

Original Article

Identification of long non-coding RNA expression profile in tissue and serum of papillary thyroid carcinoma

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Abstract: To study long non-coding RNA (lncRNA) expression profile in papillary thyroid carcinoma (PTC), a total of 86 PTC patients treated with radical resection and 60 patients with benign thyroid nodules were enrolled. Quantitative reverse transcription-polymerase chain reaction was used to detect the expression of H19, HOTAIR (HOX Antisense Intergenic RNA), MALAT1 (Metastasis associated lung adenocarcinoma transcript 1) and GAS5 (Growth Arrest-Specific 5) in tissues and serum samples. Results showed that, H19, HOTAIR and MALAT1 expression were significantly up-regulated in cancerous tissues, while GAS5 was down-regulated. Clinicopathological analyses showed no correlations between lncRNAs' expression level and clinicopathological characteristics of PTC. Further, serum H19, HOTAIR and MALAT1 levels in PTC patients were all significantly higher than that in patients with benign thyroid nodules, their detectability to distinguish PTC from benign diseases are satisfactory, illustrated by receiver operating characteristics (ROC) curve. Finally, Knockdown of MALAT1 expression significantly inhibited cell proliferation and invasion in HO8910 and Ovar3 cells, western blot assays indicated that MALAT1 may exert its function by inhibition Akt-mediated signaling pathway. In conclusion, the expression levels of the candidate lncRNAs in PTC display a similar trend in other cancer type, circulating lncRNAs level may be a potential tumor marker for PTC diagnosis, and lncRNA MALAT1 acts as a functional oncogene in PTC cell line.

Keywords: lncRNA, papillary thyroid carcinoma, diagnosis, MALAT1

Introduction

Thyroid cancer constitutes about 1% of all epithelial malignancies worldwide. But, the incidence of thyroid cancer has been steadily increasing in China and many other countries, over the past few decades [1]. Papillary thyroid carcinoma (PTC) accounts for around 90% of these cases [2]. Although most PTC shows low malignancy and invasiveness, a few cases suffer recurrence, transformation and death, it is necessary to further study the pathogenesis of PTC. Consistent with the majority of malignant neoplasms, PTC is usually associated with specific genetic abnormalities, for example the T1799A BRAF mutation in the mitogen-activated protein kinase pathway [3, 4].

Long non-coding RNAs (lncRNAs) are kinds of transcriptional products of the eukaryotic genome that are composed of more than 200

nucleotides in length [5]. Growing evidences have suggested that lncRNAs are key regulators which governing various biological processes and dysregulation of lncRNAs is associated with various types of cancers [6, 7]. However, the clinical significance of lncRNAs in PTC has not yet been fully explored. Furthermore, circulating lncRNAs have been proved to be potential biomarkers for cancer diagnosis [8, 9]. Therefore, in the present study, we would like to invest lncRNAs' expression profile in tissue and serum of PTC. To select the most appropriate candidate lncRNA biomarkers in PTC, we reviewed the literatures about the expression levels of various lncRNAs in different cancer types. As a result, four lncRNA biomarkers were selected according to their functional importance in human cancers: H19, HOTAIR (HOX Antisense Intergenic RNA), MALAT1 (Metastasis associated lung adenocarcinoma transcript 1), GAS5 (Growth Arrest-Specific 5).

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Table 1. List of primers used in PCR

Gene Name	Primer Sequence (5'-3')	Tm (°C)
GAPDH	F: CAATGACCCCTTCATTGACC	55
	R: TGGAAGATGGTATGGGATT	
HOTAIR	F: GGCGGATGCAAGTTAATAAAAC	59
	R: TACGCCTGAGTGTTCCAGAG	
MALAT1	F: GGATCCTAGACCAGCATGCC	59
	R: AAAGGTTACCATAAGTAAGTTCCAGAAAA	
GAS5	F: CTTCTGGGCTCAAGTGATCCT	60
	R: TTGTGCCATGAGACTCCATCAG	
H19	F: ATCGGTGCCTCAGCGTTCGG	60
	R: CTGTCCTCGCCGTCACACCG	

Materials and methods

Study population

PTC tissues and paired adjacent non-tumor tissues were obtained from 86 patients treated with radical resection at our institutes from Jan 2013 to Oct 2014. Tumor stage was conducted according to the 7th edition of the TNM staging system of the International Union against Cancer. Cellular differentiation was graded according to the WHO grading system. For each patient, 5 mL peripheral blood pre-operation was collected by promoting coagulation tubes, then serum was isolated and stored at -80°C. Serum samples from 60 patients with benign thyroid nodules were also collected. Ethical approval was obtained from the hospital and informed consent was obtained from all patients prior to sample examination.

RNA isolation and qRT-PCR

Total RNA was extracted from tissues and serum samples using Trizol reagent (Invitrogen) according to the manufacturer's instructions. lncRNA expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using the SYBR®Green (TaKaRa) dye detection method on ABI StepOne PCR instrument, with GAPDH as an internal control. The primers are listed in **Table 1**. The expression levels of lncRNAs were calculated using ΔCt method, where $\Delta\text{Ct} = \text{Ct}_{\text{lncRNA}} - \text{Ct}_{\text{GAPDH}}$, smaller ΔCt value indicates higher expression [10]. $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate relative expression of lncRNAs.

Cell lines and lentivirus-mediated RNA interference

Thyroid tumor cell lines (8505C, BCPAP, FT-C133) and normal human thyroid cell line (HT-ori3) were cultured in RPMI-1640 medium supplemented with 10% calf serum, 0.1 mM non-essential amino acids, 1 mM sodium-pyruvate and 1% penicillin streptomycin in a 37°C humidified incubator with 5% CO₂. The following short hairpin RNA (shRNA) 19 was used to target human MALAT1: sense: 5-CACAGGGAAA-GCGA GTGGTTGGTAA-3' and antisense: 5'-TT-ACCAACCA CTCGCTTCCCTGTG-3'. The sequence of the negative control shRNA was 5'-TTCTCCGAAC GTGTCACGT-3'. These shRNAs were synthesized and inserted into the pFH-1UGW lentivirus core vector containing a cytomegalovirus (CMV)-driven enhanced green fluorescent protein (EGFP) reporter gene; expression of the shRNA was driven by the H1 promoter. Recombinant lentivirus expressing MALAT1 small interfering RNA (siRNA) or control siRNA (siMALAT1 or siCON) was designed and produced by GeneChem (Shanghai, China).

MTT assay

The cells were seeded onto 96-well plates at a density of 2×10^3 /well in a final volume of 100 μL . After incubation for 24, 48 and 72 h, 20 μL MTT (Merck Millipore, Billerica, MA, USA) (5 mg/ml) in phosphate-buffered saline was added to each well and the cells were incubated at 37°C for a further 4 h. A total of 150 μL dimethyl sulfoxide was added to the cells in each well after the supernatants were discarded. The absorbance of each well was measured at 490 nm using a microplate reader.

Colony formation assay

A total of one thousand cells were seeded into six-well plates with 2 mL culture medium. After cultured in RPMI 1640 media supplement with 10% FBS at 37°C and 5% CO₂ for 14 days, cells were washed twice with PBS, fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted. The cloning efficiency (%) = (the number of clones/the number of seed cells) $\times 100\%$.

Transwell assay

Transwell (24-well) chambers (Costar, Cambridge, MA, USA) were used to evaluate cell inva-

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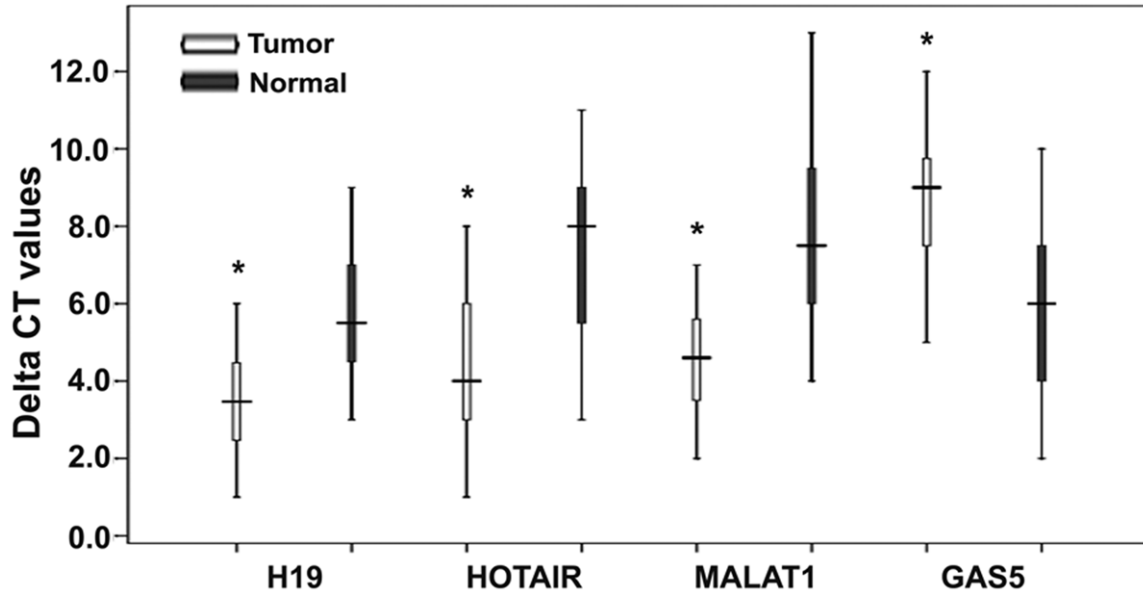


Figure 1. lncRNAs expression profile in human papillary thyroid carcinoma. Δ Ct method was used to calculate lncRNA expression, which was normalized to GAPDH, and smaller Δ Ct value indicated higher expression. Horizontal lines inside the box plots represent the median, boxes represent the interquartile range, and error bars represent 97.5th and 2.5th percentiles. * $P < 0.05$.

Table 2. Correlation between lncRNAs expression (Δ Ct, Mean \pm SD) in tissues and clinicopathological characteristics of 86 PTC patients

Variables	H19	HOTAIR	MALAT1	GAS5
Age				
<45 (n = 46)	5.519 \pm 1.173	6.345 \pm 3.232	6.395 \pm 1.478	10.783 \pm 2.013
\geq 45 (n = 40)	5.428 \pm 1.332	5.626 \pm 1.399	6.748 \pm 1.598	11.230 \pm 1.279
Gender				
Male (n = 31)	5.213 \pm 1.243	5.805 \pm 1.228	7.162 \pm 1.670	12.108 \pm 1.859
Female (n = 52)	5.624 \pm 1.434	6.322 \pm 1.675	6.220 \pm 1.402	10.361 \pm 1.770
Size				
<2 cm (n = 41)	5.119 \pm 1.134	6.184 \pm 1.292	7.100 \pm 1.282	10.172 \pm 1.713
\geq 2 cm (n = 45)	5.802 \pm 1.314	5.851 \pm 1.744	6.066 \pm 1.759	11.736 \pm 1.814
Lymph metastasis				
No (n = 62)	5.434 \pm 1.191	6.255 \pm 1.267	6.663 \pm 1.168	10.456 \pm 1.319
Yes (n = 24)	5.599 \pm 1.241	5.298 \pm 1.450	6.257 \pm 1.735	12.363 \pm 2.241
Calcification				
No (n = 45) 52.3	5.894 \pm 1.234	6.206 \pm 1.103	6.190 \pm 1.325	11.556 \pm 2.043
Yes (n = 41) 47.7	5.018 \pm 1.270	5.795 \pm 1.287	6.964 \pm 1.293	10.369 \pm 1.844

sion. Initially, fibronectin (2 μ g/filter) was dissolved in 100 μ l of MEM and poured into the upper part of the polyethylene filter (pore size, 8 μ m). The wells were coated overnight in a laminar flow hood. Then, 105 cells (in 100 μ L of growth medium) were added to the top of the filter in the upper well. The chamber was incubated for 24 h in 5% CO₂ at 37°C. Finally, attached cells in the lower section were stained with H&E, and counted using light microscopy.

Western blotting

Cell lysates were made with standard methods, then 20 μ g of protein samples were separated by 5% SDS-PAGE. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and blocked in 5% skim milk powder. The primary antibodies against CyclinD1, proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, matrix metalloproteinase 2

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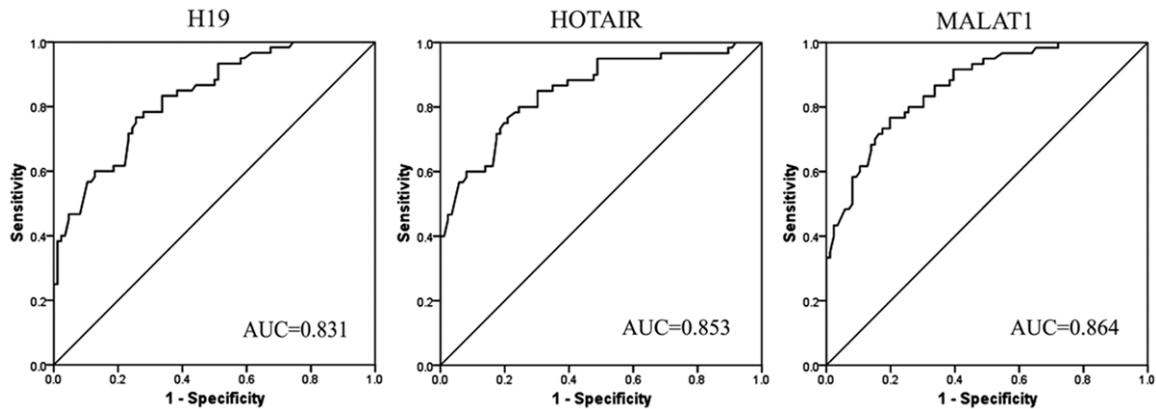


Figure 2. Evaluation of serum lncRNAs for detection of papillary thyroid carcinoma (PTC). Receiver operating characteristics (ROC) curves were drawn with the data of serum lncRNAs from 86 PTC patients and 60 patients with benign diseases.

(MMP-2), Akt, p-Akt and GAPDH were diluted according to the instructions of antibodies and incubated overnight at 4°C. Then, horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:1000 and incubated at room temperature for 2 h. The membranes were washed with PBS for three times, and the immunoreactive bands were visualized using ECL Plus Kit. Antibodies were purchased from Univ-bio Inc (Shanghai, China).

Statistical analysis

Statistical tests were carried out using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The differences of lncRNA expression between the groups were calculated with Student's t-test. Differences in frequency were assessed by Chi-square test. The receiver operating characteristics (ROC) curve was constructed to describe diagnostic specificity and sensitivity. $P < 0.05$ was taken as statistically significant.

Results

lncRNA expression profile in PTC tissues

The levels of H19, HOTAIR, MALAT1 and GAS5 were detected in 86 paired PTC tissues and adjacent normal tissues by qRT-PCR, and normalized to GAPDH. As compared with normal counterparts, H19, HOTAIR and MALAT1 expression was significantly up-regulated in cancerous tissues, while GAS5 was down-regulated ($P < 0.01$) (Figure 1). Further, clinicopathological analyses showed no correlations between lncRNA expression level and clinicopathological characteristics of PTC, such as patients' age,

gender, tumor size, lymph metastasis, intratumoral calcification (Table 2).

Serum lncRNA expression in PTC

We then detected the levels of H19, HOTAIR and MALAT1 in serums from 86 PTC patients and 60 patients with benign thyroid nodules. As expected, serum H19, HOTAIR and MALAT1 levels in PTC patients were all significantly higher than that in patients with benign thyroid nodules (ΔCt value: 4.56 ± 1.392 vs 7.17 ± 1.543 ; 5.74 ± 1.129 vs 8.22 ± 1.301 ; 3.65 ± 0.926 vs 5.82 ± 1.251 , respectively, $P < 0.001$). Then ROC curve was constructed to describe the diagnostic specificity and sensitivity of serum lncRNAs. Obviously, their detectability to distinguish PTC from benign diseases are satisfactory, area under the curves (AUC) of H19, HOTAIR and MALAT1 are 0.831 (95% CI: 0.758-0.912), 0.851 (95% CI: 0.776-0.932), 0.864 (95% CI: 0.786-0.945), respectively (Figure 2).

Knockdown of MALAT1 inhibits PTC cells proliferation and invasion

To investigate the functional role of MALAT1 in PTC cells, firstly, qRT-PCR was performed to detect the expression of MALAT1 in diverse PTC cell lines. As shown in Figure 3A, MALAT1 expression level was increased significantly in 3 cancer cell lines (8505C, BCPAP, FTC133), as compared with a human thyroid cell line (HT-ori3). Then MALAT1 siRNA was transfected in to BCPAP cell line, qPCR assays revealed that MALAT1 expression was significantly reduced (Figure 3B). MTT and colony-formation assay showed that knockdown of MALAT1 expression

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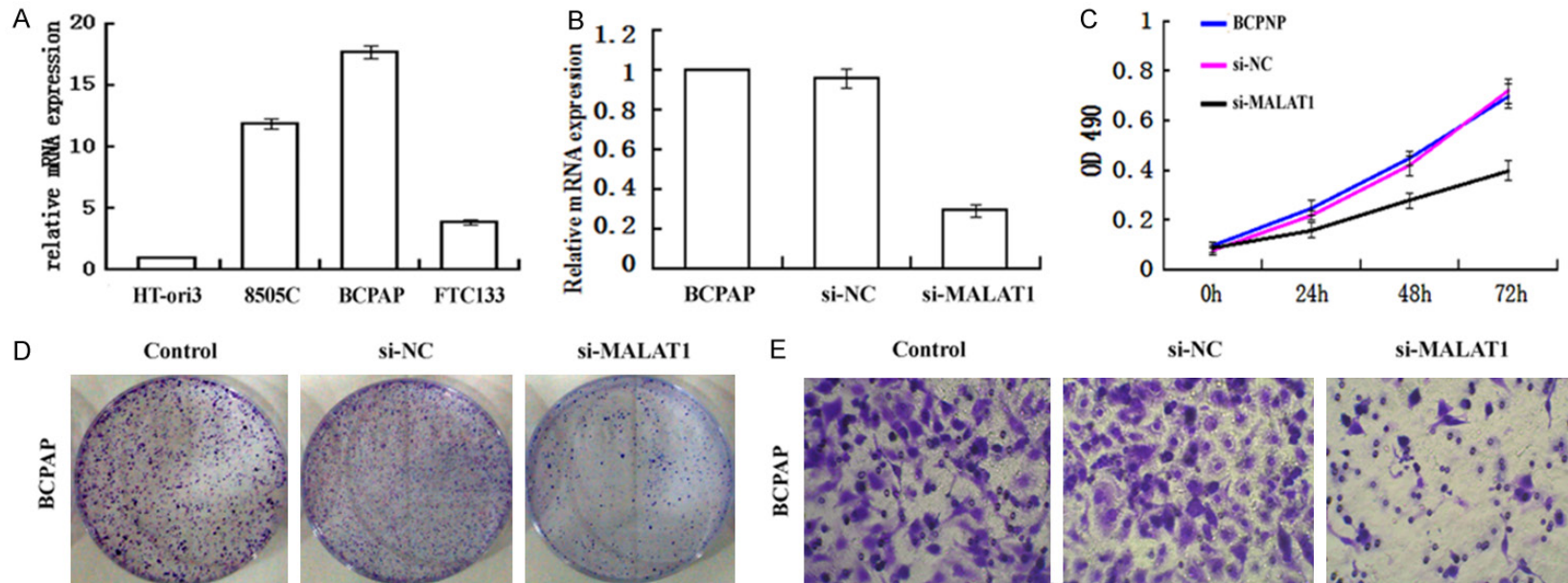


Figure 3. Knockdown of MALAT1 inhibits PTC cells proliferation and invasion in vitro. (A) Expression of lncRNA MALAT1 in 3 PTC cell lines (8505C, BCPAP, FTC133), and a human thyroid cell line (HT-ori3) was determined by qRT-PCR. (B) Knockdown efficiency was determined by qRT-PCR in BCPAP. Knockdown of MALAT1 in BCPAP cells significantly reduced their proliferative and invasive capacities, as determined by cell number counting assay (C), colony formation assay (D) and transwell assay (E).

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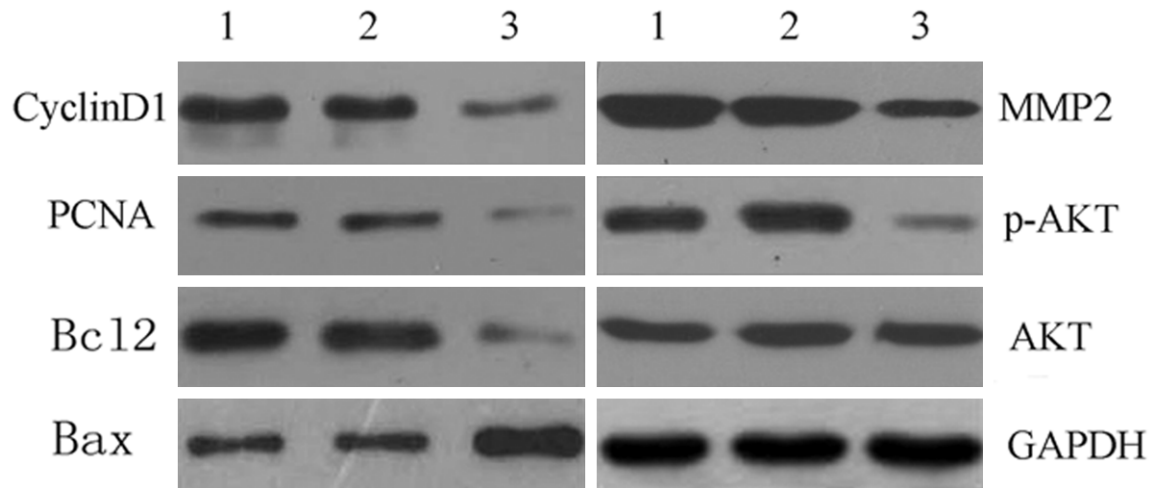


Figure 4. Mechanisms of lncRNA MALAT1 exerts its function. Signal pathway and key moderators in tumor progression were determined by western blotting.

significantly inhibited cell proliferation in BCPAP cell line compared with the controls (**Figure 3C, 3D**).

Next, transwell assays showed the number of BCPAP cells in lower section were significantly reduced in the MALAT1 knockdown group compared with the control groups (**Figure 3E**), which indicated that the expression of lncRNA MALAT1 promote cell invasion and metastasis.

Mechanisms of MALAT1 exerts its function

To analyze the expression of genes downstream of lncRNA MALAT1, western blot assays were performed (**Figure 4**). Consistent with functional characterization in vitro, knockdown of MALAT1 caused cell growth (CyclinD1, PCNA), metastasis (MMP2) and anti-apoptosis (Bcl-2) actors downregulated significantly in BCPAP cell line compared with the controls, while apoptosis factor (Bax) significantly upregulated. Moreover, p-AKT expression were significantly reduced in the MALAT1 knockdown group compared with the controls, while AKT protein expression were not changed, which indicated that MALAT1 may exert its function by inhibition Akt-mediated signaling pathway.

Discussion

With the growing use of diagnostic imaging, such as neck ultrasonography, the number of thyroid nodules identified is steadily growing. Nevertheless, only 5% of all thyroid nodules harbor malignancy; therefore, preoperative dif-

ferentiation of benign from malignant thyroid nodules is imperative [11]. Patients with suspicious thyroid nodules should undergo ultrasound-guided fine-needle aspiration biopsy (FNAB), which is a safe, straightforward and sensitive diagnostic procedure that represents an accepted standard of practice. However, FNAB continues to be limited in the differential diagnosis of indeterminate lesions, which are found in up to 20% of aspirations [12]. Only 20%-30% of the indeterminate lesions harbor malignancy and this means that up to 80% of patients with cytologically indeterminate thyroid nodules undergo thyroidectomy solely for the purpose of diagnosis. Obviously, unnecessary thyroidectomies harbor risks like laryngeal nerve injury, hypoparathyroidism, and the need for lifelong thyroid replacement. Molecular markers may help to diagnose, MicroRNA, DNA methylation or BRAF mutation status is being sought in PTC [13, 14]. In fact, the clinical application of diagnostic biomarkers remains to be realized for patients with indeterminate thyroid nodules. Growing evidences have suggested that lncRNAs are key regulators which governing various biological processes and dysregulation of lncRNAs is associated with various types of cancers [6, 7].

Increasing studies suggest that lncRNAs constitute an important component of tumor biology, representing regulatory functions including modulation of apoptosis and invasion, reprogramming of induced pluripotent stem cells, marker of cell fate and parental imprinting [5,

15]. Deregulation of individual lncRNA expression not only involve in development and progression of specific cancers, but also be significant molecules for clinical implication [16]. lncRNAs utilize varied mechanism to regulate gene expression. In general, the mechanism lncRNA regulating gene expressing could be transcriptional or post-transcriptional. Out of numerous lncRNAs, a few well characterized lncRNAs to date such as HOTAIR, H19, MALAT1, Xist, ANRIL, MEG3, and GAS5 were extensively studied in human cancers [17-20]. However, the role of lncRNAs in thyroid cancer is just beginning to be elucidated and there is a long way to go. A recent study found a novel long intergenic noncoding RNA gene PTSC2 (papillary thyroid cancer susceptibility candidate 2) was down-regulated in PTC tumors [21]. To further determined lncRNA expression profile in PTC, H19, HOTAIR, MALAT1 and GAS1 were selected. H19, one of the imprinting-associated lncRNAs, has been observed to be deregulated in hepatocellular and bladder cancer and involve in both oncogenic and tumor suppressive qualities [22, 23]. HOTAIR is found to be significantly upregulated in breast cancer and hepatocellular carcinoma and can be served as an independent predictor of prognosis [24, 25]. Abnormal expression of MALAT1 was investigated in various human cancers including breast, lung, pancreas, prostate, liver, colon, and ovarian cancer [26, 27]. Silencing of MALAT1 expression in lung cancer cells can impair cell migration ability by regulation of motility-related genes [28]. GAS5 comprises 12 exons and encodes 10 box C/D snoRNAs within its introns. Several studies found that GAS5 sensitizes cells to apoptosis and induces growth arrest [29]. In addition, the GAS5 transcript levels were found to be significantly reduced in breast and lung cancer samples compared with adjacent unaffected normal tissues [30, 31].

In the present study, we found H19, HOTAIR and MALAT1 expression were significantly up-regulated in cancerous tissues, while GAS5 was down-regulated. Further, clinicopathological analyses showed no correlations between lncRNAs' expression level and clinicopathological characteristics of PTC, which indicated that dysregulation of these lncRNAs is an early event in carcinogenesis. Thus, they can be used as potential biomarkers for PTC early diagnosis. The stability of lncRNAs in plasma/serum has been confirmed in many previous studies,

and circulating lncRNAs have also been proved as useful biomarkers for cancer diagnosis and prognosis [8, 10]. As expected, serum H19, HOTAIR and MALAT1 levels in PTC patients were all significantly higher than that in patients with benign thyroid nodules, their detectability to distinguish PTC from benign diseases are satisfactory, illustrated by ROC curve.

Functional roles of MALAT1 in PTC still remain unclear; we further explored the effects of loss of function on various aspects of PTC cell biology. Firstly, BCPAP cell line with higher MALAT1 expression was chosen. RNAi-mediated suppression of lncRNA MALAT1 significantly inhibited cells proliferation, migration and invasion. Thus, lncRNA MALAT1 plays an oncogenic role in PTC and represents a potential target for PTC treatment. As expected, western blotting showed that MALAT1 regulates the expression of factors in cell growth, invasion and apoptosis. Downregulation of p-Akt indicated that MALAT1 may exert its function by inhibition Akt-mediated signaling pathway [32]. Additional investigations are required to understand the exact molecular mechanisms by which lncRNA MALAT1 regulates these genes and signaling pathways.

In conclusions, the expression levels of the candidate lncRNAs in PTC display a similar trend in other cancer type, circulating lncRNAs level may be a potential tumor marker for PTC diagnosis, and lncRNA MALAT1 acts as a functional oncogene in PTC cell line.

Disclosure of conflict of interest

None.

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