

## ORIGINAL ARTICLE

# Diagnostic Value of Exosomal miR-148a-3p in the Serum of Patients with Differentiated Thyroid Cancer

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### SUMMARY

**Background:** The current study aims to evaluate the expression and diagnostic value of exosomal miR-148a-3p in the serum of DTC patients.

**Methods:** Exosomes were isolated from the serum of DTC patients and were identified using a transmission electron microscope. RT-PCR was performed to determine the level of exosomal miR-148a-3p in DTC patients. The possible target gene of miR-148a-3p was determined using dual luciferase reporter assay. ROC analysis was performed to determine the diagnostic value of exosomal miR-148a-3p in DTC patients.

**Results:** We identified a novel exosomal miRNA, exosomal miR-148a-3p, that was significantly decreased in the serum of DTC patients compared with that of benign thyroid tumor patients and healthy controls. Further study showed that exosomal miR-148a-3p was correlated with the malignant characteristics of DTC, including tumor diameter, lymph node metastasis (LNM), and higher TNM staging. Dual luciferase reporter assay indicated that IGF-1 was a target gene of miR-148a-3p. ROC analysis demonstrated that the AUC of exosomal miR-148a-3p was better than TgAb and Tg in DTC patients. More importantly, combined use of exosomal miR-148a-3p, TgAb, and Tg significantly enhanced the sensitivity and specificity, indicating exosomal miR-148a-3p is a sensitive biomarker in DTC patients.

**Conclusions:** Altogether, reduced exosomal miR-148a-3p was associated with the risk of DTC and may be used as a biomarker for the diagnosis of DTC.

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#### KEY WORDS

exosome, miR-148a-3p, differentiated thyroid cancer, serum

#### INTRODUCTION

Differentiated thyroid cancer (DTC) is characterized by an innocuous clinical course, accounting for 90% of all thyroid cancer [1]. However, the quality of life and survival rate are significantly reduced for DTC patients with distant metastases (DMs) [2]. Due to the occult onset of DTC, the clinical manifestations and imaging features intersect with benign thyroid lesions, which lead to a certain misdiagnosis rate and missed diagnosis rate [3,4]. Therefore, non invasive biomarkers with high sensitivity and specificity that can help identify DTC

are of great importance [5].

Exosomes are small membrane vesicles measuring 50 - 100 nm in diameter that are secreted from cells and are key regulators in intercellular communication [6]. Tumor cells secreting excessive amounts of exosomes that carry mRNA, microRNA (miR), and proteins could communicate signals to local and remote cells and tissues [7]. Accumulating evidence has shown that exosomes carrying miRs play key roles in the progression of tumors [6,8,9]. For instance, plasma exosomal miR-146b-5p and miR-222-3p are suggested to be potential biomarkers for lymph node metastasis (LNM) in papillary thyroid cancer (PTC) [10]. However, we still lack knowledge about the role of exosomal-derived miRs in the progression of DTC.

In the present study, we mainly focused miR-148a-3p, which is found to be differentially expressed in different tumors, including prostate cancer and glioblastoma [11, 12]. However, the role of miR-148a-3p in DTC patients has never been explored. The current study aims to evaluate the expression and diagnostic value of exosomal miR-148a-3p in the serum of DTC patients.

## MATERIALS AND METHODS

### Patient samples

Eighty patients with thyroid disease admitted to Tengzhou Central People's Hospital from March 2018 to August 2019 were selected as the study subjects. Inclusion criteria: 1. age  $\geq$  18 years old; 2. diagnosis confirmed by clinical examination, thyroid function test, imaging examination or cytology examination. Exclusion criteria: 1. with a history of thyroid surgery; 2. with hyperthyroidism or hypothyroidism and chronic lymphocytic thyroiditis. According to the results of fine needle aspiration and histology after surgery, 80 patients with thyroid diseases were divided into DTC and benign thyroid nodule. Among the DTC patients, 29 were male and 11 were female, with an average age of  $64.87 \pm 4.13$  years (45 - 72 years). Among the patients with benign thyroid nodule, 28 were male and 12 were female, and the average age was  $65.02 \pm 5.01$  years. There was no significant difference in age and gender between DTC and benign thyroid nodule ( $p > 0.05$ ). In addition, 50 healthy controls were recruited at the physical center of Tengzhou Central People's Hospital at the same time. Details of all the participants were shown in Table 1. Blood samples (5 mL) from elbow vein were collected and centrifuged at 1,500 g for 15 minutes. Serum was separated and stored in refrigerator at  $-70^{\circ}\text{C}$  for future use. The study was approved by the ethics committee of the hospital, and all patients signed the informed consent.

### Isolation of exosomes

GET™ Exosome Isolation Kit (GET301-10, Genexosome Technologies, Freehold, NJ, USA) was used to isolate exosomes according to the instructions. Presence of isolated EV were validated using an HT-7700 trans-

mission electron microscope (Hitachi High-Technologies, Tokyo, Japan) (bar = 50 nm).

### RNA isolation

To extract RNA from the exosomes, a RNeasy Micro Kit (Qiagen, Hilden, Germany) was used in strict accordance with the instructions. The concentration and the purity of RNA samples were determined by measuring the optical density (OD) 260/OD280.

### qPCR

RNA reverse transcription was performed according to the instructions of QuantiTect Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). SYBR Green Super mix (Bio Rad Laboratories, Inc., Hercules, CA, USA) was used for quantitative PCR according to the instructions. PCR reaction system was as follows:  $95^{\circ}\text{C}$  for 30 seconds, 45 cycles of 5 seconds at  $95^{\circ}\text{C}$  and 30 seconds at  $60^{\circ}\text{C}$ . Relative miRNA expression was normalized to U6 using the  $2^{-\Delta\Delta\text{Cq}}$  method [13].

### Dual luciferase reporter assay

To search for the possible target gene of miR-148a-3p, TargetScan (<https://www.targetscan.org>) was used. The 3'untranslated region (3'UTR) of Insulin-like growth factor-1 (IGF-1) containing the binding site of miR-148a-3p was cloned into the pmirGLO plasmid. After 293T cells were seeded for 24 hours, miR-148a-3p or scramble were cotransfected with blank pmirGLO or pmirGLO-IGF-1-3'UTR using vigofect (Vigorous, Beijing, China) according to the instructions. The luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (E1910; Promega).

### Statistical analysis

Data were expressed as the mean  $\pm$  standard error. Each experiment was carried out with three replicates. Multiple comparisons were performed using one-way analysis of variance followed by Tukey's multiple comparison test. ROC analysis was carried out to explore the diagnostic value of exosomal miR-148a-3p in the serum of DTC patients.  $p < 0.05$  was considered to indicate a statistically significant difference. The data were analyzed using SPSS software, version 20.0 (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY, USA).

## RESULTS

### Isolation of exosomes from the serum of DTC patients

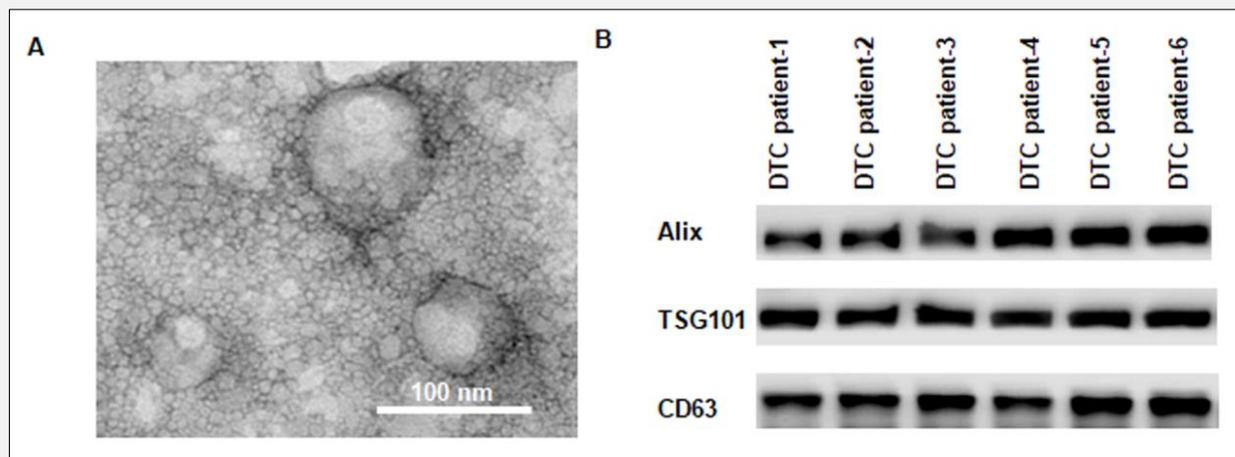
As shown in Figure 1A, the exosomes were isolated from the serum samples of DTC patients, with the diameter around 100 nm. Western blot assay showed that the protein markers of exosomes, including Alix, TSG-101 and CD63, were identified in the serum samples of DTC patients (Figure 1B).

**Table 1. Demographic and clinical features of PTC patients, benign nodule patients, and healthy control individuals.**

Variables	DTC (n = 40)	Benign nodule (n = 40)	Healthy control (n = 50)
Gender (M/F)	29/11	28/12	32/18
Age (years)	64.87 ± 4.13	65.02 ± 5.01	63.48 ± 6.32
Tumor size			
≤ 2 cm	28	38	
> 2 cm	12	2	
LN metastasis			
Yes	18		
No	22		
Multifocal tumor			
Yes	15		
No	25		
TNM stage			
I/II	26		
III/IV	14		
Tg (ng/mL)	158.24 ± 20.86	90.15 ± 10.37	5.67 ± 1.34
TgAb (IU/mL)	1,165.29 ± 230.50	632.78 ± 150.10	50.45 ± 10.87
TSH (mIU/mL)	60.35 ± 10.97	12.22 ± 3.45	0.56 ± 0.12

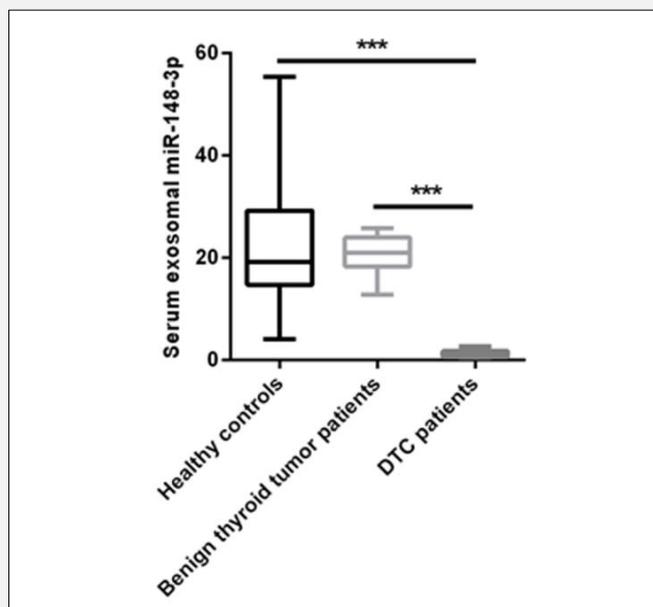
**Table 2. Correlation between exosomal miR-148a-3p and clinical features in DTC patients.**

Variables	Exosomal miR-148a-3p	p
Gender		0.458
M	1.40 ± 0.70	
F	1.10 ± 0.84	
Age		0.888
≤ 42	1.25 ± 0.36	
> 42	1.19 ± 0.94	
Tumor diameter		< 0.001
≤ 2 cm	1.76 ± 0.61	
> 2 cm	0.86 ± 0.65	
Capsular infiltration		0.876
Yes	1.24 ± 0.42	
No	1.18 ± 0.87	
Lymph node metastasis		< 0.001
Yes	1.71 ± 0.66	
No	0.87 ± 0.57	
TNM staging		< 0.001
I/II	1.94 ± 0.55	
III/IV	0.89 ± 0.58	



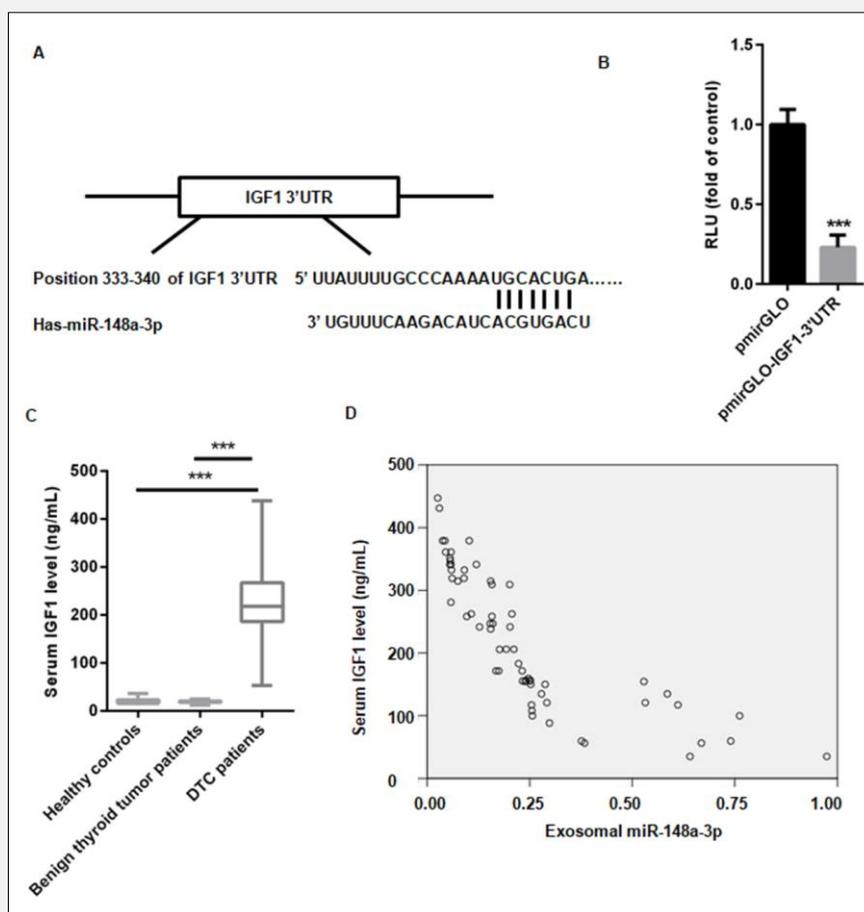
**Figure 1. Exosomes were isolated from the serum of DTC patients.**

(A) Transmission electron microscope showed that the exosomes were isolated from the peritoneal serum samples of DTC patients, with the diameter around 100 nm. (B) Western blot assay showed that the protein markers, including Alix, TSG101 and CD63, were identified in the serum samples of DTC patients.



**Figure 2. RT-PCR analysis showed that exosomal miR-148a-3p was significantly decreased in the serum of DTC patients compared with that of benign thyroid tumor patients and healthy controls.**

\*\*\*  $p < 0.001$  vs. as indicated.



**Figure 3.** IGF-1 was a target gene of miR-148a-3p.

(A) Based on TargetScan, a conserved binding site was identified in the 3'UTR of IGF-1, a well-known oncogenic gene in thyroid cancer. (B) Dual luciferase reporter assay showed that miR-148a-3p significantly suppressed the relative luciferase activity of pmirGLO-IGF-1-3'UTR. (C) The level of serum IGF-1 was significantly enhanced in DTC patients compared with that of benign thyroid tumor patients and healthy controls. (D) Pearson's correlation assay indicated that exosomal miR-148a-3p negatively correlated with serum IGF-1. \*\*\* p < 0.001 vs. as indicated.

**Decreased exosomal miR-148a-3p in the serum of DTC patients**

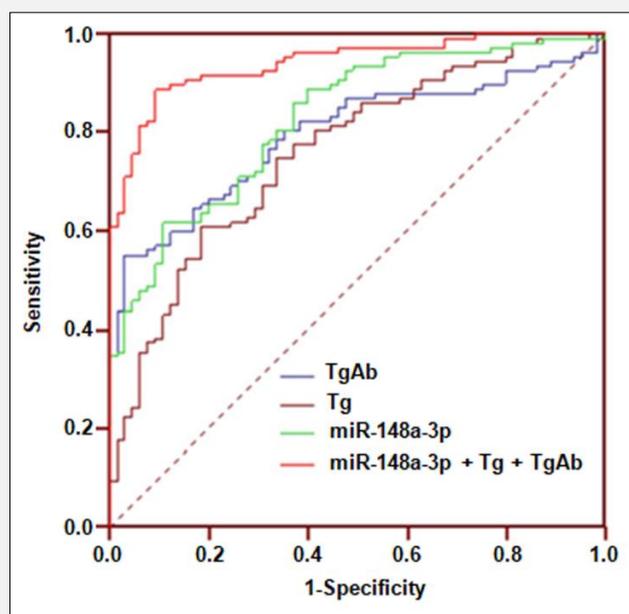
After isolation of exosomes from the serum of DTC patients, we quantified the level of exosomal miR-148a-3p in DTC patients. As shown in Figure 2, exosomal miR-148a-3p was significantly decreased in the serum of DTC patients compared with that of benign thyroid tumor patients and healthy controls. But no significant difference of exosomal miR-148a-3p was found between benign thyroid tumor patients and healthy controls (Figure 2).

**Correlation between exosomal miR-148a-3p and clinical features in DTC patients**

We then analyzed the level of exosomal miR-148a-3p according to the clinical characteristics of DTC patients. As shown in Table 1, the level of exosomal miR-148a-3p was not related to gender, age, and capsular infiltration in DTC patients. In contrast, significant reduction of exosomal miR-148a-3p was identified in DTC patients with tumor diameter > 2 cm, lymph node metastasis (LNM), and higher TNM staging (Table 2).

**IGF-1 was a target gene of miR-148a-3p**

These above findings lead us to further explore the possible target gene of miR-148a-3p. Based on TargetScan, a conserved binding site was identified in the 3'UTR of



**Figure 4.** ROC analysis was carried out to explore diagnostic efficiency of exosomal miR-148a-3p in DTC patients.

IGF-1, a well-known oncogenic gene in thyroid cancer [14] (Figure 3A). Dual luciferase reporter assay showed that miR-148a-3p significantly suppressed the relative luciferase activity of pmirGLO-IGF-1-3'UTR (Figure 3B). These data indicated that IGF-1 was a target gene of miR-148a-3p. We then evaluated the contents of serum IGF-1 in DTC patients, benign thyroid tumor patients and healthy controls. As shown in Figure 3C, the level of serum IGF-1 was significantly enhanced in DTC patients compared with that of benign thyroid tumor patients and healthy controls. Pearson's correlation assay indicated that exosomal miR-148a-3p negatively correlated with serum IGF-1 ( $r = -0.756$ ,  $p < 0.001$ ) (Figure 3D).

#### Diagnostic efficiency of exosomal miR-148a-3p in DTC patients

Previous studies have indicated that TgAb and Tg are important biomarkers for the diagnosis of DTC patients [15,16]. We then compared the diagnosis efficiency of exosomal miR-148a-3p with TgAb and Tg in DTC patients and benign thyroid tumor patients. ROC analysis showed the AUC of exosomal miR-148a-3p was 0.828 (95% CI: 0.763 - 0.881), with the sensitivity and specificity of 88.8% and 90.8%, respectively. The AUC for Tg was 0.795 (95% CI: 0.727 - 0.853), with the sensitivity and specificity of 55.1% and 96.9%, respectively. In addition, the AUC of TgAb was 0.759 (95% CI: 0.688 - 0.821), with the sensitivity and specificity of

60.7% and 81.5%, respectively. In contrast, the AUC for combined use of exosomal miR-148a-3p, TgAb, and Tg was 0.941 (95% CI: 0.894 - 0.971), with the sensitivity and specificity of 88.8% and 90.8%, respectively. Obviously, combined use of exosomal miR-148a-3p, TgAb, and Tg improved the diagnostic efficiency between DTC patients and benign thyroid tumor patients.

## DISCUSSION

The incidence rate of DTC is high, and the etiology of the disease is complex, which may be related to many factors such as diet, environment and heredity [17,18]. Because of the occult disease and the lack of typical clinical manifestations, it is easily confused with benign nodules [19]. Therefore, early differential diagnosis of DTC is the focus of clinicians.

Exosomes are small vesicles that are released by cancer cells and transfer mRNA, microRNA, and proteins from donor cells to recipient cells [20,21]. In the present study, we identified a novel exosomal miRNA, exosomal miR-148a-3p, that was significantly decreased in the serum of DTC patients compared with that of benign thyroid tumor patients and healthy controls. Further study showed that exosomal miR-148a-3p was correlated with the malignant characteristics of DTC, including tumor diameter, LNM, and higher TNM staging. These data suggested that exosomal miR-148a-3p

plays a key role in carcinomatosis in DTC patients. Furthermore, we explored the possible target gene of miR-148a-3p in the progression of DTC. For the first time, we found IGF-1 was a target gene of miR-148a-3p. The oncogenic role of IGF-1 has been widely reported in various cancers [22,23]. In thyroid cancer, significantly higher concentrations of IGF-1 were identified compared with that of controls [24]. We propose that decreased exosomal miR-148a-3p promoted the progression of cancer by enhancing the production of IGF-1 in DTC patients.

TgAb is an important thyroid tissue antibody, which is an important indicator for the diagnosis of thyroid diseases [25]. Its level is closely related to the degree of thyroid function damage and serum TGAb was a risk factor for DTC [26]. Tg is a glycoprotein secreted by thyroid follicular epithelial cells, which is the precursor of thyroxine synthesis and is an important tumor marker in DTC patients [27,28]. However, both benign and malignant thyroid diseases may lead to the increase of Tg level, but the serum Tg value of some thyroid cancer patients may also be normal [29]. Therefore, the serum Tg value does not have specificity between benign and malignant thyroid diseases [30]. Here, we analyzed the diagnostic value of exosomal miR-148a-3p. Our data showed that the AUC of exosomal miR-148a-3p was better than TgAb and Tg in DTC patients. More importantly, combined use of exosomal miR-148a-3p, TgAb, and Tg significantly enhanced the sensitivity and specificity, indicating exosomal miR-148a-3p is a sensitive biomarker in DTC patients.

Here, it is interesting to consider whether this new marker can be used in standard clinical care, especially when calculating the work load and the costs of determination of exosomal miR-148a-5p. At present, the isolation of exosomes and the quantification of miR-148a-5p are costly when we use the commercial kits. For clinical application, the reduction of costs is necessary. In addition, large samples are necessary to validate the diagnostic value of exosomal miR-148a-5p in thyroid cancer patients.

In summary, reduced exosomal miR-148a-3p was associated with the risk of DTC and may be used as a biomarker for the diagnosis of DTC.

#### Declaration of Interest:

We declare no conflicts of interest.

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