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Quantitation of thyroid-stimulating hormone receptor mRNA with real-time PCR for early diagnosis of papillary thyroid microcarcinoma

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Recently, the value of thyroid-stimulating hormone receptor(TSHR) mRNA as a molecular marker in diagnosis of thyroid cancer has been greatly explored. However, its role in detecting thyroid microcarcinoma has never been reported. In this study, we aim to investigate the value of TSHR mRNA in diagnosis of papillary thyroid microcarcinoma(PTMC).

To do that, TSHR mRNA levels were measured by real time PCR in 30 healthy subjects and 72 patients (benign thyroid nodules, n=38; PTMC, n=34). TSHR mRNA levels were then compared with cancer volume in surgical specimen.

We found that TSHR mRNA levels in PTMC were significantly higher than benign thyroid nodules($P<0.01$). At a cutoff value of 0.71 ng/μg total RNA, the TSHR mRNA correctly classified 77.8% of patients preoperatively (sensitivity= 76.5%; specificity= 79.4%). Comparatively, it performed better in larger PTMC of 0.5-1.0cm than <0.5cm(sensitivity, 83.4%*vs*72.4%; specificity, 88.5%*vs*74.1%). Combining TSHR mRNA and ulstrasound examination correctly detect 97.1% of PTMC and could spared surgery in 86.8% of patients with benign thyroid nodules.

Thus, TSHR mRNA measured with real time PCR enhances the preoperative detection of PTMC in patients with thyroid nodules, which dramatically reduces unnecessary surgeries.

Key words: thyroid-stimulating hormone receptor, papillary thyroid microcarcinoma, diagnosis

In recent years, the incidence of papillary throid cancer as well as PTMC has greatly increased [1–3]. Thyroid microcarcinoma has been defined as thyroid cancer less than 10 mm in diameter, usually papillary (papillary thyroid microcarcinoma, PTMC) and accounts for more than 50% of all detected papillary thyroid cancer [4, 5]. Though, most of PTMC has a quite benign clinical course associtated with a good prognosis, there are also many cases of PTMC with bulky cervical lymph node metastasis, distant metastasis and even cancer-related death [6–8].

Thus, the early detection of PTMC is very necessary for us in order to take measures promptly. Ultrasound(U/S) and fine needle aspiration cytology(FNAC) are two cornerstrones in diagnosis of papillary thyroid cancer. According to the guidelines of both American Thyroid Association and European Thyroid Association, thyroid nodules smaller than 1.0 cm should be considered for evaluation only when there are suspicious ultrasound findings such as a round, solid, hypoechoic nodule with microcalcifications and/or irregular border, or a history of familial thyroid cancer or head and neck irradiation [9, 10]. However most PTMC don't present a typical ultrasound character, which usually results in missing some malignant thyroid nodule. In ***addition***, the high rate of inadequate cytology specimens during the FNAC of thyroid cancer with small size also limits the accurate diagnosis of PTMC [10, 11]. Recently, molecular marker like THSR , have been explored as alternative methods for detecting thyroid cancer [12–14], but it has never been explored in diagnosis of PTMC. In the current study, we specially investigate the usefullness of TSHR as a marker for preoperative diagnosis of PTMC among people with small thyroid nodules by quantitative detection of TSHR mRNA in peripheral blood.

Patients and methods

Patients. The study consisted of 30 healthy subjects without a history of thyroid disease and 72 patients with thyroid nodules. All patients with thyroid nodules no more than 1.0 cm in diameter were recruited during 2007–2008 who came into our hospital for surgery because of malignant hint from ultrasound examination or enlargening within short term or strong request from patients to remove the thyroid nodules. Written informed consent was obtained from each study participant, and the study protocol was approved by our

institutional review board. Blood samples were taken during the preoperative routine examinations.

Ultrasound (U/S) was performed as a routine diagnostic work-up of patients with thyroid nodular disease. U/S features of thyroid nodules considered suspicious for possible thyroid malignancy included irregular shape, ill-defined margins, solid structure,hypervascularity, fine internal calcifications [15]. None of the patients received FNAC due to objective limitions. All patients were finally diagnosized as PTMC or benign nodular goiter as comfirmed by pathological examination after surgery.

TSHR mRNA measurement. Briefly, 3ml venous blood was collected, and the mononuclear cells were separated by Ficoll-Hypaque gradient(Invitrogen, California). Total RNA was extracted with TRIzol reagent (Life Technologies, Rockville, MD). Quality of RNA was tested in every sample by performing RT-PCR for the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For RT-PCR, we used an in-cycle fluorescent detection system(Rotorgene 3000; Corbett Research, Sydney, Australia) and one-step Quantitect SYBR Green kit (QIAGEN Inc., Valencia, CA). The assay was performed in duplicate using 1μg RNA according to the manufacturer's recommendations. The primer sequences for TSHR mRNA and for GAPDH have been described previously [16]. Each sample was also quantitated for GAPDH mRNA in a separate reaction tube within the same run. For quantitation, total RNA extracted from throid tissue was used as a reference preparation to produce a standard (calibration) curve consisting of four concentrations ranging from 0.12–5.2 ng total RNA. A positive and negative control was included in each amplification reaction. The subject samples were normalized for the amount of RNA loaded into each reaction tube. Results are reported as reference preparation equivalent TSHR mRNA ng/μg of total RNA. The estimated functional assay sensitivity is 0.12 ng/μg of total RNA with a CV of 8%.

Statistical analysis. Statistical analysis was performed using SPSS13.0 (SPSS Inc,

Chicago, Illinois). All subject groups demonstrated positively skewed distributions and therefore data are expressed as medians and interquartile ranges(25–75th percentiles) unless otherwise specified. The Kruskal-Wallis test and the Wilcoxon rank sum test were used to investigate the group differences at an overall α□0.05.

Results

Characteristics of the patients. According to the final surgical pathology, 34 of the 72 patients had papillary thyroid cancer and 38 patients had benign thyroid nodules. All patients included in this study don't have a family history of thyroid disease. The characteristics of these patients are summarized in Table 1.

Quantitative PCR analysis of TSHR mRNA. Firstly, we demonstrated that a significant difference exists among the three groups in our study with Wilcoxon rank sum test. Then we compared the TSHR mRNA value between PTMC and benign thyroid nodule group as well as healthy control and benign thyroid nodule (Fig 1, table 2). We found that TSHR mRNA levels of PTMC was significantly higher than that of benign thyroid nodules (P<0.01). Benign thyroid nodule has a greater value of TSHR mRNA compared with healthy control(P<0.01). Since there were only two cases of lymph node metastasis, we didn't compare the difference of TSHR mRNA between primary and metastatic PTMC. Furthermore, we determined that a cutoff

Table 1. Characteristics of the patients

	Healthy control	Benign nodules	PTMC
No of subjects	30	38	34
Age(median)	38	39	42
Female/male	2.0	2.5	2.8
Diameter of nodules /		0.4-1.0cm	0.1-1.0cm
Node positive	/	/	2
Distant metastasis	/	/	0

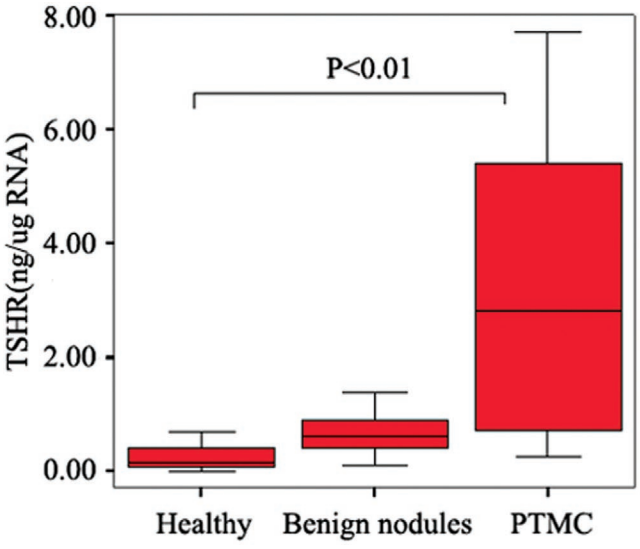

Fig 1. Real time PCR analysis of TSHR mRNA levels in healthy control, benign nodules and PTMC. The median values of TSHR mRNA and 25%-75% ranges are shown by boxed areas.

Table 2. TSHR mRNA levels in healthy control, benign nodules and PTMC

	Healthy control	Benigh nodules	PTMC
TSHRmRNA	0.15(0.048-0.40)	0.60(0.38-0.90)	2.80(0.72-5.45)
T value	/	648.50[a]	986.00[b]
P value	/	<0.01	<0.01

[a]compared with healthy control
[b]compared with benign nodules

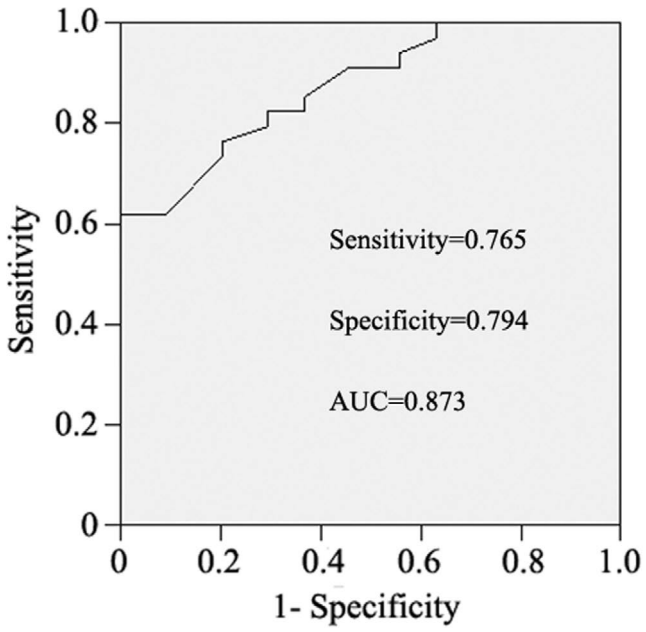

Fig 2. ROC curves for preoperative TSHR mRNA concentrations. A cutoff of 0.71 ng/μg total RNA is used to calculate diagnostic sensitivity and specificity that are listed along with the area under the curve (AUC).

TSHR mRNA value of 0.71 provided an optimal sensitivity of 76.5% and specificity of 79.4% respecitively according to the receiver operating characteristic (ROC) curve(Fig 2). With this cutoff, we could preoperatively correctly predict 26 of the total 34 PTMC patients and 30 of the 38 benign thyroid nodules patients. The positive predictive value(PPV) and negative predictive value(NPV) of TSHR in diagnosis of thyroid disease reached 65.0% and 75.0% respectively(Table 3). Only 1 of 30 healthy volunteers gave a false positive result, implying a 96.7% specificity in differentiating healthy people from PTMC patients.

Evaluation of the correlation of TSHR mRNA performance with the size of thyroid nodules and combined application with U/S. To further evaluate TSHR mRNA as a marker to differeciate PTMC from benign thyroid nodules, we compared the diagnostic performance of TSHR mRNA between patients with different size thyroid nodules. We found TSHR mRNA performs better in larger PTMC of 0.5-1.0 cm than less than 0.5cm in both sensitivity and specificity. When combined with U/S, TSHR mRNA yield a sensitivity as high as 97.1% and a specificity of 82.6% in diagnosis of PTMC(Table 3).

Table 3. Diagnostic performance of TSHR mRNA alone or in conjunction with U/S

	PTMC		Benign nodule	
	Sensitivity (%)	PPV (%)	Specificity (%)	NPV (%)
Total	76.5	65.0	79.4	75.0
Nodule size				
≤0.5cm	72.4	63.1	74.1	73.5
>0.5cm	83.4	72.3	88.5	79.2
TSHR+U/S	97.1	86.8	86.8	97.9

Discussion

Circulating tumor cells (CTCs) from solid tumors have been proved to be detectable in patient blood circulation and are considered a 'real-time' biopsy in monitoring the disease [17]. In this study, for the first time we investigated the application of quantatitive detection of TSHR mRNA using peripheral blood CTC in the diagnosis of PTMC. We demonstrate that this method is sensitive and specific in differenciating PTMC from benign thyroid nodules.

Use of TSHR mRNA as a marker in diagnosis of thyroid cancer has been reported before [12–14]. By optimizing the design of specific primers, Su-Ynn Chia et al has reported that measuring circulating TSHR mRNA with real time PCR enhances the preoperative detection of differentiated thyroid cancer in patients with thyroid nodules [18]. In this study, we used the same pair of primers to assess the value of TSHR mRNA in diagnosis of PTMC and correlated the TSHR mRNA levels directly with the final pathological result after surgery . We believe that such a molecular diagnosis could be a good compensation for current deficiency in preoperatively evaluating thyroid nodules less than 1.0cm. Though far from perfect, our data show quantitation of TSHR in peripheral blood circulation is sensitive and specific, especially for nodule between 0.5 to 1.0cm. More importantly, its sensivitity amounts to nearly 100% if combined with U/S. It's a promising method to help us discover PTMC preoperatively as early as possible.

Actually, other molecular markers such as thyroglobulin(Tg), MUC1, HMGI et al, have also been explored as alternative methods for detecting thyroid cancer with variable success and suffers from the lack of specificity [19–21]. However, TSHR has been proved to be a relatively highly sensitive and specific marker for both primary and recurrent thyroid cancer(16,18,22). Further work is needed to explore the combined use of different molecular markers in order to improve the diagnosis power of PTMC preoperatively.

Due to the limited patients recruitted into this study, we didn't make a comparison between PTMC at different stage such as node positive or distant metastasis even. But our study does show that the larger of the nodule, the more sensitive and specific of TSHR mRNA in diagnosis of PTMC. This is inconsistent with a previous report, which found no significant correlation between tumor size and TSHR mRNA levels in node-negative patients(18). We believed such a disparity is caused by the different size range of thyroid nodules each study focused on. When confined only nodules within 1.0cm, the PTMC might significantly express more TSHR mRNA than benign countparts and real time PCR used in our study

is able to capture the difference by a large margin within this range. That may explain why TSHR mRNA yields a better sensitivity in our study.

In conclusions, we have shown that quantitative TSHR mRNA assay is helpful in the preoperative diagnosis of PTMC, which is hard to differentiate from benign thyroid nodules by using FNA or U/S technology only. Surely, our assay is unlikely to replace the role of those conventional methods. Future studies are needed to explore the combined application of molecular marker with FNA and U/S in preoperatively detecting PTMC.

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