Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;35:1934-1942
DOI: 10.1159/000374002
Published online: March 26, 2015
Accepted: January 28, 2015

1421-9778/15/0355-1934$39.50/0

Original Paper

Aberrant Expression of miRNA and mRNAs in Lesioned Tissues of Graves' Disease

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Key Words
MicroRNA • MRNA • Graves' disease (GD)

Abstract
Background and Aims: Abnormal microRNA (miRNA) expression is found in many diseases including autoimmune diseases. However, little is known about the role of miRNA regulation in Graves' disease (GD). Here, we simultaneously detected different expressions of miRNA and mRNAs in thyroid tissues via a high-throughput transcriptomics approach, known as microarray, in order to reveal the relationship between aberrant expression of miRNAs and mRNAs spectrum and GD. ***Methods:*** Totally 7 specimens of thyroid tissue from 4 GD patients and 3 controls were obtained by surgery for microarray analysis. Then, 30 thyroid specimens (18 GD and 12 controls) were also collected for further validation by quantitative real-time PCR (qRT-PCR). ***Results:*** Statistical analysis showed that the expressions of 5 specific miRNA were increased significantly while those of other 18 miRNA were decreased in thyroid tissue of GD patients (FC≥1.3 or≤0.77 and $p<0.05$). In addition, the transcription of 1271 mRNAs was up-regulated, while the expression of 777 mRNAs transcripts was down-regulated (FC≥2.0 or≤0.5 and $p<0.05$). Furthermore, integrated analysis of differentially expressed miRNA and their target mRNAs demonstrated that 2 miRNA (miR-22 and miR-183) were increased while their potential target mRNAs were decreased. 3 miRNA (miR-101, miR-197 and miR-660) were decreased while their potential target mRNAs were increased. The above findings from microarray screening were confirmed by qRT-PCR in more samples. The results were consistent with those observed in the microarray assays. ***Conclusion:*** Our study highlights the possibility that miRNA-target gene network may be involved in the pathogenesis of GD and could provide new insights into understanding the pathophysiological mechanisms of GD.

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Introduction

Graves' disease (GD), a common organ-specific autoimmune disorder that affects thyroid glands, is characterized by diffuse goiter and hyperthyroidism with or without the associated ophthalmopathy and dermopathy [1]. Over the past few decades, the incidence of GD has increased dramatically due to the widespread changes in our lifestyle [2]. The etiology of GD is the complex interplay of genetic, environmental and endogenous factors and up to date, the exact roles of these factors in the pathogenesis of GD remains to be elucidated. Recently, the pivotal role of abnormal inflammation and autoimmune response in the development of GD attracts abundant attention. The immunological pathogenesis of GD is, to some extent, similar to that of other common autoimmune diseases (AIDs), such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and inflammatory bowel disease (IBD) [3].

MicroRNA (miRNA) are endogenous, single-stranded, small non-coding RNAs with about 21-23 nucleotides in length. MiRNA control gene expression by binding complementary sequences of target mRNAs and negatively regulate at the post-transcriptional level through translational repression or degradation of mRNA targets [4]. Plenty of evidence has demonstrated that miRNA play a crucial role in maintaining immune system development and function [5-7], and are implicated in numerous immunological disorders, especially in ordinary AIDs. Previous studies have found that several AIDs such as RA [8, 9], SLE [9, 10] and psoriasis [11] share the same miRNA dysregulation as miR-155 and miR-146a. The common miRNA detected in different AIDs suggest that aberrant expression of miRNA may also implicate in the development of GD. In addition, the unique miRNA expression profiles have been identified in other AIDs. For example, miR-326 is highly correlated with disease severity in patients with MS [12], while miR-192 expression is dramatically decreased in active ulcerative colitis and predominantly localized to colonic epithelial cells [13]. However, there is no data available on miRNA regulation in diseased tissue of GD, an organ-specific AID. Therefore, in this study, we investigated miRNA and mRNAs expression profiles in thyroid tissue of GD patients and controls in order to reveal the relationship between aberrant expression of miRNA and mRNAs spectrum and GD. Finally, we explored whether GD is associated with a specific miRNA signature.

Materials and Methods

Tissue samples

GD patients were diagnosed on the clinical manifestations, supplemented with the biochemical tests of thyroid function, thyroid ultrasound, and histological examination [14]. All the patients had hyperthyroidism, goiter and positive thyroid-stimulating hormone receptor antibody (TRAb). Thyroid function were measured by radioreceptor assay (Union Medical&Pharmaceutical Technology Limited Company, Tianjin, China). The controls were euthyroid without goiter, and showed negative antibodies against thyroid components and no other autoimmune diseases (Table 1). This study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and all patients and control participants gave written informed consent.

Methods

RNA isolation and quantification. The tissue samples were cryo-pulverized and homogenized using the Biopulverizer and Mini-Bead-Beater-16 (Biospec, USA). Total RNA was harvested using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. RNA was isolated from 7 thyroid tissues for microarray analysis, and also 30 individual tissues were isolated for qRT-PCR validation test. The quality and quantity of extracted RNA were assessed by the NanoDrop 1000 (Thermo Scientific, the USA).

MicroRNA microarray analysis. Seven RNA samples were labeled with the kit of miRCURY ™ Array Power Labeling (Exiqon, the USA), accordance with the manufacturer's instructions. After evaluating the labeling efficiency, the labeled RNA samples were hybridized on the miRCURY™ LNA (locked nucleic acid, LNA) Array (the 6th generation, Exiqon, the USA) and totally 1μg RNA was added in the reaction mixture for the microarray labeling. Three independent hybridizations for each sample were performed on chip with each miRNA spotted in quadruplicate. For each test sample and control sample, two hybridizations were performed by using a reversal fluorescent strategy. The original data signals were scanned using GenePix 4000B scanner (635 nm, Axon Instruments) , then the "normalized data" for each miRNA were further analyzed [the "normalized data"= (foreground-background)/median] by GenePix Pro V6.0 software (Axon

Table 1. Clinical features of GD patients in this study. *M-male; F-female. **Data are shown as Mean±SD

	Microarray		qRT-PCR		
	GD	Control	GD	Control	Normal range
n	4	3	18	12	
Gender(M/F)*	1/3	0/3	3/15	2/10	
Age(M±S)**	24.5±10.4	35.7±5.5	29.3±12.4	37.2±10.7	
Free T4	28.2±10.2	13.9±1,2	35.4±41.8	15.8±2.2	9.05-25.5pmol/L
Free T3	14.6+±3.2	6.4±0.2	13.1±11.3	6.3±0.7	2.91-9.08pmol/L
TSH	0.076±0.01	1.7±1.0	0.08±0.05	2.1±0.9	0.25-5uIU/mL
TRAb	Positive	Negative	Positive	Negative	

Table 2. Specific primers used for qRT-PCR validation

Gene	GeneBank accession	Primer sequence
Hsa-miR-22	NR_029494(85bp)	AAGCTGCCAGTTGAAGAACTGT
Hsa-miR-101	NR_029516(75bp)	CGGTACAGTACTGTGATAACTGAA
Hsa-miR-183	NR_029615(110bp)	CGTATGGCACTGGTAGAATTCAC
Hsa-miR-197	NR_029583(75bp)	TTCACCACCTTCTCCACCCAGC
Hsa-miR-660	NR_030397(97bp)	CGTACCCATTGCATATCGGAGTT
EDA	NM_001399	F-AGCAATTCAAGTCAAGAATGATCT R-GTGCCGTCCACCAGTACC
GATM	NM_001482	F-AATGGCTGATGAGCTTTATAACC R-TGTAACCTGGCTTCTCTGTGC
AKAP12	NM_005100	F-AAGTCATTGTCACAGAGGTTGG R-CGATTATTTCGGGTGTCTCCT
ZFPM2	NM_012082	F-TAACAAAGGTGATGATGAAGGAAT R-AACCACTTGGGACCAGCAG
PBX3	NM_006195	F-ATCGGCGACATCCTCCAC R-TCCTCTGATGCTGAGACCTGT
CILP	NM_003613	F-TGTGTTGGGGAGACAGACGA R-GGACGGGCACATACACGGT
SDC1	NM_001006946	F-CCCCTGAAGATCAAGATGGCT R-CGGTGGGTTCTGGAGACG

Instruments). Differentially expressed miRNA were identified through fold-change screening between the two groups. miRNA level with 1.3-fold difference in normalized expression and statistical significance in Welch t-test ($p<0.05$) between GD and control was considered as differentially expressed miRNA.

Target mRNAs for individual miRNA were predicted using
miRanda (http://www.microrna.org/microrna/home.do),
miRBase (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/)
and TargetScan (http://www.targetscan.org/). The overlapped data in these three databases were selected as target genes.

MRNA microarray analysis. Totally 5μg RNA of 7 individual samples were amplified and labeled using the Superscript ds-cDNA synthesis kit (Invitrogen, the USA) and NimbleGen One-color DNA labeling kit (Roche, the USA). Then the labeled cDNA was hybridized with NimbleGen Human 12×135K microarrays (Roche, the USA) for gene expression analysis. After hybridization and washing, the slides were rapidly scanned with the Axon GenePix 4000B scanner. Original data were extracted as pair files and normalized using NimbleScan v2.5 Software and then further analyzed with Agilent GeneSpring GX software (Version 11.5.1). Individual genes with 2.0-fold difference in normalized expression and statistical significance in Welch t-test ($p<0.05$) between control and GD were considered as differentially expressed genes.

Biological functional analysis was preformed as Gene Ontology (GO) categories and pathway analysis based on Gene Ontology (http://www.gneontology.org) and the latest KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) database, respectively.

Docking study of differentially expressed miRNA and mRNAs. It is worth noting that miRNA-mRNA functional pair is generally defined as consisting of anticorrelation with each other in terms of expression. Thus, the predicted target genes were further comparative analyzed with mRNA microarray analysis, in order to confirm that those miRNA predicted targets were truly expressed in thyroid tissue.

Quantitative RT-PCR. Totally 1μg RNA of 30 individual samples were reversely transcribed into cDNA using One Step PrimeScript® miRNA cDNA Synthesis kit (TakaRa, China) and Primescript RT reagent kit (TakaRa, China). The following PCR amplification of cDNA was performed with SYBR Premix Ex Taq Ⅱ (TakaRa, China) in the presence of specific primers shown below (Table 2). The U6 and β-actin expression were used as internal controls. The fluorescence signals were analyzed using ABI7300 software

and the data were calculated with comparative CT method for relative quantification. All samples were processed in triplicates.

Statistical Analyses

All data are expressed as mean±SD. SPSS 13.0 was used for statistical analysis. The means of multiple samples were analyzed by the Mann-Whitney U-test, and the interclass comparison was tested by independent-samples t test or Mann-Whitney test. A difference with $p<0.05$ was considered as significant.

Results

Differential miRNA and mRNAs expressions in microarray analysis

We tested miRNA from thyroid tissue of patients and controls by miRCURY™ LNA Array. Totally 2662 capture probes were detected in this test. Using a threshold of 1.3-fold or greater change, 283 miRNA were differentially expressed in GD patients and controls and only 23 out of 283 differentially expressed miRNA differed significantly ($p<0.05$). Among these, 5 miRNA were up-regulated, including Hsa-miR-22, -183, -625*, -3613-3p and hsv2-miR-H20 while 18 miRNA were down-regulated, including Hsa-miR-29a*, -92b, -361-5p, -101, -708, -1179, -210, -192, -505, -29c*, -660, -26a, -324-3p, -4324, -455-5p, -197, -191 and hsa-let-7b*(Table 3). The heat map of miRNA microarray analysis is shown in Fig. 1A. Then, in order to better understand the role of the regulated miRNA in GD patients, miRBase, miRanda and targetscan algorithms were used to identify the predicted target genes of the significantly regulated miRNA, and the overlapped data in these three databases were selected as target genes. As a result, totally 363 genes were found to be those significantly regulated miRNA' target genes (full data not shown here).

MRNAs were tested by NimbleGen Human 12×135K microarrays containing a total of 27963 human gene transcripts. With a threshold of 2.0-fold or greater change, totally 2042 genes (1271 overexpressed and 771 lowexpressed) were significantly differentially expressed in GD patients in comparison with the controls ($p<0.05$). The heat map of mRNAs microarray shown the cluster analysis which arrange samples into groups based on their expression levels and allow us to hypothesize the relationships among samples (Fig. 1B). Then, we used GO project to classify the significantly regulated genes according to biological process (BP), molecular function (MF) and cellular component (CC), we found that up-regulated genes in GD were associated with several significantly enriched GO terms involved in the immune system. For example, enrichment analysis of BP term revealed that top annotation cluster of up-regulated genes involved immune system process, immune response, and lymphocyte activation (Fig. 2A). Consistent with GO categories, pathway analysis also found that the major signal transduction biological pathways in up-regulated transcripts were mainly associated with or participated in inflammatory and immune-related diseases, such as SLE, staphylococcus aureus infection, and osteoclast differentiation (Fig. 2B). Unfortunately, either GO or pathway analysis of down-regulated genes suggested any meaningful accumulation features.

Table 3. All the differentially expressed miRNA in thyroid tissues from Graves' disease patients and normal controls

miRNA	Fold change	P value
Hsa-miR-3613-3p	1.83	0.02
Hsa-miR-183	1.85	0.04
Hsa-miR-625*	2.73	0.03
Hsa-miR-22	2.91	0.04
hsv2-miR-H20	1.82	0.03
hsa-let-7b*	0.27	0.02
Hsa-miR-29a*	0.58	0.03
Hsa-miR-92b	0.34	0.03
Hsa-miR-361-5p	0.51	0.00
Hsa-miR-101	0.45	0.01
Hsa-miR-708	0.15	0.00
Hsa-miR-1179	0.26	0.04
Hsa-miR-210	0.41	0.01
Hsa-miR-192	0.32	0.03
Hsa-miR-505	0.38	0.02
Hsa-miR-29c*	0.49	0.04
Hsa-miR-660	0.50	0.04
Hsa-miR-26a	0.46	0.02
Hsa-miR-324-3p	0.38	0.04
Hsa-miR-4324	0.65	0.04
Hsa-miR-455-5p	0.21	0.04
Hsa-miR-197	0.24	0.00
Hsa-miR-191	0.58	0.04

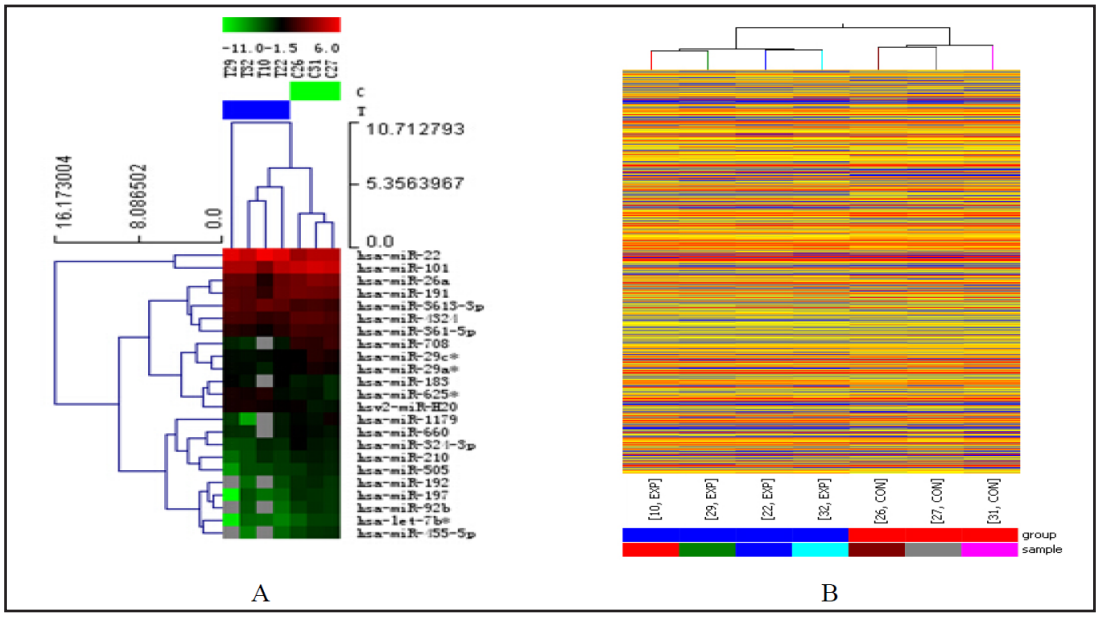

Fig. 1. Heat map of the miRNA and mRNA microarray expression data from thyroid glands of GD patients. (A) Hierarchical clustering results of differentially-expressed miRNA. Each row represents the expression profile of an miRNA across 7 samples and each column represents a sample. Red and green colors indicate higher and lower expression levels of the miRNA, respectively. (B) Hierarchical clustering result of all target mRNA expression. "Red" indicates high relative expression, and "Blue" indicates low relative expression.

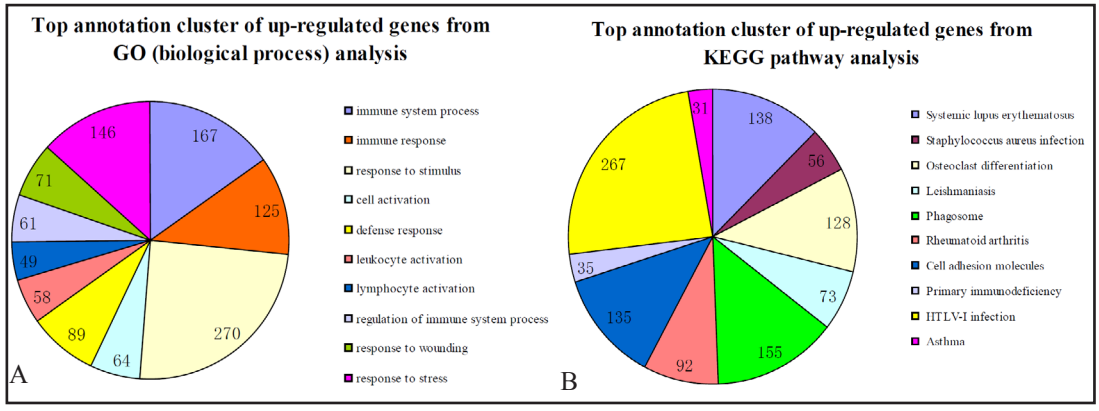

Fig. 2. Gene Ontology and pathway enrichment of genes significantly deregulated in Graves' disease.

Docking analysis of miRNA-mRNA interaction

To achieve high-confidence miRNA-mRNA associations and to evaluate the impact of each miRNA on the gene expression, the predicted target genes of each miRNA were identified and combined with the mRNA transcriptome from GD patients and controls. Based on the concomitant reversal of expression patterns of miRNA and its related target mRNA genes, 5 miRNA and their related 8 predicted target genes were identified. The final results revealed that miR-22 and miR-183 were significantly overexpressed in GD patients while their predicted target genes EDA (ectodysplasin A, EDA), GATM (glycine amidinotransferase, GATM), MLLT4 (myeloid/lymphoid or mixed-lineage leukemia translocated to 4, MLLT4), AKAP12 (A kinase anchor protein 12, AKAP12) and ZFPM2 (zinc finger protein multitype 2, ZFPM2) were decreased, respectively. Similarly, miR-101, miR-197 and miR-660 were obviously decreased while their predicted target genes PBX3 (pre B cell leukemia homeobox 3, PBX3), CILP (cartilage intermediate layer protein, nucleotide pyrophosphohydrolase, CILP) and SDC1 (syndecan 1, SDC1) were increased correspondingly in thyroid tissue of GD patients.

Table 4. Significant differentially expressed miRNAs and their predicted target mRNAs expressions

miRNAs	miRNA microarray		qRT-PCR		mRNAs	mRNA microarray		qRT-PCR	
	fold change	p value	fold change	p value		fold change	p value	fold change	p value
hsa-miR-22	2.911	0.04	1.33	0.037	EDA	0.44	0.001	0.2	0.015
					GATM	0.43	<0.001	0.26	0.039
					MLLT4	0.46	<0.001		
hsa-miR-183	1.854	0.045	2.36	0.005	AKAP12	0.469	<0.001	0.22	0.003
					ZFPM2	0.331	<0.001	0.18	0.002
hsa-miR-101	0.447	0.011	0.67	0.037	PBX3	2.214	<0.001	2.26	0.007
hsa-miR-197	0.239	0.002	0.65	0.015	CILP	3.454	0.011	4.58	0.001
hsa-miR-660	0.501	0.043	0.54	0.015	SDC1	2.535	<0.001	4.73	0.028

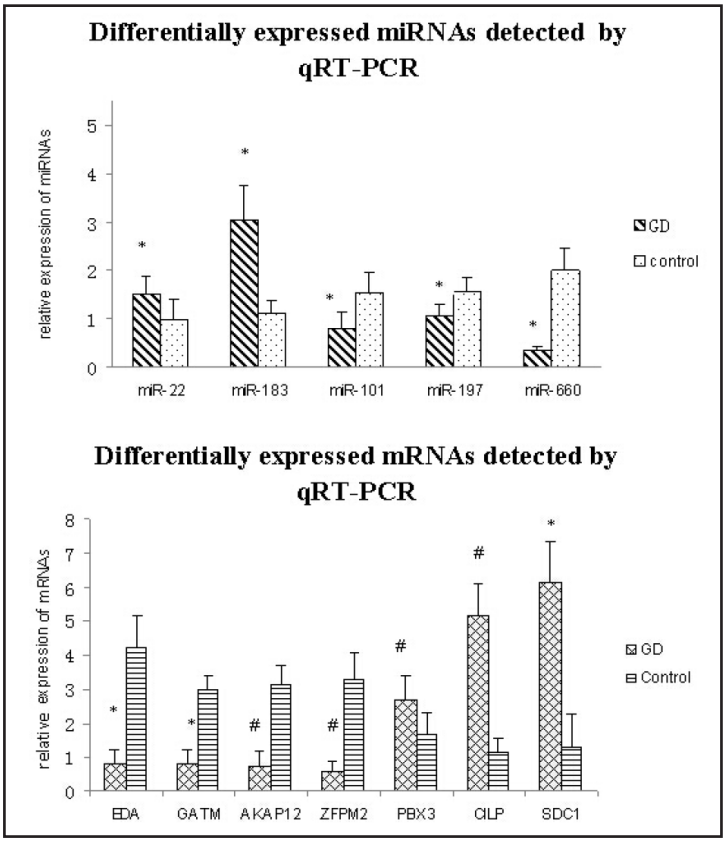

Fig. 3. Differentially expressed genes verified by qRT-PCR analysis. The expended samples obtained from 18 GD and 12 controls were used to verify the significantly regulated miRNA and their related target mRNAs by qRT-PCR. *p<0.05, #p<0.01.

Validation of miRNA and their predicted target genes by real time qRT-PCR

To further confirm the results of microarray test, we determined differentially expressed miRNA (miR-22, miR-101, miR-183, miR-197, miR-660) and their potential target mRNAs (EDA, GATM, AKAP12, ZFPM2, PBX3, CILP and SDC1) by qRT- PCR. Collectively, the data indicated that the expression profiles of these miRNA and mRNAs were consistent with those observed in the microarray assays (Table 4, Fig. 3).

Discussion

The etiology of Graves' disease remains elusive. Our's and other investigators found genetic factors and immunologic imbalance are implicated in its pathogenesis [15-18]. MiRNA is a newly discovered molecule which has been explored extensively in many diseases. Results from our mRNA microarray analysis revealed that totally 1271 transcripts were significantly increased and 771 decreased. GO and pathway analysis were used to describe the role of these differentially expressed genes in GD. In regard to biological process, one of GO terms, nearly all the top 10 annotation clusters of up-regulated genes were related to

immune regulation, such as immune system process, immune response, and immune cell activation. Consistent with GO analysis, signal transduction pathway analysis also revealed that in most cases, up-regulated mRNAs participated in inflammatory and autoimmune diseases, such as SLE, RA and asthma. But we haven't found significant enrichment of down-regulated genes in specific biological process both from GO and pathway analysis. It seems that these up-regulated genes may serve as causative genes in GD, mainly mediating the immune processes including lymphocyte infiltration and autoantibody production, and finally initiate a cascade of pathological events in the development of GD.

Numerous studies have already revealed that different cancers have different miRNA profiles [19], which indicate that miRNA have their own characteristics of cell- or tissue-specific expression pattern. This feature was also found in our study. It demonstrated that GD also has unique miRNA expression profiles in its target organs. In our study, totally 23 miRNA were significantly differentially expressed in thyroid *in situ* of GD, and 5 (including miR-22, -183, -3613-3p, -625* and hsv2-miR-H20) out of 23 were up-regulated while 18 (miR-101, -197, -29a*, -92b, -361-5p, -708, -1179, -210, -192, -505, -29c*, -26a, -324-3p, -4324, -455-5p, -191, -660 and hsa-let-7b*) down-regulated. Overall, these differentially expressed miRNA were greatly distinct from those of control subjects as well as other AIDs. For instance, some of the most common immune-related miRNA such as miR-155 [20], miR-146a [21] and miR-17-5p [22] involved in AIDs were not found to differ in GD in our study. Meanwhile, different miRNA expressions detected in our study were also rarely reported in other AIDs. When reviewing the previous studies, only a few significantly regulated miRNA obtained in our research were found to be associated with other immune or autoimmune disorders. For example, Jernås and colleagues found a decreased level of miR-197 in MS patients compared with controls [23] and Ninomiya et al. found that miR-197-3p was significantly reduced in primary billiary cirrhosis [24]. Dai et al. found miR-101 was markedly unregulated in splenic T, but not in B cells from MRL-lpr mice, which was one of murine lupus model [25] and Rigby et al. revealed dysregulation of miR-101 in SLE [26]. Tissue-specific miRNA expression pattern in GD may confer them as diagnostic biomarkers or therapeutic targets in the near future. Furthermore, an interesting phenomenon found in our study is that the majority of miRNA expression were decreased in GD, which was unanimously linked to the predominant increasedly expressed mRNAs in thyroid tissue. This scenario is very similar to what has been found in many malignant tumors [27-29] as well as in some AIDs [30].

The docking analysis, after combining miRNA and mRNAs expression data, revealed that two highly expressed miRNA are in accordance with their down-expressed potential target genes. These genes are miR-22 and its predicted target genes EDA, GATM and MLLT4 as well as miR-183 and its predicted target genes AKAP12 and ZFPM2. Three decreased miRNA are in accordance with their increased potential target genes. These genes contain miR-101 and its predicted target gene PBX3, miR-197 and its predicted target gene CILP, as well as miR-660 and its predicted target gene SDC1. qRT-PCR was performed to further confirm the results from microarray analysis, and the results were consistent with those of the microarray assays. Further study should be conducted to verify the relationship between these miRNA and their potential targets in order to assess whether these genes can be regarded as "star genes" in diagnosis, treatment and prognosis of GD.

However, the results we obtained are different from those of the study done by Liu et al. [31]. The alternative explanation for this discrepancy can be attributed to the differences between the materials maintained in individual studies. There is non-overlapped miRNA expression between Liu's and ours study. The phenomenon that miRNA expression profile from peripheral blood is different from that of diseased tissue is also found in other diseases, such as IBD, RA, SLE and psoriasis [22, 32-38]. In addition, our results are also different from study done by Bernecker et al., in which they confirmed significant variations of miR-200a and miR-155 in CD4+ T-cells and CD8+ T-cells from AITD patients [39]. The reason for this divergence not only due to the different materials but also the different methods. They referred to previous literature to verify the "selected miRNA". These discrepancies studies consistently imply the importance of miRNA in GD. Further investigations with various avenues could be used to understand the relationship between miRNA and pathogenesis of GD in-depth, such as circulating miRNA in AITD detected by Yamada et al. [40].

In summary, we employed GD tissue and displayed the unique profiles of differentially expressed miRNA and mRNAs. Although our study is preliminary, we believe that our findings provide a basis for further investigation of the pathogenic process and its associated signal

transduction pathways in GD. Apparently, modulation of those related miRNA transcription may aid in new therapies for the prevention and treatment of GD in clinic.

Acknowledgments

We are very grateful to Professor Shao-qiang Zhang in the Department of Head and Neck Surgery, the First Affiliated Hospital, Medical School of Xi'an Jiaotong University, for providing thyroid specimens. Supported by the National Natural Science Foundation of China (No. 81270871).

Disclosure Statement

The authors report that they have no conflicts of interest.

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