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miR-199a-3p downregulation in thyroid tissues is associated with invasion and metastasis of papillary thyroid carcinoma

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ABSTRACT

Background and aims: miR-199a-3p may play an important role in tumour inhibition. The aim of the present study was to determine any link between miR-199a-3p expression in thyroid tissues and clinicopathologic features, as well as to assess potential usefulness of miR-199a-3p in prediction for invasion and metastasis of papillary thyroid carcinoma (PTC).

Methods: A total of 188 tissue samples (136 PTCs, 52 normal thyroid tissue) were collected. We measured the levels of miR-199a-3p with quantitative reverse-transcriptase PCR (RT-qPCR) in all subjects. In addition, the correlation between the expression levels of miR-199a-3p and clinicopathological factors was explored.

Results: qRT-PCR indicated that the expression levels of miR-199a-3p was 7.1 (95% CI, 3.9–12.4) in PTCs, which was significantly lower than that of in the normal thyroid tissues 31.4 (95% CI, 15.4–44.3) (p = 0.002). Receiver operating characteristic curve (ROC) analyses revealed that miR-199a-3p could be promising biomarkers for PTC, with relatively high area under the curve (AUC) values was 0.87 (95% CI, 0.66–0.90; p = 0.001). Low miR-199a-3p expression levels were linked to TNM stage (p = 0.026), extra-thyroidal extension (p = 0.02), lymph node (LN) metastasis (p = 0.036), distant metastasis (p = 0.002) and recurrence of LN metastasis (p = 0.03).

Conclusions: Our data suggest that downregulation of miR-199a-3p in thyroid tissues is linked to invasion and metastasis of PTC and may be a potential target for therapeutic intervention.

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KEYWORDS
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Introduction

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy, accounting for more than 80% of all thyroid cancers [1]. In general, the prognosis of patients with PTC is believed to be relatively good. However, lymph node (LN) metastasis or aggressive tumour behaviour, such as local invasion and distant metastasis, are seen in some cases. Cervical LN metastasis is common in PTC; it is associated with recurrence and is a prognostic factor in older patients [2]. Some study results have shown that clinicopathologic factors including extrathyroidal extension, tumour size, sex, multifocality, lymphatic invasion, and desmoplastic reaction are associated with poor prognosis of PTC [3–5]. The focus of the present research was the identification of biomarkers indicating the aggressiveness of PTC.

MicroRNAs (miRNAs, miRs) play critical roles in the regulation of genes involved in controlling tumour pathogenesis and progression. miRs bind to the 3′-untranslated region (3′-UTR) of specific gene mRNAs and participate in the regulation of many biological functions in tumour cells [6]. miRNAs involvement in human cancer is well established: they represent a new level of gene regulation and a potential tool as diagnostic, prognostic and therapy response biomarkers [7]. The main miRNAs alteration in cancer cells is abnormal expression. Accordingly, recent reports defined miRNA profiles that could discriminate between PTC and normal thyroid tissue, suggesting their value as diagnostic markers [8, 9].

miR-199a is a phylogenetically conserved miRNA whose precursors miR-199a-1 and miR-199a-2 map in human genome to different loci, respectively on chromosome 19 and on chromosome 1. From both hairpin precursors, two mature sequences are produced: miR-199a-5p and miR-199a-3p. MiR-199a-2 is also reported as a member of miR-199a-2/214 cluster [10].

MicroRNA-199a-3p (miR-199a-3p) is downregulated in several human malignancies, such as ovarian carcinoma, hepatocellular carcinoma, colorectal cancers, osteosarcoma and prostate cancer [11–16]. Clinical and pathological survey indicated that high expression of miR-199a-3p was significantly associated with deep wall invasion in

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colorectal cancer [14]. However, in osteosarcoma, miR-199a-3p downregulation plays an important role in the development of metastasis and recurrence [15]. In prostate cancer (PCa), the expression level of miR-199a-3p is inversely correlated to tumour stage and Gleason score of PCa [16]. Minna et al. [17] reported that miR-199a-3p showed under-expression in PTC specimens and cell lines. Furthermore, miR-199a-3p restoration in PTC cells impairs migration and proliferation, suggesting miR-199a-3p as a tumour suppressor miRNA in PTC.

We believe that assessment of miR-199a-3p may be a useful tool in the prediction of invasion and metastasis of PTC. Accordingly, we hypothesis that the expression of miR-199a-3p in PTC is linked to key clinicopathological indicators.

Materials and methods

136 snap-frozen PTCs and 52 normal thyroid were studied. All the patients were diagnosed by fine needle aspiration cytology and histopathological examination before surgery. The normal thyroid samples were obtained from patients with pathologies other than thyroid cancer. All the tissue samples were taken from the surgical specimens immediately after excision and directly snap frozen in liquid nitrogen, stored at −80 °C and later used for extraction of RNA. The mean tumour size was 17.3 mm. All analysed specimens were sampled from primary tumours treated surgically at the Department of Thyroid surgery of the affiliated hospital of Qingdao University between January 2013 and December 2015. Each patient signed an informed consent form prior to their surgical procedure, in which they approved the collection of fresh thyroid samples to be used for medical research. The clinicopathologic variables collected included sex, age, tumour size, extra-thyroidal extension(structures involved), extra-thyroidal extension (surrounding structures involved: Perithyroid muscle and soft tissues only, Recurrent laryngeal nerve, Trachea, Esophagus, Carotid artery sheath and Jugular vein), encapsulation, multicentricity, LN metastasis and distant metastasis. We classified the cancer stage according to the TNM staging system of the seventh edition of the AJCC Cancer Staging Manual (followed the NCCN guidelines on thyroid carcinoma).

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and finally resuspended in 60 µL of pre-heated (95 °C) nuclease-free water. The measurement of the expression levels of miR-199a-3p was performed using miRNA sequence specific primers (Applied Biosystems Inc., Foster City, CA) by the real time-RT-PCR-based detection methodology. Briefly, 10 ng of total RNA was reverse transcribed using High-Capacity cDNA Archive kit (Applied Biosystems Inc., Foster City, CA) followed by amplification on ABI 7500 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). All RT-PCR reactions were performed in triplicate Small nucleolar RNA RNU6 was used as endogenous control for the normalisation of RNA input. miR-199a-3p expression levels were calculated by relative quantitation using the ABI 7500 Real-Time PCR SDS 1.2 software (Applied Biosystems Inc., Foster City, CA).

The data were analysed statistically using the statistical package for social sciences (SPSS) software version 22 (SPSS Inc., USA). Mann–Whitney test was used to compare the miR-199a-3p levels between two groups of subjects. The association of the miR-199a-3p levels with the clinicopathological parameters of PTC patients was assessed using chi-squared test. Receiver's operating characteristic (ROC) curve was constructed to determine the discriminating efficacy of the miR-199a-3p between healthy individuals and patients with PTC. Differences of $p < 0.05$ were considered statistically significant.

Results

The clinicopathological characteristics of the subjects are shown in Table 1. The two groups were age and sex matched. The expression of miR-199a-3p in 136 PTC tissues and in 52 normal thyroid tissues as determined by quantitative RT–PCR are shown in Figure 1. The expression levels of miR-199a-3p was 7.1 (95% CI 3.9–12.4 in PTCs, significantly lower than that of in the normal thyroid tissues 31.4 (95% CI, 15.4–44.3) ($p = 0.002$).

To determine the diagnostic value of miR-199a-3p in PTCs, an ROC curve was constructed (Figure 2). Comparing the relative miR-199a-3p levels in controls and patients, the AUC was 0.87 (95% CI, 0.66–0.90; $p = 0.001$). According to the ROC curve, the relative miR-199a-3p of 8.24 was defined to be the optimal cutoff value for differentiating PTC patients and controls (Youden's index). With this cutoff value for miR-199a-3p, the sensitivity, specificity, positive and negative predictive values and accuracy values of 68.4% (95% CI, 56.7–78.3), 100% (95% CI, 76.3–99.5), 100% (95% CI, 84.6–99.6), 40.3% (95% CI, 29.4–60.2) and 75.3% (95% CI, 61.8–80.6), respectively, were achieved. These results clearly showed that the detection of miR-199a-3p in the tissues should provide a new complementary tumour marker for PTC.

To determine whether down-regulated miR-199a-3p can distinguish aggressive from non-aggressive phenotype of PTC, we determined a cutoff value of 8.24 to distinguish high miR-199a-3p value patients from low value patients. At this level, 93 patients were categorised into the low miR-199a-3p group, and 43 patients were categorised into the high miR-199a-3p group. The miR-199a-3p level and clinical and pathological characteristics in PTCs was showed in Table.1. Low miR-199a-3p expression was associated with TNM stage ($p = 0.026$), extra-thyroidal extension ($p = 0.02$), LN metastasis ($p = 0.036$), distant metastasis ($p = 0.002$) and recurrence of LN metastasis ($p = 0.03$). These results clearly showed that the detection of miR-199a-3p in the PTC could reflect metastasis and predict prognosis.

Table 1. Associations between miR-199a-3p level and clinicopathologic factors in PTCs.

Clinicopathological feature	Number	miR-199a-3p levels		*p* value
		High	Low	
Sex				0.437
Male	29	15	14	
Female	107	78	29	
Age (year)				0.543
≤50	55	24	31	
>50	81	69	12	
TNM stage (AJCC)				0.026
I + II	47	39	8	
III + IV	89	54	35	
Tumour size (cm)				0.194
≤1	31	21	10	
1<, <2	73	50	23	
≥2	32	22	10	
Extra-thyroidal extension				0.006
Structures involved	23	13	10	
Surrounding structures involved	29	8	21	
No extra-thyroidal extension	84	72	12	
Encapsulation				0.214
Yes	28	9	19	
No	108	84	24	
Multicentricity (the number of PTCs was >2				0.317
Yes	33	19	14	
No	103	74	29	
Lymph node (LN) metastasis				0.036
Yes	110	73	37	
No	26	20	6	
Distant metastasis				0.002
Yes	8	1	7	
No	128	92	36	
Recurrence of LN metastasis				0.03
Yes	12	2	10	
No	124	91	33	

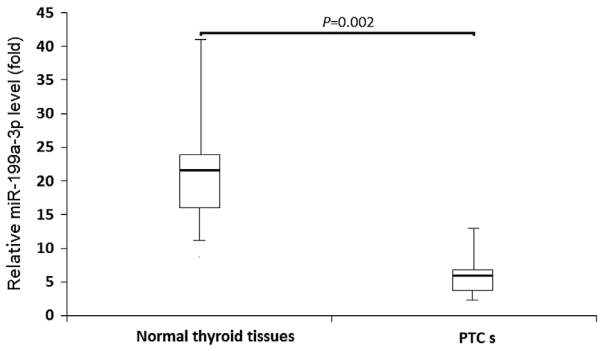

Figure 1. The expression levels of miR-199a-3p in PTC tissues and normal thyroid tissues.

The levels of miR-199a-3p were significantly lower in cancer tissues (p = 0.002). The upper and lower limits of the boxes and the lines inside the boxes indicate the 75th and 25th percentiles and the median, respectively. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively.

Discussion

With the increased diagnosis of predominantly indolent PTC, biomarkers that can efficiently select the minority of patients with aggressive tumours are urgently needed. This would allow personalised treatment planning to adequately treat the minority with aggressive tumours and avoid overtreatment in the majority with indolent disease.

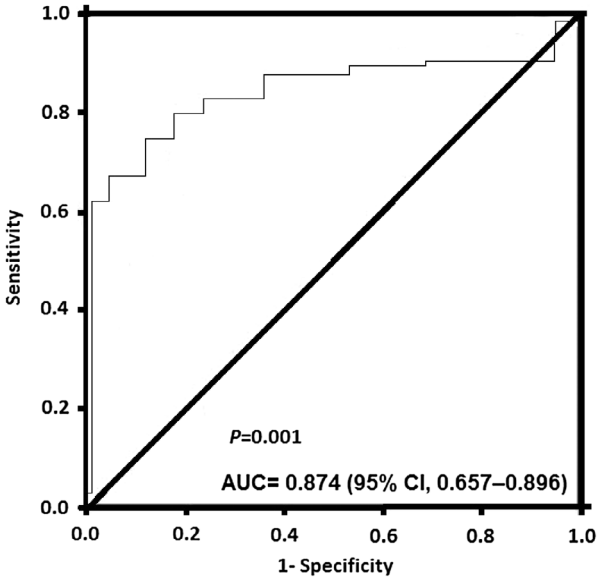

Figure 2. The role of miR-199a-3p in PTC diagnosis.

The receiver-operating characteristic curve analysis of miR-199a-3p expression levels for discriminating PTC (n = 136) and controls (n = 52).

Fine needle aspiration biopsy (FNAB) and cytological assessment have been a cornerstone of diagnostic thyroid nodule management since the 1980s and this basic preoperative assessment has substantially reduced the number of patients sent for diagnostic surgery for nodules that ultimately prove to be benign [18]. Large FNAB studies applying the United States National Cancer Institute (NCI) Bethesda classification scheme have confirmed the malignancy rates for follicular neoplasm or suspicious for follicular neoplasm and suspicious for malignancy cited at the NCI Conference, but occurrence of malignancy for patients undergoing diagnostic surgery for atypia of undetermined significance or follicular lesion of undetermined significance was much more variable (7–48%), suggesting that watchful waiting might not be the best approach [19]. Ideally, FNAB and cytological interpretation should be done by specialists with much experience of the procedures to reduce the variability noted in the atypia of undetermined significance or follicular lesion of undetermined significance category, but because of the many patients with thyroid nodules undergoing assessment, this recommendation might not be practical.

Identification of suitable molecular markers to guide surgery or watchful waiting for patients with indeterminate FNAB of thyroid nodules has been the so-called holy grail of thyroid nodule research for more than 20 years. Many potential markers and combinations of markers have been studied in thyroid tissues and FNAB specimens [20, 21].

Several studies have assessed the usefulness of different molecular-marker tests to predict final histopathological findings in many patients with preoperative

indeterminate FNAB cytology [22, 23]. The most studied marker is BRAF. The $BRAF^{V600E}$ mutation has been shown to be associated with aggressive clinical behaviours of PTC by some investigators [24]; however, the evidence is still inconclusive, with contradictory reports [25]. Other molecular markers being investigated include cell-cycle regulators p27, p21, and cyclin D1 and immunohistochemical markers such as CEACAM-1, OPN, and E-Cadh [26, 27]. None of these markers have reached wide acceptance in clinical practice so far, including BRAF.

He et al. were the first to apply a miRNA profiling capability to PTC [28]. One of the significant findings was that in PTC, the key differentially expressed miRNAs appeared to be overexpressed, although global under-expression of miRNA was normally associated with cancers in general. This finding has since been confirmed in other reports [29, 30]. Pallante et al. also showed that miR-221 and miR-222 were significantly overexpressed in PTC tissue compared with normal thyroid tissue of the contralateral, unaffected lobe [31]. In their study, the third overexpressed miRNA reported was miR-181b [31]. Further confirmation of these findings was achieved by quantitative RT-PCR comparing the miRNA precursors in an external cohort of 39 PTC and 8 follicular adenoma tissue samples.

Ren et al. has recently reported that upregulation of miR-21 was associated with poor clinicopathological characteristics in patients with osteosarcoma [32]. In patients with pancreatic ductal adenocarcinoma (PDAC), serum miR-124 levels were significantly decreased in these patients and serum miR-124 levels have utility as diagnostic biomarkers in patients with PDAC [33]. In the present study, we found miR-199a-3p was low expressed in the PTC tissues than the normal thyroid tissue, which could identify individuals with PTC to exclude healthy individuals. ROC analysis identified miR-199a-3p as being suitable for use as PTC biomarkers. Notably, miR-199a-3p showed reliable diagnostic specificity and sensitivity.

Following reports on the role that miRNAs play in PTC tumourigenesis, and thus their potential diagnostic utility, investigation also progressed to testing the prognostic ability of miRNA expression profiling. Yip et al. demonstrated that miR-146b and miR-222 were overexpressed in aggressive PTC, defined as PTC associated with distant metastasis or recurrence. They also demonstrated downregulation of miR-34b and miR-130b in the PTC samples associated with aggressive biology [34]. Lee et al. reported that miR-146b and miR-222 were overexpressed in PTC with recurrence and were more strongly associated with PTC recurrence than BRAF expression [35]. miR-199a-3p, a miR previously reported to be decreased in hepatocellular carcinoma [12] and osteosarcoma [15]. Furthermore, miR-199a-3p may have a tumour suppressor function in human osteosarcoma and PTC [15, 17]. In the current study, we found that decreased miR-199a-3p expression in PTC specimens with advanced disease stage and indicated that it may be a useful prognostic marker for PTC. In addition, we have shown that decreased miR-199a-3p expression in PTC cells was easily metastasised to regional LN or distant organs or recurrence of LN metastasis as well as involved in extra-thyroidal extension, further supported that decreased miR-199a-3p is involved in PTC invasion and metastasis.

This study represents an advance in biomedical science because it shows that downregulation of miR-199a-3p is associated with thyroid tumour invasion and metastasis and it may be a potential target for therapeutic intervention.

Disclosure statement

No potential conflict of interest was reported by the authors.

Summary table

What is known about this subject

- A tissue biomarker is needed for diagnosis and discrimination in PTC.
- miR-199a-3p is down-expressed in tissues in PTC.
- Whether tissue miR-199a-3p could predict invasion and metastasis of PTC is unknown.

What this study adds

- miR-199a-3p was down-expressed in tissues in PTC.
- miR-199a-3p could discriminate PTC from normal controls.
- Down-regulation of miR-199a-3p is associated with invasion and metastasis in PTCs.

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