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MiR-7-5p and *miR-451* as diagnostic biomarkers for papillary thyroid carcinoma in formalin-fixed paraffin-embedded tissues

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MiR-7-5p and *miR-451* are important members of the small RNA family, which have been shown to be significantly downregulated in various human tumors and play a key role in the occurrence and development of tumors. However, little is known about their role in endocrine malignancies. This study aimed to investigate the diagnostic value of *miR-7-5p* and *miR-451* levels in formalin-fixed paraffin-embedded tissues of papillary thyroid carcinoma (PTC) patients, as well as the relationship between clinicopathological characteristics and the two miRNAs. Quantitative real-time PCR (qRT-PCR) was performed to detect the expression levels of *miR-7-5p* and *miR-451* in 101 PTC tissues and in 40 nodular goiter tissues (controls). *MiR-7-5p* and *miR-451* levels were significantly downregulated in PTC patients compared with controls ($P < 0.001$). *MiR-7-5p* expression was further downregulated in tumors with larger diameter and advanced tumor stages (all $P < 0.05$). Moreover, the two miRNAs showed great capability of discriminating PTC patients from controls with 89.5% *(miR-7-5p)* and 76.8% (*miR-451*) diagnostic accuracy. Furthermore, according to univariable/multivariate logistic regression, *miR-7-5p* was significantly associated with PTC ($P < 0.001$). In conclusion, *MiR-7-5p* and *miR-451* may be used as potential diagnostic biomarkers for identification and validation of PTC patients. Moreover, *miR-7-5p* appears to be associated with the aggressiveness of PTC.

1. Introduction

Thyroid carcinoma (TC) is the most frequent malignancy of the endocrine system with a steadily increasing incidence rate recorded over recent years (Yu et al. 2016). According to the American cancer society, there will be 53,990 newly diagnosed TC cases in the USA by 2018, resulting in 2,060 deaths (Siegel et al. 2017). Moreover, by 2030, TC will replace the colorectal cancer as the fourth leading cancer diagnosis (Rahib et al. 2014). Based on different degrees of differentiation, TC includes four major histological subtypes: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), poorly differentiated thyroid carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (Kondo et al. 2006). Among these, PTC remains the most prevalent subtype, accounting for more than 80% of all cases (Yuksel et al. 2008). Although the majority of PTC patients have a good prognosis, approximately 5% of PTC cases manifest invasive tumors, such as capsule invasion, lymph node metastasis, advanced stage of tumor, and 5-20% of patients who have undergone thyroidectomy show signs of regional recurrence, which contribute to poor prognosis and shorter overall survival (Hua et al. 2016; Tang et al. 2015). Hence, it is of utmost importance to investigate the mechanism underlying carcinogenesis and progression in PTC and to develop potential diagnostic biomarkers that could identify high-risk aggressive PTC in the early stages.

MicroRNAs (miRNAs or miRs) are a class of small (19-25 nucleotides), endogenous, non-protein-coding, single-stranded RNAs. They were firstly discovered in *Caenorhabditis elegans* in the 1990s (Mattick 2001), and currently there are more than 2,000 miRNAs sequenced in humans (Fuziwara and Kimura 2017). By binding to the 3′-untranslated regions (3′-UTR) of specific target mRNA, miRNAs regulate post-transcription gene expression, and have an important role in several biological functions, either in physiological or pathological conditions (Boufraqech et al. 2016). Plentiful studies have indicated that miRNAs modulated many cellular processes, such as growth, senescence, proliferation, differentiation, invasion, apoptosis, migration and cell cycle regulation (Boufraqech et al. 2016; Mutalib et al. 2016; Cao et al. 2016). Aberrant expression of miRNAs is closely related to human cancer initiation and progression (Aragon Han et al. 2015), including thyroid carcinoma (Liu et al. 2017). Additionally, miRNAs functioning as diagnostic and therapeutic tools have been reported in various cancers due to their peculiar biological functions, and they could be measured in both formalin-fixed paraffin-embedded (FFPE) tissues and serum or other biological materials due to their relatively stable property (Wójcicka et al. 2016). *MicroRNA-7 (miR-7)* is a 23 nucleotide (nt), duplex, evolutionarily conserved

Abbreviations
3′-UTR: 3′-Untranslated Regions; AJCC: American Joint Committee on Cancer; ATC: Anaplastic thyroid carcinoma; EMT: Epithelial-to-mesenchymal transition; FFPE: Formalin-fixed paraffin-embedded; FNAB: Fine-needle aspiration biopsies; FTC: Follicular thyroid carcinoma; LNM: Lymph node metastasis; *miR-7: microRNA-7; miR-451: microRNA-451*; nt: nucleotide; PDTC: poorly differentiated thyroid carcinoma; PTC: papillary thyroid carcinoma; qRT-PCR: quantitative real-time PCR; ROC: receiver operating characteristic curve; TC: thyroid carcinoma; TNM: Tumor-Node-Metastasis

miRNA which contains *miR-7-5p* and *miR-7-3p* strand, where the former one is most commonly investigated, and usually referred to simply as "*miR-7*" (Horsham et al. 2015b). Multiple studies have verified that *miR-7-5p*, functioning as a tumor suppressor gene, is downregulated in various cancers and is involved in decreasing cell proliferation and metastasis by regulating target molecules (Wang et al. 2016; Bi et al. 2017). *MicroRNA-451(miR-451)* is also characterized as a critical tumor suppressor which could inhibit invasion and metastasis through reversing the epithelial-to-mesenchymal transition (EMT) process (Chen et al. 2014). Additionally, studies have revealed that the downregulation of *miR-451* is negatively correlated with certain aggressive clinicopathological features of cancers, such as lymph node metastasis (LNM), as well as Tumor-Node-Metastasis (TNM) stages (Wang et al. 2011). However, to date, little is known about the expression levels of *miR-7-5p* and *miR-451* in FFPE tissues of thyroid carcinoma patients and about their clinical significance.

The aims of the present study were to investigate the expression of *miR-7-5p* and *miR-451* in FFPE tissues of PTC patients; to analyze the association between the two miRNAs and clinicopathological characteristics in PTC patients, and to confirm the diagnostic value of *miR-7-5p* and *miR-451* as potential molecular biomarkers for PTC.

2. Investigations and results

2.1. Expression and correlation of miR-7-5p and miR-451 in FFPE tissues of PTC patients

Both *miR-7-5p* and *miR-451* expression levels were significantly downregulated in PTC patients compared with controls ($P < 0.001$, Fig. 1a and 1b). The median level of *miR-7-5p* was 0.028 in PTC patients, and 0.193 in controls; and the median level of *miR-451* was 0.035 and 0.110 in PTC patients and in controls, respectively (Table 1). Afterwards, Spearman correlation analysis indicated high association between *miR-7-5p* and *miR-451* expression as illustrated in the scatter plot (r = 0.537, $P < 0.001$, Fig. 1c).

Table 1: M*iR-7-5p* and *miR-451* expression levels and their diagnostic significance in papillary thyroid carcinoma

	Median in cancer patients	Median in controls	*P*-value	AUC	Cutoffs	Sensitivity (%)	Specificity (%)
miR-7-5p	0.028	0.193	< 0.001	0.895	0.0712	80.2	82.5
miR-451	0.035	0.110	< 0.001	0.768	0.0507	62.4	82.5

Abbreviations: AUC, area under the curve

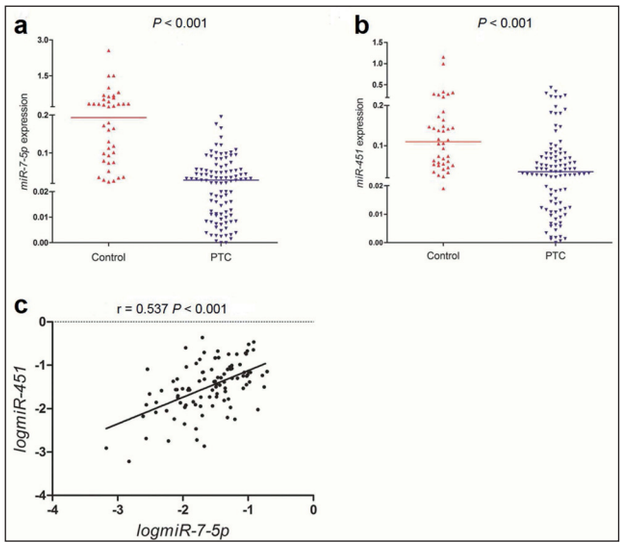

Fig. 1: PTC tissues exhibit low levels of miR-7-5p and miR-451 expression. a MiR-7-5p and b miR-451 expression levels in PTC tissues and nodular goiter (control) tissues; median expression level of each miRNA in both control and PTC group were represented as marked. c The Spearman correction analysis implied high association between miR-7-5p and miR-451, both expression levels presented after log10 transformation, with r = 0.537, $P < 0.001$ between the two miRNAs as indicated in the scatter plot. PTC: papillary thyroid carcinoma.

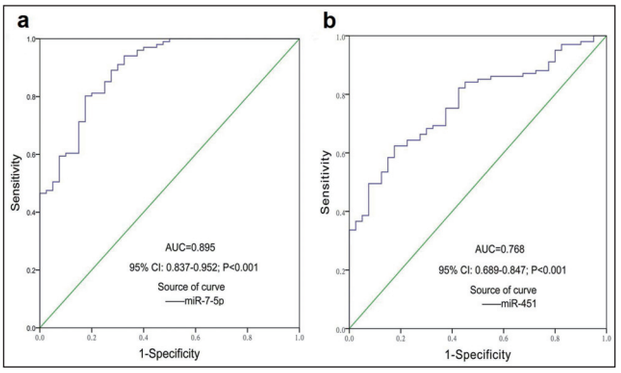

Fig. 2: ROC curve analysis for the expression of miR-7-5p and miR-451 in PTC patients and controls. The AUC indicates the accuracy for distinguishing PTC patients from controls in terms of sensitivity and specificity. a ROC curve of miR-7-5p showed 0.895 AUC with 80.2% sensitivity and 82.5% specificity, $P < 0.001$. b ROC curve of miR-451 showed 0.768 AUC with 62.4% sensitivity and 82.5% specificity, $P < 0.001$. AUC, area under the ROC curve; ROC, receiver-operator characteristic.

2.2. High specificity and sensitivity of miR-7-5p and miR-451 for determination of papillary thyroid carcinoma

Next, ROC analysis was employed to evaluate the diagnostic capability of *miR-7-5p* and *miR-451* expression for discriminating between 101 PTC patients and 40 controls. As shown in Table 1 and Fig. 2, *miR-7-5p* indicated 0.895 AUC (95% CI: 0.837, 0.952, $P < 0.001$), and the results showed 80.2% sensitivity and 82.5% specificity in distinguishing PTC patients from controls at the cut-off value of 0.0712 (Fig. 2a); while *miR-451* showed 0.768 AUC (95% CI: 0.689, 0.847, $P < 0.001$) with 62.4% sensitivity and 82.5% specificity at the cut-off value of 0.0507 (Fig. 2b).

2.3. Relationship between miR-7-5p and miR-451 expression and clinicopathological characteristics in PTC patients

According to the cut-off values of *miR-7-5p* and *miR-451*, patients were divided into two groups: low (< 0.0712) / high (> 0.0712) *miR-7-5p* expression level group, and low (< 0.0507) / high (> 0.0507) *miR-451* expression level group, respectively. Interestingly, significantly lower *miR-7-5p* level was observed in tumors with larger diameter, compared with subgroup with smaller tumors [90.2% (37/41) vs. 73.3% (44/60), P=0.044]. Additionally, according to AJCC stage, III/IV PTC patients showed significantly lower *miR-7-5p* expression than I/II PTC patients [93.3% (28/30) vs. 74.6% (53/71), $P = 0.032$]. Yet, there was no significant difference between low and high expression groups of *miR-7-5p* in gender, age, lymph node metastasis, capsule invasion, and multifocality ($P > 0.05$, Table 2). Also, no significant association was observed between *miR-451* low and high groups and these clinicopathological characteristics ($P > 0.05$, Table 2).

2.4. Low-expression of miR-7-5p as an independent risk factor of increasing the development of PTC

M*iR-7-5p* and *miR-451* expression levels were also significantly correlated with PTC by univariable logistic regression ($P < 0.001$, Table 3). Additionally, the correlation between *miR-451* and PTC was greatly attenuated in multivariate regression analysis which incorporated two selected miRNAs, while *miR-7-5p* still appeared closely associated with PTC ($P < 0.001$, Table 4).

Table 2: Clinicopathological characteristics and expression of *miR-7-5p* and *miR-451* in papillary thyroid carcinoma

Patients' characteristics	Cases-n	*miR-7-5p* expression level			*miR-451* expression level		
		Low-N (%)	High-N (%)	*P*	Low-N (%)	High-N (%)	*P*
Gender							
Male	18	13(72.2)	5(27.8)	0.343	10(55.6)	8(44.4)	0.594
Female	83	68(81.9)	15(18.1)		53(63.9)	30(36.1)	
Age (years)							
<45	48	36(75)	12(25)	0.225	30(62.5)	18(37.5)	>0.999
≥45	53	45(84.9)	8(15.1)		33(62.3)	20(37.7)	
Tumor diameter*(cm)							
<1.9	60	44(73.3)	16(26.7)	0.044#	36(60)	24(40)	0.676
≥1.9	41	37(90.2)	4(9.8)		27(65.9)	14(34.1)	
AJCC Stage							
I/II	71	53(74.6)	18(25.4)	0.032#	43(60.6)	28(39.4)	0.656
III/IV	30	28(93.3)	2(6.7)		20(66.7)	10(33.3)	
Lymph node metastasis							
Absent	41	32(78)	9(22)	0.8	24(58.5)	17(41.5)	0.537
Present	60	49(81.7)	11(18.3)		39(65)	21(35)	
Capsule invasion							
Absent	69	54(78.3)	15(21.7)	0.596	45(65.2)	24(34.8)	0.508
Present	32	27(84.4)	5(15.6)		18(56.2)	14(43.8)	
Multifocality							
Absent	78	63(80.8)	15(19.2)	0.772	52(66.7)	26(33.3)	0.141
Present	23	18(78.3)	5(21.7)		11(47.8)	12(52.2)	

Notes: *Median of tumor diameter at surgery was 1.9 cm in this cohort.
#$P < 0.05$, relative to high expression group by Chi-square test or Fisher's exact test.
Abbreviations: AJCC, American Joint Committee on Cancer.

3. Discussion

Papillary thyroid carcinoma is the most prevalent subtype of thyroid carcinoma, thus contributing to the increasing incidence of thyroid malignancies (Lee et al. 2014). There is plenty of evidence that the aberrant expression of miRNAs is associated with initiation and progression of PTC, and thus could be used as a diagnosis and prognosis tool for better management of PTC patients (Wei et al. 2016). In PTC, *miR-221, miR-222, and miR-146,* which are the most studied miRNAs, have been shown to be significantly up-regulated in PTC compared with benign tissues. In addition, they have been shown to be associated with aggressive pathological features, such as larger tumor diameter, lymph node metastasis, and advanced AJCC stage (Chruścik and Lam 2015; Yip et al. 2011). Little is known about *miR-7* and *miR-451* expression and potential clinical value in FFPE tissues of PTC patients. In the present study, we detected the expression levels of *miR-7-5p* and *miR-451* in FFPE tissues of PTC patients, and we explored their potential diagnostic value in PTC.

Over recent decades, studies have suggested that *miR-7-5p* is very important for development of organs such as brain, and pancreatic islets (Horsham et al. 2015a). *MiR-7-5p* has shown to be significantly downregulated, and to act as tumor suppressor in a series of malignancies such as lung cancer (Zhao et al. 2015), colorectal carcinoma (Nagano et al. 2016), adrenocortical carcinoma (Glover et al. 2015), breast cancer (Shi et al. 2015), carcinoma of uterine cervix (Hao et al. 2015). Yue et al. (2016) have confirmed that *miR-7* expression is significantly downregulated in fresh frozen tissues of 32 PTC patients, compared with normal adjacent tissues and healthy thyroid tissues, as well as human PTC cell lines by qRT-PCR. Consistent with these results, in the present study we also confirmed that *miR-7-5p* was significantly downregulated in FFPE tissues of PTC patients, compared with controls. *MiR-451*, an important member of miRNAs family, located on chromosome 17 at 17q11.2, has shown to be widely dysregulated in various human cancers (Li et al. 2015a). Li et al. (2015b) have found that plasma *miR-451a* is elevated in the PTC cases, compared with benign nodules or the healthy controls. Zhang et al. (2017) have confirmed that *miR-451* is significantly downregulated in both FFPE tissues and sera from PTC patients, compared with controls. Consistent with Zhang's results, using FFPE tissues, we revealed that *miR-451* expression level was significantly downregulated in PTC patients compared with controls.

Table 3: Univariable logistical model of *miR-7-5p* and miR-451

	Regression coefficient (β)	SE	Wald.χ2	*P*-value	Exp (β)
miR-7-5p	-2.949	0.485	36.935	< 0.001	0.052
miR-451	-2.056	0.464	19.632	< 0.001	0.128

Abbreviations: SE, standard error; Exp, odd ratio value; β, beta

Table 4: Multivariate logistical model of *miR-7-5p* and miR-451

	Regression coefficient (β)	SE	Wald.χ2	*P*-value	Exp (β)
miR-7-5p	-2.520	0.520	23.492	< 0.001	0.080
miR-451	-1.036	0.545	3.609	0.057	0.355

Abbreviations: SE, standard error; Exp, odd ratio value; β, beta

Additionally, only one study has investigated the diagnostic accuracy of *miR-7* and *miR-451* in thyroid fine-needle aspiration biopsies (FNAB) reporting that in up to 30% of cases it is difficult to distinguish between benign or malignant thyroid neoplasms (Kitano et al. 2011). In the present study, we performed the ROC curve to assess the diagnostic ability of *miR-7-5p* and *miR-451* expression, where the AUC was 0.895 (95% CI: 0.837, 0.952) in *miR-7-5p*, while *miR-451* showed 0.768 AUC (95% CI: 0.689, 0.847), both of them having high specificity and sensitivity. Our results suggested that both these miRNAs had potential diagnostic value, and may serve as molecular biomarkers for identification of PTC patients, which was also consistent with previous study. Moreover, we also explored the relationship between *miR-7-5p* and *miR-451* and PTC using univariable/multivariate logistic regression. The results of both univariable and multivariate logistic regression suggested that *miR-7-5p* was significantly correlated with PTC. These finding confirmed that the *miR-7*-5p might be an independent risk factor promoting the development of PTC. These results suggested that *miR-7* and *miR-451* may have a vital role in PTC tumorigenesis.

Several studies have reported that the expression of *miR-7* and *miR-451* are associated with certain aggressive clinicopathological characteristics, emphasizing the role in the metastasis cascade of the two miRNAs (Wang et al. 2013). Hua et al. *(2016)* have demonstrated that *miR-7* expression level is negatively correlated with tumor size and numbers of metastatic lymph nodes in 20 snap-frozen PTC tissues. Moreover, it has been shown that advanced stage of thyroid tumors have lower expression level of *miR-7* compared with early stage tumors (Yue et al. 2016). Partially consistent with these results, in our experiment, low-expression of *miR-7-5p* level was related with larger tumor diameter and advanced cancers. These findings suggested that *miR-7-5p* was associated with partly aggressive features of PTC, and thus might have a potential role in tumor initiation and progression. On the other hand, Wang et al. (2013) have found that *miR-451* is significantly upregulated in PTC with lymph node metastasis compared with PTC with non-lymph node metastasis. Nonetheless, in the present study, there was no significant association between *miR-451* expression and lymph node metastasis in PTC patients. These results could be explained with different sample sizes in two studies.

In conclusion, the downregulations of *miR-7-5p and miR-451* in FFPE tissues of PTC patients could serve as potential diagnostic biomarkers for discovery and identification of papillary thyroid carcinoma. *MiR-7-5p* may be conducive to identify some aggressive traits of PTC that could be used for better management of papillary thyroid carcinoma.

4. Experimental

4.1. Patients and clinical specimens

This study was approved by the Ethic Committee of the Affiliated People's Hospital of Jiangsu University. A total of 141 FFPE tissue specimens were examined including 101 PTC and 40 nodular goiters as controls. All FFPE tissues used in this study were from patients who underwent thyroidectomy from May 2012 to October 2016 at the Affiliated People's Hospital of Jiangsu University. None of the patients had received chemotherapy or radiation therapy before surgery. Patient consent and specimen collection were performed according to the guidelines of the Affiliated People's Hospital's protocol. The diagnosis of PTC was confirmed for all 101 patients based on clinicopathological findings which were verified as PTC by two pathologists. Additionally, the classifications of PTC patients were based on the TNM system of American Joint Committee on Cancer (AJCC). All the clinical and pathological information of patients were obtained from electronic medical records and are displayed in Table 2. Informed consent was obtained from all individual participants included in the study.

4.2. RNA extraction and reverse transcription

Total RNA was isolated from FFPE tissues of 101 PTC patients and 40 controls using the RecoverAll™ Total Nucleic Acid Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was generated by reverse transcription using miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

4.3. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out according to the manufacturer's instructions using the miScript SYBR Green PCR kit (Qiagen, Hilden, Germany) with miScript Universal primer and miRNA-Specific forward primer: 5′-GGAAGACTAGTGATTTTGTTGT-3′ (*miR-7-5p*), 5′-AACCGTTACCATTACTGAGTT-3′ (*miR-451*) on ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification procedure was as follows: an initial denaturation step of 95 °C for 15 min followed by 40 cycles of a denaturation step at 94 °C for 15 s, an annealing step at 55 °C for 30 s, and an extension step of 72 °C for 34 s. At the end of the PCR cycles, melting program (95 °C for 15 s, 60 °C for 60 s and 95 °C for 15 s) was performed to validate the specificity of the expected PCR product. The relative expression levels of *miR-7-5p* and *miR-451* were calculated by the comparative $2^{-\Delta\Delta Ct}$ method using *U6* (Forward: 5′-GTGCTCCCTGCTTCGGCAGCACATATAC-3′, Reverse: 5′-AAAAATATGGAACGCTTCACGAATTTG-3′) small nuclear RNA level for internal control. All samples were performed in triplicates.

4.4. Statistical analysis

All statistical analyses were calculated using SPSS version 20.0 (IBM SPSS Inc, Chicago, IL, USA) and Prism version 5.0 (GraphPad software, California, USA). The Mann-Whitney test was carried out to analyze the differential expression of *miR-7-5p* and *miR-451* between PTC patients and controls. Spearman correlation coefficient was used to analyze the correlation of *miR-7-5p* and *miR-451* expression. The receiver operating characteristic curve (ROC) analysis was utilized to assess diagnostic significance of the two miRNAs. The Chi-square test or Fisher exact test was used to further analyze the correlation between *miR-7-5p* and *miR-451* expression and clinicopathological characteristics. The correlation between PTC and the selected miRNAs was explored using univariable logistic regression, together with multivariable logistic regression. All *P*-values were two-sided, and statistically significance was defined as *P*-value less than 0.05.

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Conflicts of interest: None declared.

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