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ORIGINAL ARTICLE

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Circulating microRNA-144-3p and miR-762 are novel biomarkers of Graves' disease

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Abstract

Purpose Recently, it has been confirmed that circulating miRNAs play an important role in disease pathogenesis and can be biomarkers of many autoimmune diseases. However, the knowledge about circulating miRNAs in Graves' disease (GD) is very limited. In this study, we aimed to identify circulating miRNAs as potential biomarkers of GD.

Methods We recruited 68 participants who met the criteria for GD and healthy controls. The expression profile of miRNAs in plasma was detected using microarrays. We found five interesting miRNAs were differentially expressed between GD and control group and futher validated their relative expression by quantitative real-time PCR. According to their putative target genes predicted by the TargetScan database, we also performed Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses to predict their potential functions and related pathways.

Results Microarray data showed that five miRNAs were differentially expressed in GD and control plasma samples. Among them, miR-16-1-3p, miR-122-5p, miR-221-3p, and miR-762 were upregulated in GD ($P < 0.001$). In validation stage, we found miR-144-3p was significantly decreased and miR-762 was markedly upregulated in GD plasma ($P < 0.01$). In addition, miR-762 expression was positively associated with levels of FT3 ($r = 0.307$, $P = 0.038$) as well as TRAb ($r = 0.302$, $P = 0.042$). The receiver-operating characteristic (ROC) curve analysis showed that both miR-144-3p and miR-762 displayed good sensitivity and specificity in discriminating the GD patients from the rest of subjects with the area under the ROC curve (AUC) of 0.761 ($P = 0.001$, 95% CI = 0.648–0.875) and 0.737 ($P = 0.001$, 95% CI = 0.618–0.857), respectively. Combination of miR-144-3p and miR-762 could better discriminate GD patients from healthy controls with AUC of 0.861 ($P < 0.001$, 95% CI = 0.775–0.947).

Conclusions We first demonstrated that aberrant levels of plasmic miR-144-3p and miR-762 were associated with GD, which may be biomarkers for GD diagnosis.

Keywords Graves' disease · MicroRNA · Plasma · Biomarker

Introduction

Graves' disease (GD) is a common subtype of autoimmune thyroid disease (AITD). Its clinical manifestations mainly include hyperthyroidism, diffuse thyroid enlargment (goiter), and some extra-thyroidal complications like ophthalmopathy and skin abnormalities in affected individuals [1]. In GD, immune system is triggered by a complex interaction between genetic and environmental factors, producing thyrotropin receptor antibody (TRAb) or thyroid-stimulating antibodies (TSAb), which can mimic thyroid-stimulating hormone function, stimulate the TSH receptor, and lead to

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hyperthyroidism [1, 2]. Recently, the pivotal significance of epigenetic modifications in immune disorders have captured more and more attention, including GD.

MicroRNAs (miRNAs), small, single-stranded RNAs, are ~22 nucleotides in length. They can downregulate the expression of genes encoding proteins by inhibiting mRNA translation or promoting target RNA degradation [3]. Extensive studies have indicated that miRNAs exhibit important regulatory roles in many physiological and pathological processes, including organism development [4], metabolism [5], viral infection [6], and immune response [7]. Although the majority of miRNAs are measurable intracellularly, a certain number of miRNAs can also be identified in a variety of body fluids, such as plasma, saliva, tears, urine, and cerebrospinal fluid [8–10]. Among different fluid types, these circulating miRNAs have different expression profiles and are quite stable [11].

The literature has demonstrated that levels of specific circulating miRNAs in blood are associated with various pathological conditions, and extracellular miRNAs can act as promising non-invasive biomarkers for certain human diseases [12]. Two reports have confirmed that circulating miRNAs show different profiles in autoimmune diseases, including rheumatoid arthritis (RA) and multiple sclerosis (MS) [13, 14]. However, the knowledge about the role of circulating miRNAs in GD is very limited, though different miRNAs extracted from serum or plasma have been showed to be associated with GD [15–19].

In the present study, we investigated differentially expressed circulating miRNAs in GD patients and normal controls using miRNA microarray. We further validated the differentially expressed circulating miRNAs by quantitative real-time PCR (qRT-RCR) analysis. We also performed the gene ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and the receiver-operating characteristic (ROC) curve analysis to identify whether these circulating miRNAs can act as potential biomarkers for GD diagnosis and the possible mechanism of circulating miRNAs in GD.

Subjects and Methods

Subjects

We recruited 49 patients with GD and 39 healthy controls from Jinshan Hospital. GD was diagnosed based on the clinical manifestations, supplemented with thyroid functional results and ultrasound findings. All patients included in the GD group met the diagnostic criteria for GD and had not received any treatment, and had no other systemic autoimmune disease or infection. The inclusion criteria for normal controls were the absence of autoimmune diseases, and the gender and age of them were matched with the GD group. All the subject were consecutively enrolled. Plasma samples were collected from these patients and healthy controls for study use. We performed thyroid function test in all patients included in our study, which contained free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (TSH), thyrotrophin receptor antibody (TRAb), antithyroglobulin antibody (TGAb), and thyroperoxidase antibody (TPOAb). Written informed consents were obtained from all participants. The study was approved by the ethics committee of Jinshan Hospital of Fudan University.

RNA isolation from plasma

We extracted the RNA from plasma of every sample with the use of a miRNeasy Serum/Plasma Kit (Qiagen, cat. no. 217184) according to the manufacturer instructions. Ce_miR-39_1(MS00019789), a synthetic RNA spike, was added into total RNA as the normalization control. RNA quality was measured by Nanodrop 2000 instrument (Thermo Scientific). Isolated miRNA was then converted to complementary DNA (cDNA) using a miScript II RT Kit (Qiagen, cat. no. 21816). Prepared cDNAs were stored at −80 °C freezer.

miRNA microarray screening

Plasma samples from three GD patients and three healthy controls were used for microarray analysis. The miRNA microarray chip was provided by LC Sciences. All the experimental procedures were conducted by a commercial corporation (Lianchuan, Hang Zhou, China). After subtracting the background, data were analyzed and a locally weighted regression filter was then performed to normalize the signals. Differentially expressed miRNAs were determined through fold change ≥ 2.0, P-value < 0.05 filtering. The differentially expressed miRNAs with a total signal density ≥ 1000 were selected to generate the heatmap with the use of MultiExperiment Viewer software (v4.0, 2006).

GO and KEGG analysis

The target mRNAs of differentially expressed miRNAs between patients and healthy control groups were predicted by TargetScan database. Functional analysis of these predicted mRNAs, including GO and KEGG analysis, were performed in DAVID database (http://david.abcc.ncifcrf.gov).

Real-time PCR

The expression levels of differentially expressed miRNAs were quantified by use of the miScript SYBR Green PCR Kits

(Qiagen, cat. no. 218075) according to the recommended thermocycling conditions. Primers for each abnormally expressed miRNA, including has-miR-16-1-3p, has-miR-122-5p, has-miR-221-3p, has-miR-144-3p, and has-miR-762 (cat. nos. MS MS00008806, MS00003416, MS00003857, MS00020328, and MS00037695, respectively) were provided from Qiagen. To ensure a single PCR product of the primers, melt curve analyses were performed. ABI7300 software was utilized to analyze the fluorescence signals, and $2^{-\Delta\Delta Ct}$ method was used for relative quantification. Each reaction was processed in triplicates.

Statistical analysis

All data of this study were presented as mean ± standard deviation (SD). Hierarchical clustering was performed on the samples using a euclidean distance and average linkage clustering to generate a heatmap and clusters for the samples. The comparison of the different expression of miRNAs between two groups was performed by two independent-sample *T* test or nonparametric test. The correlation analysis between miRNAs expression and thyroid function of GD patients was conducted by the nonparametric spearman's test. ROC curve and AUC were also established for discriminating GD patients from healthy controls. We used SPSS 17.0 for statistical analysis. *P*-values < 0.05 were considered to be statistically significant.

Results

Clinical characteristics of participants

Table 1 shows clinical characteristics of the enrolled participants. A total of 88 participants including 49 GD patients and 39 healthy subjects were recruited in our study. The patients with GD had increased serum FT3, FT4, and TRAb levels. There were 69.4% female in GD group with age 37.52 ± 14.66 years, 69.2% female in HC group with age 34.08 ± 8.87 years, which showed no difference between two groups.

Table 1 Clinical characteristics of patients with Graves' disease (GD) and healthy controls (HC)

	GD	NC
Number	49	39
Age (years)	37.52 ± 14.66	34.08 ± 8.87
Male	15	12
Female	34	27
FT3 (pmol/L)	20.64 ± 14.81	4.26 ± 0.78
FT4 (pmol/L)	58.95 ± 31.69	14.94 ± 1.90
TSH (mIU/L)	< 0.005	2.38 ± 0.93
TgAb (+)	38 (77.55%)	0
TPOAb (+)	41 (83.67%)	0
TRAb (IU/L)	13.57 ± 12.10	1.01 ± 0.24

Data are expressed as mean ± standard deviation

FT3 free T3, *FT4* free T4, *TRAb* anti-TSH receptor antibody, *TPOAb* thyroperoxidase antibody, *TgAb* antithyroglobulin antibody

microRNA expression profiling

miRNA profiling microarray revealed that five miRNAs were differentially expressed in GD compared with controls ($P < 0.05$). Among them, miR-16-1-3p, miR-122-5p, miR-221-3p, and miR-762 were upregulated in GD, while miR-144-3p was downregulated in GD, as shown by hierarchical clustering Heatmap (Fig. 1).

Analysis of miRNA target genes

Five hundred putative target genes of the five validated miRNAs were found in patients with GD and healthy controls by TargetScan. GO analysis showed that the target genes of differentially expressed miRNAs were enriched in regulation of transcription from RNA polymerase II promoter, RNA metabolic process, cellular biosynthetic process, and gene expression in biological process (BP) terms (Fig. 2a). KEGG pathway analysis showed that the target genes of differentially expressed miRNAs were involved in the p53 signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, and ubiquitin-mediated proteolysis (Fig. 2b).

Microarray result validation

We then retested the above mentioned five miRNAs in the plasma of 31 GD patients and 31 healthy controls using qRT-PCR. We verified that miR-144-3p was significantly

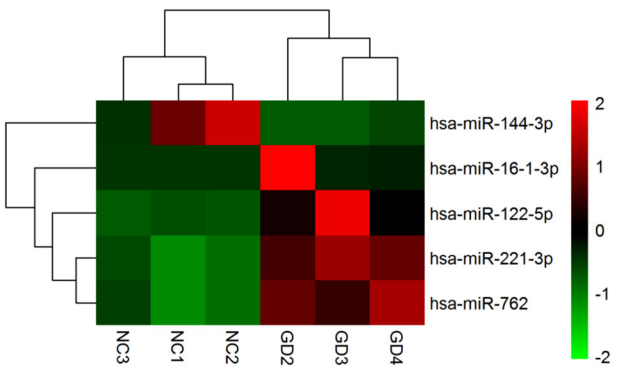

Fig. 1 Differentially expressed miRNAs ($P < 0.05$) were analyzed by hierarchical clustering of values of miRNA microarray signals. Red: upregulation; green: downregulation. The heatmap shows five differentially expressed miRNAs in patients with Graves' disease (GD) compared with normal contols (NC), using miRNA array data

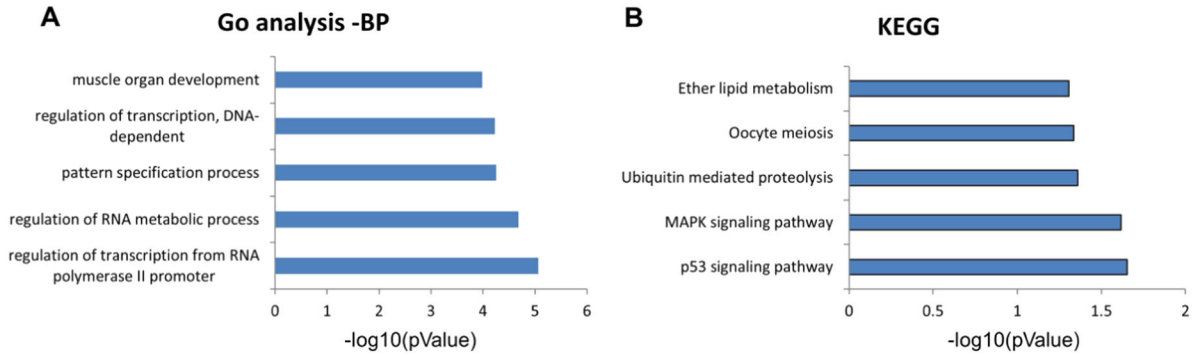

Fig. 2 The most enriched GO categories **a** and the most enriched pathway **b** of the differentially expressed target genes of the differentiall expressed miRNAs. GO gene ontology, KEGG Kyoto encyclopedia of genes and genomes

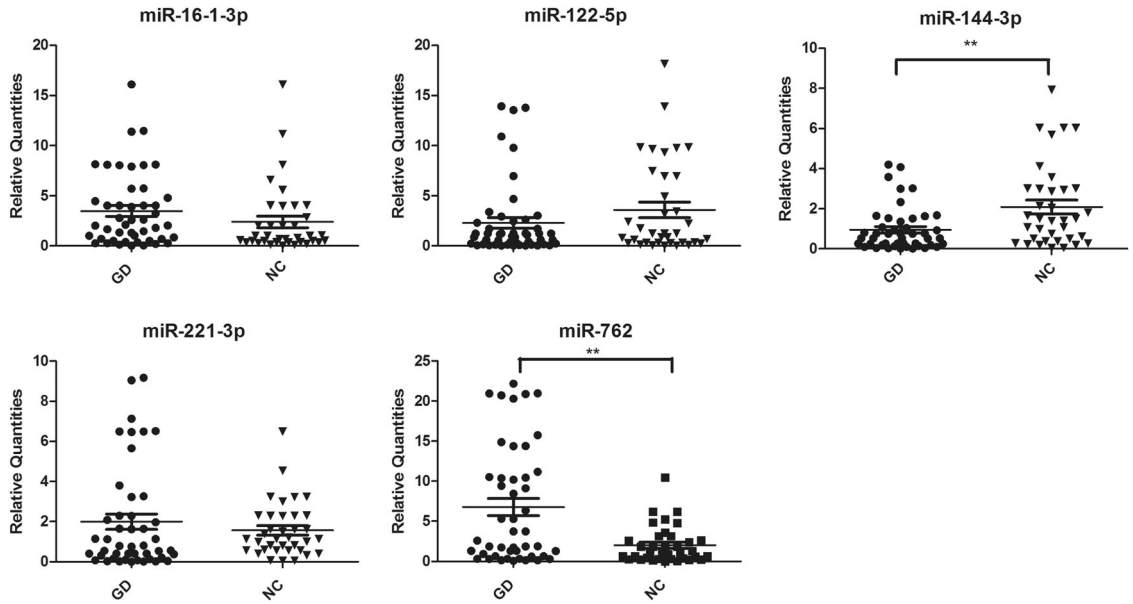

Fig. 3 Differential serum levels of five miRNAs from Graves' disease (GD) patients and healthy controls (**$P < 0.01$)

decreased and miR-762 was markedly upregulated in GD plasma ($P < 0.001$, Fig. 3).

Association of the plasma miRNAs with clinical features

To explore the association of plasma miRNA levels with clinical features and assess their clinical utility, we investigated the relationship between the miRNAs and the levels of FT3, FT4, and TRAb (Fig. 4). Spearman analyses revealed that miR-762 was positively associated with FT3 ($r = 0.307$, $P = 0.038$) as well as TRAb ($r = 0.302$, $P = 0.042$).

Evaluation of the validated miRNAs as biomarkers for the diagnosis of GD

We generated ROC curves to estimate the sensitivity and specificity for each miRNA. As shown in Fig. 5, both miR-144-3p and miR-762 displayed good sensitivity and specificity in discriminating the GD patients from normal individuals with the area under the ROC curve (AUC) of 0.761 ($P = 0.001$, 95% CI = 0.648–0.875) and 0.737 ($P = 0.001$, 95% CI = 0.618–0.857), respectively. Combination of miR-144-3p and miR-762 could better discriminate GD patients from healthy controls with AUC of 0.861 ($P < 0.001$, 95% CI = 0.775–0.947).

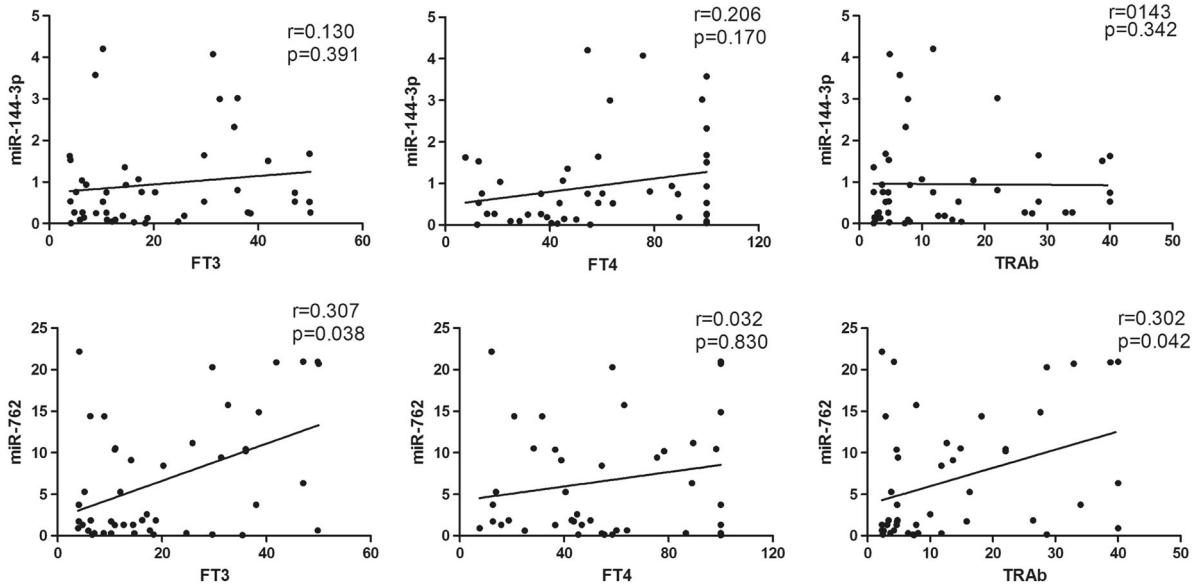

Fig. 4 Linear relationships of serum miR-144-3p and miR-762 relative expression with FT3, FT4, and TRAb levels. Spearman analyses revealed that miR-762 was positively associated with FT3 ($r = 0.307$, $P = 0.038$) as well as TRAb ($r = 0.302$, $P = 0.042$). FT3 free triiodothyronine, FT4 free thyroxine, TRAb thyrotrophin receptor antibody

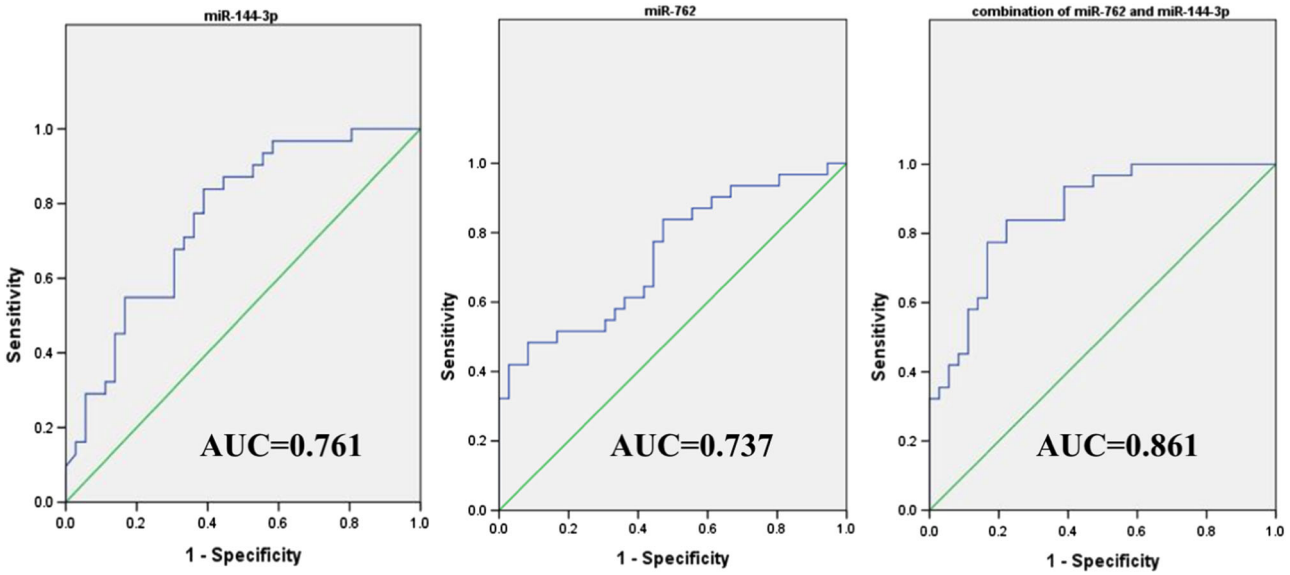

Fig. 5 The receiver operating characteristic (ROC) curve analysis of miR-144-3p, miR-762, and combination of the two miRNAs in the discrimination of Graves' disease (GD) patients from healthy controls with area under the curve (AUC) of 0.761, 0.737, and 0.861, respectively

Discussion

In this study, we evaluated the expression pattern of miRNAs by microarray in the plasma from patients with GD. We identified that five miRNAs, including four upregulated miRNAs (has-miR-122-5p, 16-1-3p, 221-3p, and 762) and one downregulated miRNA (has-miR-144-3p) were differentially expressed in patients with GD and healthy controls. Our KEGG pathway analysis showed that majority of the target genes of those miRNAs were involved in the p53 signaling pathway and MAPK signaling pathway, which have been closely linked with inflammation, and they have been implicated in many inflammatory or autoimmnune diseases. For example, Jiang et al. found that IL-22-induced miR-122-5p can promote keratinocytes proliferation through downregulating the expression of Spry2 and thus plays an important role in the pathogenesis of psoriasis [20]. The miR-122-5p expression in serum was

significantly decreased during relapse in relapsing–remitting multiple sclerosis (RRMS) compared with healthy controls [21]. miR-221-3p was found to be statistically different between SLE patients with and without lupus nephritis (LN) [22]. Circulating miR-221-3p was also significantly upregulated in pediatric multiple sclerosis (PedMS) compared with pediatric controls (PCs) [23].

When we validated these miRNAs in more expanded samples by quantitative real-time PCR, we found that miR-144-3p was decreased and miR-762 was increased in patients with GD. Studies have shown that miR-144-3p is downregulated in many kinds of carcinoma and is associated with tumor proliferation, migration, angiogenesis, progression, and poor survival, mainly including renal cell carcinoma [24], glioblastoma [25], lung adenocarcinoma [26], hepatocellular carcinoma [27], and multiple myeloma [28]. But the association of miR-144-3p with autoimmnue diseases is not clear. Only one study found that miR-144-3p expression in plasma was downregulated in immune thrombocytopenic purpura (ITP) patients both in initial screen with a miRNA microarray assay and subsequent quantitative real-time PCR validation [29]. Consistent with these results, our study first found that circulating miR-144-3p expression was significantly decreased in GD group, and miR-144-3p had a moderate sensitivity and specificity to distinguish those GD patients from healthy controls, indicating that miR-144-3p is involved in the pathogenesis in GD and can act as a potential biomarker for GD. The pathogenic role of miR-144-3p in GD is not yet clear, which needs to be explored in the future.

So far, there is no research on the role of miR-762 in autoimmune diseases. Our study first found that circulating miR-762 expression was significantly increased in patients with GD. The mechanism of the role of miR-762 in GD is not yet clear. But a study has shown that miR-762 overexpression in breast cancer cell lines and specimens can increase both the cell proliferation and invasion by targetting interferon-regulatory factor 7 (IRF7) [30]. IRF-7 gene is localized on the human chromosome 11p15.5 and can interact with the MyD88 adaptor protein of Toll-like receptor (TLR) signaling [31]. IRF7 can induce a large amount of type I IFN production after being activated by TLR7 and TLR9 [32]. INF-α, a type 1 INF, has also been strongly linked with the occurrence of AITD for its involvement in antibody-mediated immune responses [33, 34]. Furthermore, we found that miR-762 expression was positively correlated with FT3 and TRAb, which are two important indexes for diagnosis, severity, and prognosis of GD [35]. Our ROC analysis also showed that miR-762 had a moderate sensitivity and specificity to distinguish GD patients from healthy controls. All these findings suggest that miR-762 may participate in the pathogenesis of GD and may be a novel biomarker for the diagnosis of GD.

As far as we know, several studies have examined the relationship between GD and circulating miRNAs, but the elevated or decreased miRNAs to be explored were quite different from each to other studies. For example, one study of Japan found that in female GD patients, the serum levels of miR-16, miR-22, miR-375, and miR-451 were increased than those of healthy subjects [15]. Another study revealed that the levels of serum miR-1a were significantly decreased in GD with atrial fibrillation (AF) group compared with GD group [16]. Circulating miR-23b-5p and miR-92a-3p were increased while let-7g-3p, and miR-339-5p were decreased in GD patients in remission compared with intractable GD patients [17]. Serum miR-210 in GD patients was significantly higher than that of healthy controls, but miR-155 and miR-146a were lower [18]. Serum levels of miR-142-3p, miR-21-5p, miR-96-5p, and miR-301-3p in GD patients were significantly increased compared with healthy controls [19]. However, our study showed that circulating miR-762 expression was increased and miR-144-3p expression was significantly decreased in GD group compared with healthy controls. The differences between the results of these studies can be attributed to a number of factors, such as subjects included in the study, their ethnicity, region, environment factor, sample size, and chip manufacturer. These factors all contribute to the difference in results. And it is important for a good research to validate such prelimilary data in the next steps.

Our study also has limitations. On the one hand, in the present study, plasma samples from only three GD patients and three healthy controls were used for microarray analysis. If the *P*-value was adjusted, there would be no differentially expressed miRNAs between GD patients and healthy controls. Therefore, differentially expressed miRNAs were determined through fold change ≥ 2.0, *P*-value < 0.05 filtering instead of adjusted *P*-value. On the other hand, it is difficult to distinguish those changes of the miRNA are from hyperthyroidism or abnormal autoimmunity, since we didn't follow-up the patients when their thyroid function were normalized.

In conclusion, we first find that plasma miR-762 and miR-144-3p may serve as potential markers for GD. However, studies with larger samples are still needed to verify the potential roles of them in the GD pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and consent to participate This work was approved by the Ethics Committee of Jinshan Hospital, Fudan University. Written informed consents were obtained from all participants.

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