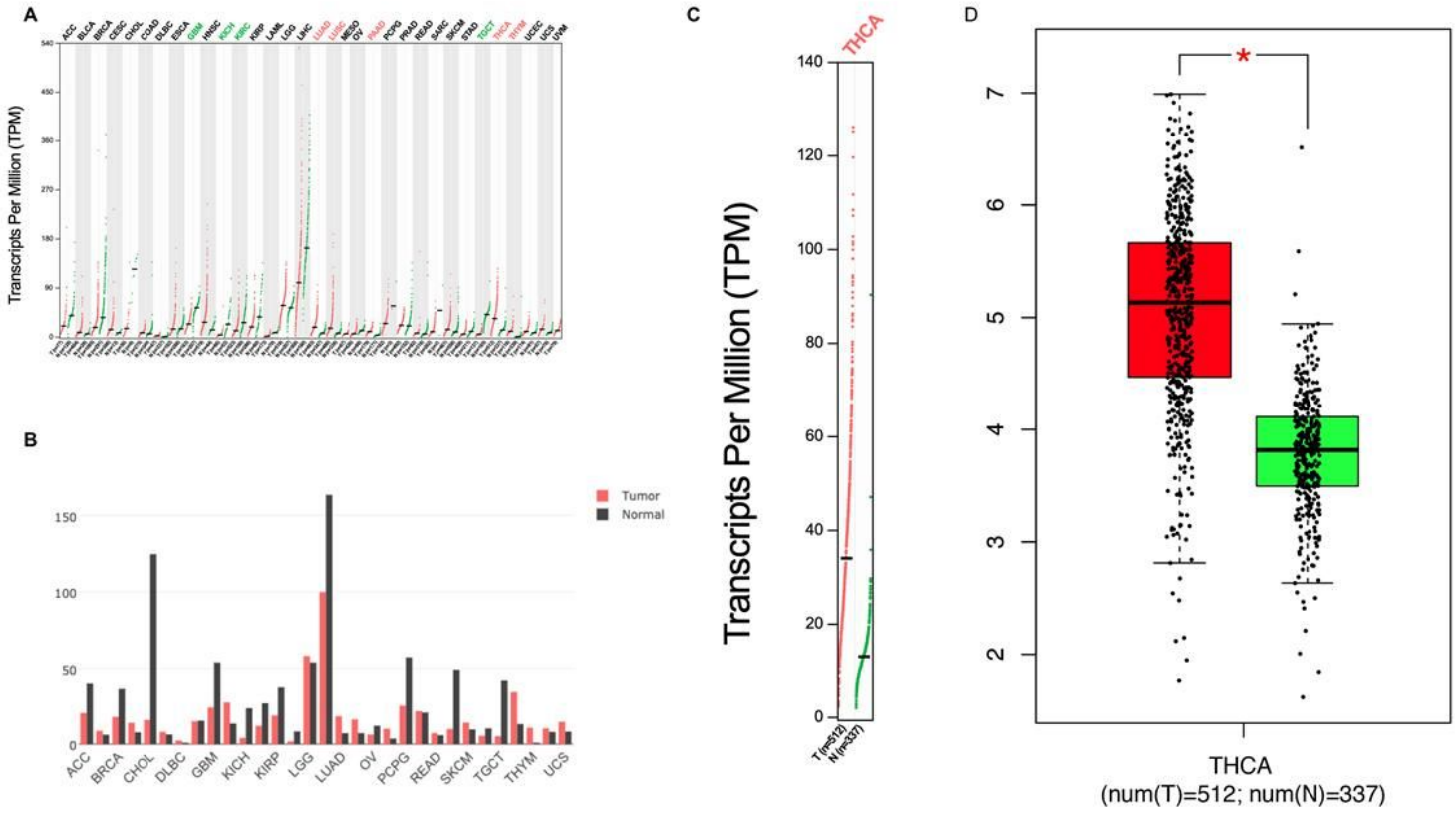

Figure 1

The expression profile of PC in GEPIA A. The expression of PC in all tumor samples and paired normal tissues. (Dot plot) Each dot represents expression of samples. B. The expression of PC in all tumor samples and paired normal tissues. (Bar plot) The height of bar represents the median expression of certain tumor type or normal tissue. C. The expression of PC in THCA. (Dot plot) |Log2FC|=1 and P < 0.01 D. The expression of PC in THCA. (Box plot) |Log2FC|=1 and P < 0.01 Note: GEPIA□Gene Expression Profiling Interactive Analysis□T, tumor; N, normal; ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangio carcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid

Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

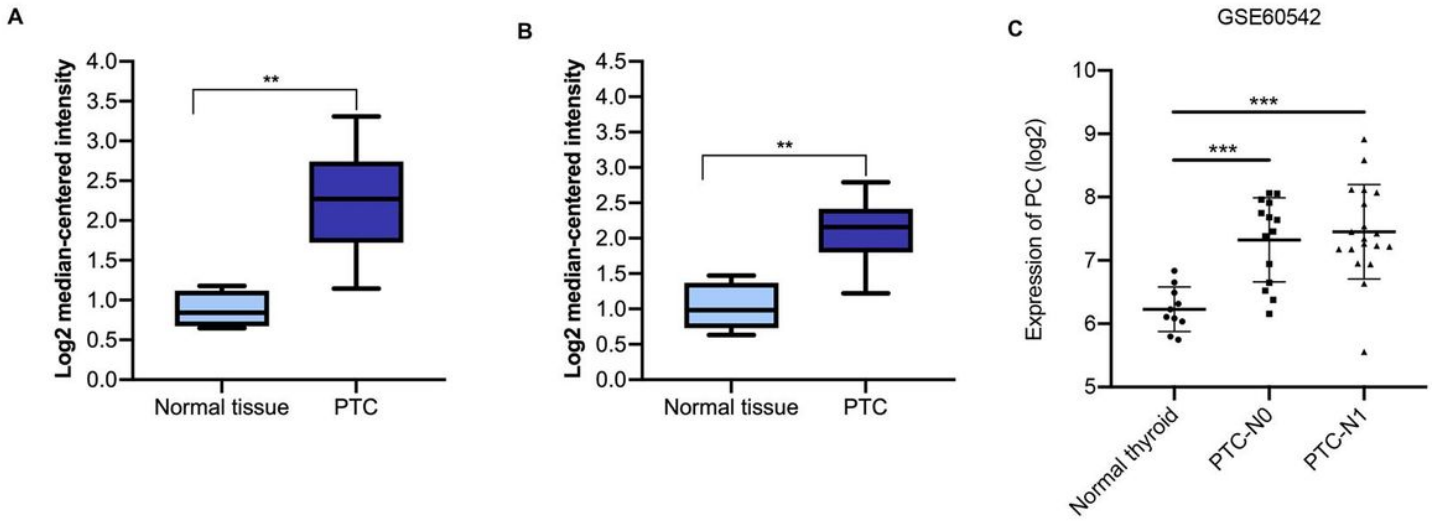

Figure 2

The transcription levels of PC in ONCOMINE and GEO datasets A. PC level in thyroid cancer dataset (Vasko's dataset), **P < 0.01. B. PC level in thyroid cancer dataset (He's dataset), **P < 0.01. C. PC level in thyroid cancer dataset (GSE 60542) Note: PCpyruvate carboxylasePTCpapillary thyroid carcinoma; PTC-N0, papillary thyroid carcinoma without lymph metastasis; PTC-N1, papillary thyroid carcinoma with lymph metastasis.

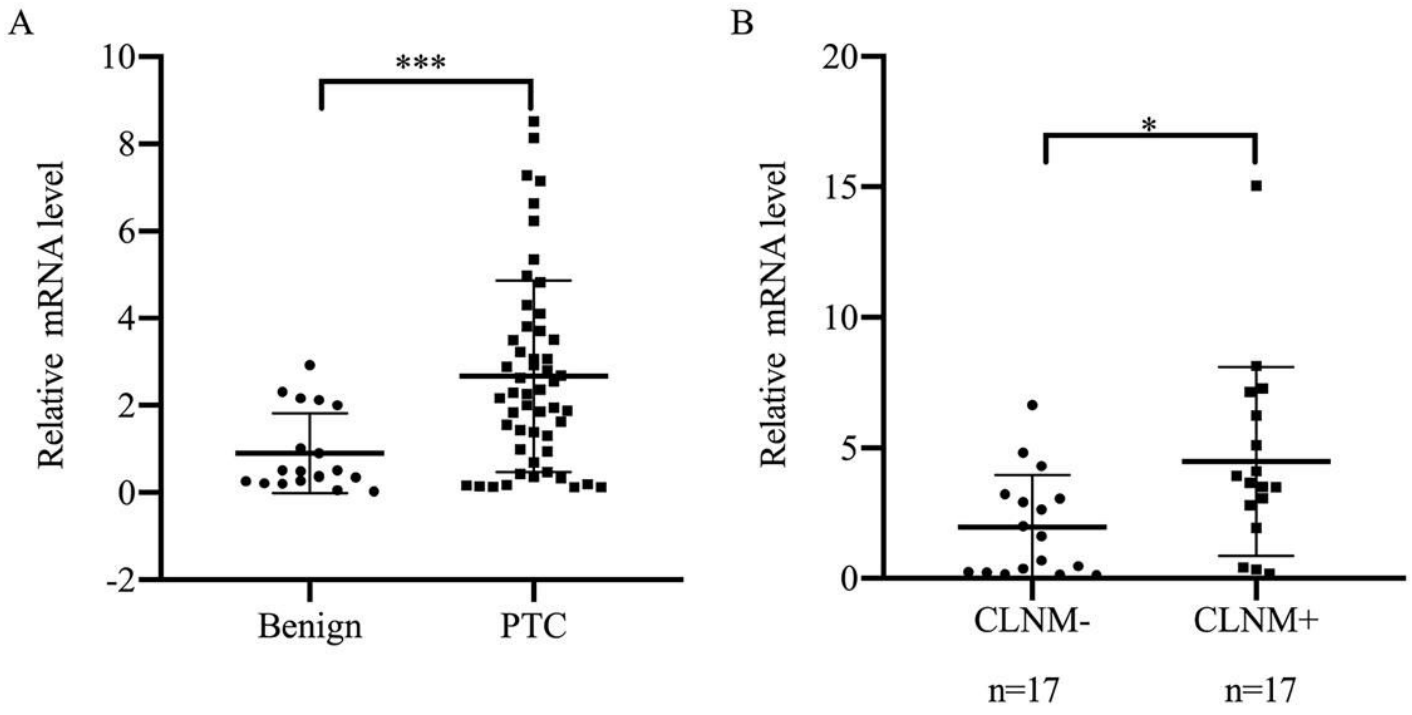

Figure 3

RT-PCR analysis of PC mRNA expression level in thyroid FNA wash-out fluid A. Expression of PC in 52 PTC FNA wash-out fluid samples and 19 benign nodule FNA wash-out fluid samples based on cytologically diagnosed were examined using RT-PCR assays. **P < 0.01. B. Expression of PC in 34 surgically diagnosed PTC with or with CLNM. *P < 0.05. Note: CLNM, central lymph nodal metastasis.

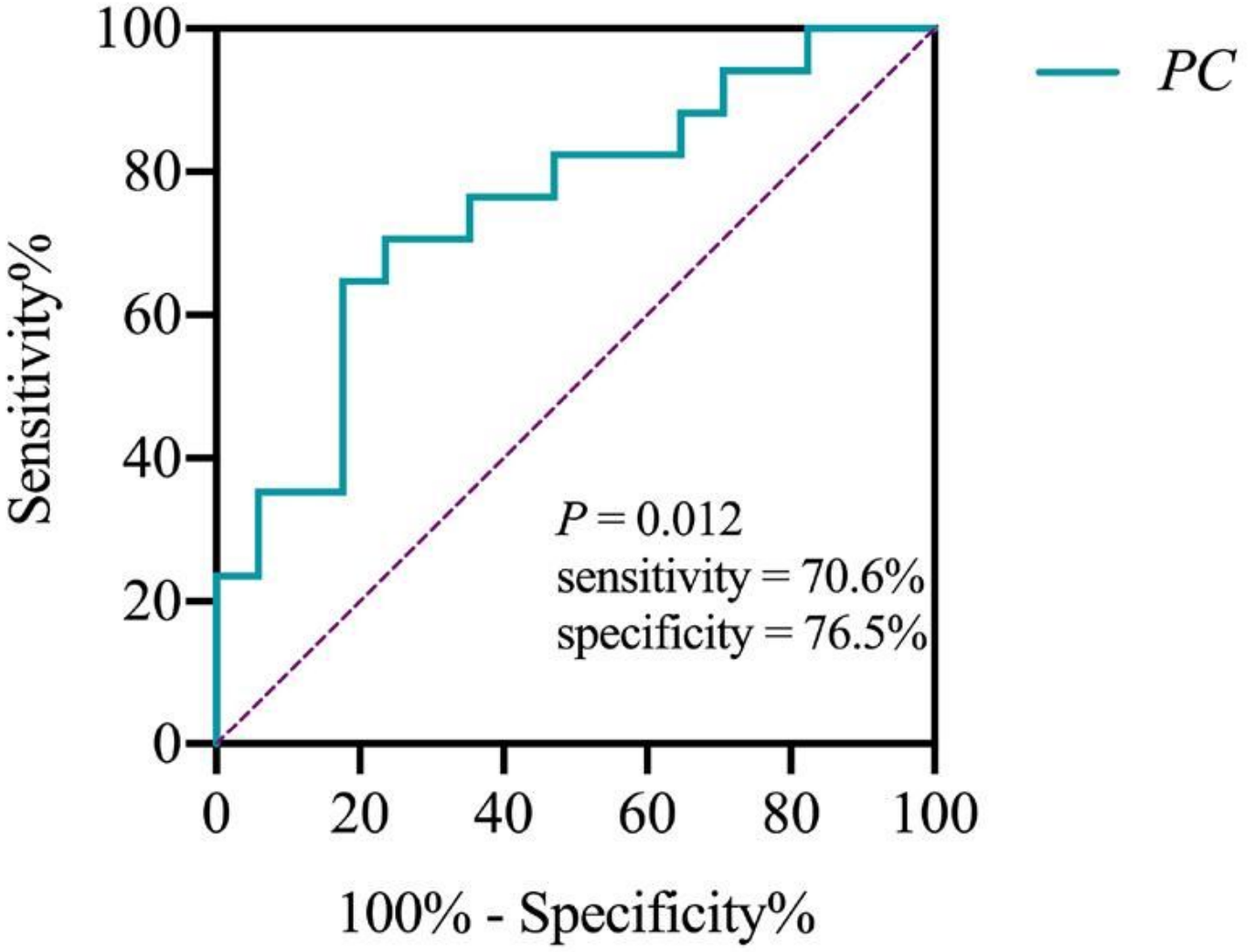

Figure 4

Area under the receiver operating characteristic curve (AUC) for identify of PTC with CLNM using PC mRNA expression level The area under the ROC curve was 0.751. (P = 0.012, 95% CI was 0.585 ~ 0.917). Note: CLNM, central lymph nodal metastasis; PC, pyruvate carboxylase; CI, confidence interval.