Expression Profile and Bioinformatics Analysis of Circular RNAs in Patients with Vitiligo

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Purpose: Circular RNAs (circRNAs) are abundant, stable, and evolutionarily conserved noncoding RNAs with impacts on cell proliferation, differentiation, invasion, apoptosis, and immunity by acting as an miRNA sponge. This study aimed to investigate the expression of circRNAs in vitiligo and analyze the differentially expressed circRNAs (DEcircRNAs) bioinformatically.

Patients and Methods: Biopsies of five lesional and five nonlesional skins of patients with vitiligo and five healthy skins (control) were harvested in this study. The expression profiles of circRNAs and DEcircRNAs were determined by microarray analysis and qRT-PCR. Bioinformatics analysis was used to predict target genes of DEcircRNAs binding to miRNAs and their underlying functions. Meanwhile, a competing endogenous RNA (ceRNA) network was constructed using Cytoscape.

Results: A total of 817 and 508 DEcircRNAs were identified in lesional and nonlesional skins of patients with vitiligo, respectively. The results of hsa_circRNA_000957 and hsa_circRNA_101798 validation were consistent with our microarray analysis. Furthermore, 32 miRNA response elements (MREs) and related target genes of DEcircRNAs were identified, whose main functions were involved in the pathogenesis of vitiligo. Hsa_circRNA_000957 and hsa_circRNA_101798 might be candidate biomarkers for vitiligo.

Conclusion: This study provides scientific clues for understanding the mechanism of vitiligo.

Keywords: vitiligo, circular RNAs, microRNAs, miRNA response element, ceRNA

Introduction

Vitiligo is an acquired depigmented skin disease characterized by the patchy depigmentation of skin, mucosa, and hair, which is caused by the deficiency of functional melanocytes. However, the true pathogenesis of vitiligo is still unclear. At present, vitiligo is considered to be a polygenic genetic disease driven by a combination of genetic and environmental factors.1 The estimated global prevalence of vitiligo varies from 0.5% to 2%.2 The clinical classification of vitiligo is generally divided into non-segmental, segmental, mixed and undetermined types. The former includes generalized, universal, acrofacial and mucosal types.3 Melanocyte self-destruction through autoimmunity (mainly cellular immunity) is one of the main theories to explain the pathogenesis of nonsegmental vitiligo (NSV). However, the molecular mechanism underlying immune imbalance in vitiligo has not been fully elucidated. In recent years, the genome-wide association study and its extension have been applied to the systematic analysis of genetic variation and could elucidate complex skin diseases. In addition, genome-wide microRNA (miRNA) research with gene regulation function has been widely used to reveal the genetic and immune-related pathogenesis of vitiligo.4,5 The identification of sensitive and effective biomarkers can help further improve the pathogenesis, diagnosis, and treatment of vitiligo because the cellular immune destruction of melanocytes is vital in inflammation and cell signaling processes.

Since the discovery of noncoding RNAs (ncRNAs), an increasing number of studies on ncRNAs have indicated the involvement of miRNAs in the pathogenesis and development of the molecular pathology of vitiligo.5,6 Circular RNAs (circRNAs) are special endogenous ncRNAs with covalent closed loop structures and without a 5’ cap and a 3’ polyadenylated tail, which are abundant, conservative, and stable. The extensive, conservative, and tissue-specific properties of circRNAs indicate that they may become a new marker for disease detection in the future.7 CircRNAs.
act as a miRNA sponge by binding to miRNAs with miRNA response element (MREs) and hence influence gene regulation and expression. In addition, many studies have shown that circRNAs are abnormally expressed in many human diseases, thus being vital in the pathogenesis and diagnosis of these diseases. Some circRNAs regulate inflammation, cell proliferation, and immunity of various cells involved in vitiligo. Recent studies on circRNAs focus mainly on cancer, neurological diseases, and cardiovascular diseases. Autoimmune diseases (AIDs) are mainly local or systemic inflammatory symptoms caused by an abnormal immune response to auto-antigens. The regulatory roles of circRNAs on AIDs have been reported, such as systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis, and multiple sclerosis. Oxidative stress may induce the pathogenesis of vitiligo by mediating melanocyte damage through competing endogenous RNA (ceRNA) regulation mechanism. However, the expression of circRNAs has not been analyzed in patients with vitiligo. The present study was performed to describe differentially expressed circRNAs (DEcircRNAs) in vitiligo and evaluate the potential function of representative candidate circRNAs.

**Materials and Methods**

**Participants and Tissue Samples**

Five patients with vitiligo and five healthy humans from the Department of Dermatology, the Second Hospital of Dalian Medical University, were enrolled in the present microarray-based circRNA profiling study. All procedures involving human participants were approved by the Medical Ethics Committee and performed in agreement with the Declaration of Helsinki Guidelines. Informed consent was obtained from all individual participants. The patients involved in this study were consistent with the diagnostic criteria of vitiligo. Systemic therapy (glucocorticoids, immunosuppressive agents, and biological agents), phototherapy, or topical therapy (corticosteroid agents, vitamin D analogs, and tacrolimus) were not used within 3 months before the collection of specimens. No congenital diseases, acquired autoimmune diseases, other immune-related diseases, coagulation disorder, or other inappropriate surgical diseases were reported. The samples of lesional and nonlesional skins from five patients with vitiligo underwent scalpel excision under local anesthesia. The same procedure was used for the five healthy human skin samples used as control. The control group consisted of five healthy adult volunteers, whose age, sex, and site of extraction were matched with those in the vitiligo group. The details about patients with vitiligo and healthy volunteers are shown in Table 1. After excision, the tissue samples were immediately stored in liquid nitrogen until the case collection was completed for RNA extraction.

<table>
<thead>
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<td>Limb</td>
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**Abbreviations:** N.A., not applicable; F, female; M, male; T, test; C, control.
CircRNA Microarray Analysis

Microarrays

Arraystar Human Circular RNA Microarray V2.0 (Arraystar Inc., MD, USA) was used to screen DEcircRNAs between lesional skins of vitiligo (T1-1, T2-1, T3-1, T4-1, and T5-1), nonlesional skins of vitiligo (T1-2, T2-2, T3-2, T4-2, and T5-2), and normal healthy skins (C_1, C_2, C_3, C_4, and C_7), which covered 13,617 circRNA probes on the microarray.

RNA Labeling and Hybridization

Total RNAs from five lesional skins, five nonlesional skins of patients with vitiligo, and five normal healthy skins were extracted with TRIzol (Life Technologies, CA, USA) following the manufacturer’s protocol. The purity and quantity of total RNAs were determined with the NanoDrop ND-1000 (NanoDrop, DE, USA). Sample labeling and array hybridization were performed following the manufacturer’s protocol (Arraystar Inc.). Briefly, total RNAs were digested with RNase R (Epicentre, WI, USA) to remove linear RNAs and enrich circRNAs. Then, the enriched circRNAs were amplified and transcribed into fluorescent cRNAs by a random priming method (Arraystar Super RNA Labeling Kit; Arraystar Inc.). An RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to purify the labeled cRNAs, and NanoDrop ND-1000 was used to measure the concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA). Further, 1 μg of each labeled cRNA was fragmented with 5 μL of 10 × blocking agent and 1μL of 25 × fragmentation buffer. Then, the mixture was heated at 60°C for 30 min. Finally, 25 μL of 2 × hybridization buffer was added to dilute the labeled cRNAs. Subsequently, 50 μL of hybridization solution was dispensed into the microarray gasket slide and transferred to the Arraystar Human Circular RNA Microarray V2.0 (8×15K, Arraystar). The slides were incubated at 65°C for 17 h in an Agilent hybridization oven (Agilent SureHyb Microarray Hybridization Chamber, Agilent Technologies, CA, USA). The hybridized arrays were washed in staining dishes (Thermo Shandon, MA, USA) with a gene expression wash buffer kit (Agilent Technologies), fixed, and scanned with an Agilent Scanner G2505C (Agilent Technologies).

Data Acquisition and Analysis

Intensity data were extracted from the scanned images with the feature extraction software (version 11.0.1.1; Agilent Technologies). R software limma package (Shanghai Biotechnology Corporation, Shanghai, China) was used to process the quantile normalization of raw data and subsequent data. After raw data processing, low-intensity filtering was performed, and the circRNAs containing flags in “P” or “M” (defined by GeneSpring software) in at least 5 out of 15 samples were retained for further differential analyses. DEcircRNAs with statistically significant differences between two groups were identified using volcano plot filtering or fold change (FC) filtering. Hierarchical clustering was performed to show the distinguishable expression pattern of circRNAs among the samples. The statistical significance of the difference was conveniently estimated using the \( t \) test. CircRNAs having FC \( \geq 1.2 \) and \( P \) values \( \leq 0.05 \) were selected as significantly DEcircRNAs.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) was performed to evaluate the expression of the reference gene β-actin, two upregulated genes hsa_circRNA_101798 and hsa_circRNA_403250, and three downregulated genes hsa_circRNA_000957, hsa_circRNA_402437, and hsa_circRNA_091420 in three lesional and nonlesional skins of patients with vitiligo and three normal healthy skins so as to validate microarray data. The cDNAs were synthesized with 3 μg total RNA using specific primers by the Superscript Reverse Transcription System (Invitrogen, CA, USA). The qRT-PCR reaction system was in a total volume of 10 μL of the mixture, including 5 μL of 2 × master mix (Arraystar), 0.5 μL of 10 μM PCR forward primer, 0.5 μL of 10 μM PCR reverse primer, 2 μL of diluted first-strand cDNA, and 2 μL of ddH2O. The primers of specific genes designed by Primer Premier 5.0 software are listed in Supplementary Table 1. The cycling program is 95°C for 10 min, followed by 40 amplification cycles at 95°C for 10 s and 60°C for 1 min (QuantStudio 5 Real-time PCR System, Applied Biosystems). The melt curve analysis was carried out after PCR to determine primer specificity, and the relative level of each circRNA was calculated using the standard
curve method. The Student t test (two-tailed) was used for data analysis. P < 0.05 indicated a statistically significant difference.

**Prediction of Target Genes and Construction of ceRNA Network**

NCBI PubMed and circBase were used for the data mining analysis of DEcircRNAs.

Besides being nuclear transcriptional factors, circRNAs are crucial in regulating miRNAs through MREs of miRNAs incompletely bound to the miRNA sponge. MRE prediction of DEcircRNAs validated by qRT-PCR in lesional or nonlesional skins of patients with vitiligo and the potential target gene mRNAs was performed bioinformatically using Arraystar’s home-made miRNA target prediction software (Arraystar) based on TargetScan (http://www.targetscan.org/vert_71) and miRanda (http://www.microrna.org/microrna/home.do) algorithm. The predicted targets dysregulated in vitiligo were used to construct a circRNA-miRNA-mRNA interaction network using Cytoscape (http://www.cytoscape.org/).

**Gene Ontology, Kyoto Encyclopedia of Genes and Genomes Pathway Analyses of Target Genes**

The Gene Ontology (GO) project provides a controlled vocabulary to describe gene and gene product attributes in any organism (http://www.geneontology.org). Biological process (BP), cellular component (CC), and molecular function (MF) were covered in the GO database and used for differentially expressed mRNAs (DEmRNAs). The P value produced by topGO denoted the significance of GO term enrichment in DEmRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system, which is used to analyze gene function and genome information. The analysis of KEGG enrichment of DEmRNAs helps in finding out the biological regulatory pathways that have changed significantly under experimental conditions. The P value (EASE-score, Fisher-P value, or hypergeometric P value) denotes the significance of the pathway correlated with the conditions. The lower the P value, the more significant the GO term/or pathway (P value ≤0.05 recommended).

**Results**

**DEcircRNAs in Vitiligo**

A circRNA microarray was used to screen DEcircRNAs so as to examine the expression of circRNAs in lesional or nonlesional skins of five patients with vitiligo and five normal healthy skin tissues. The distribution and the degree of dispersion of the hybridization data were evaluated with a box plot. No abnormal distributions of data were found in the 15 samples after log2 normalization in the box plot (Figure 1A). The difference between lesional or nonlesional skin tissues of patients with vitiligo and healthy skin tissues was shown in the scatter plot of the circRNA expression profile (Figure 1B).

A total of 817 DEcircRNAs (426 upregulated and 391 downregulated; FC ≥1.2 and P ≤0.05) were identified in lesional skins of patients with vitiligo compared with healthy skin tissues. Further, 508 DEcircRNAs (146 upregulated and 362 downregulated) were identified in nonlesional skins of patients with vitiligo compared with healthy skin tissues. Also, 82 DEcircRNAs (65 upregulated and 17 downregulated) were identified in lesional skins compared with nonlesional skins of patients with vitiligo. After removing the repeated data, 1132 DEcircRNAs were identified in the three groups, including 538 upregulated and 594 downregulated circRNAs (Figure 2). Volcano plots were constructed to show the DEcircRNAs intuitively between the two groups with two important indicators (FC/P value) (Figure 1C). Hierarchical clustering showed that the expression level of circRNA was clearly distinguished in the associated heat map. The relative expression of circRNA was displayed with a color scale: red for upregulated expression and green for downregulated expression (Figure 3). The top 10 upregulated and downregulated circRNAs based on FC in this study are displayed in Supplementary Tables 2-4. In addition, the origin of the DEcircRNAs and their distribution on human chromosomes were analyzed. The statistical classification revealed that 538 upregulated circRNAs included 493 exonic, 21 intronic, 20 sense overlapping, 2 antisense, and 2 intergenic, while 594 downregulated circRNAs included 484 exonic, 44 intronic, 42 sense overlapping, 21 antisense, and 3 intergenic. Most of the circRNAs in the microarray expression profile were derived from exons, with no significant difference between the upregulated
and downregulated circRNAs \( (P>0.05) \). The DEcircRNAs were widely distributed on all chromosomes, including X chromosome (Figure 4).

**Validation of Selected circRNAs in Vitiligo**

Five circRNAs of the DEcircRNAs were selected for further verification on the basis of raw intensity, \( P \) value, and FC from microarrays. The expression of five candidates was validated by qRT-PCR in the lesional or nonlesional skins of patients with vitiligo and normal healthy skin tissues. The melt curve analysis was carried out after PCR to determine primer specificity, and the relative expression of each circRNA was calculated using the standard curve method. As a result, three circRNAs (hsa_circRNA_000957, hsa_circRNA_091420, and hsa_circRNA_402437) were downregulated and hsa_circRNA_101798 was upregulated in the lesional or nonlesional skins of patients with vitiligo compared with normal healthy skin tissues, which were consistent with microarray results. However, the expression of hsa_circRNA_403250 in the lesional skins of patients with vitiligo was not consistent with the microarray result. Finally, hsa_circRNA_000957 and hsa_circRNA_101798 were validated with statistical significance in the lesional and nonlesional skins of patients with vitiligo compared with healthy skin tissues, but with no significant difference between lesional and nonlesional skins of patients with vitiligo (Figure 5).
Target Genes Predication and Network Construction

Target miRNAs and mRNAs of the DEcircRNAs validated with statistical significance were predicted with Arraystar’s home-made miRNA target prediction software based on the TargetScan and miRanda algorithm. Further, 29 MREs (hsa-miR-3197, hsa-miR-4649-5p, hsa-miR-7113-5p, hsa-miR-3937, hsa-miR-1913, hsa-miR-637, hsa-miR-6886-5p, hsa-miR-4493, hsa-miR-711, hsa-miR-6889-5p, hsa-miR-608, hsa-miR-4713-5p, hsa-miR-4532, hsa-miR-6729-5p, hsa-miR-6887-3p, hsa-miR-7112-5p, hsa-miR-4497, hsa-miR-661, hsa-miR-4713-5p).

Figure 2 The shared DEcircRNAs between the three subgroups in the Venn diagram.

Figure 3 Hierarchical clustering showed the expression level of circRNAs in the three groups in the associated heat map. The relative expression of circRNAs was displayed using the color scale of the fold changes (log2 scaled): red for upregulation (strong intensity) and green for downregulation (low intensity). Each row represents a circRNA, and each column represents a skin tissue from a patient with vitiligo or healthy control. (A) CircRNAs in 15 sample tissues of patients with vitiligo or healthy controls. (B) DEcircRNAs between Test1 and control. (C) DEcircRNAs between Test2 and control. (D) DEcircRNAs between Test1 and Test2. Test1, nonlesional skins of patients with vitiligo; Test2, lesional skins of patients with vitiligo. Control: normal healthy skin tissues.
Figure 4 Types of DEcircRNA (A) and distribution of DEcircRNAs on human chromosomes (B).

Figure 5 Validation of the relative expression of five selected circRNAs using qRT-PCR. After validation, hsa_circRNA_000957 was downregulated and hsa_circRNA_101798 was upregulated with statistical significance in the lesional or nonlesional skins of patients with vitiligo compared with healthy skin tissues. Test1, nonlesional skins of patients with vitiligo; Test2, lesional skins of patients with vitiligo. Control, normal healthy skin tissues. *P < 0.05, **P < 0.01.
GO and KEGG Pathway Analysis of the Target Genes

Target mRNAs of the DEcircRNAs with statistical significance were included in GO and pathway analyses to explore their potential functions in the pathogenesis of vitiligo. The results of the GO enrichment of the target mRNAs are shown in Supplementary Table 5A–C. The association of these mRNAs with BP, MF, and CC in the GO database were analyzed. The top three enriched GO terms were “response to thyroid hormone”, “sphingosine biosynthetic process”, and “serine family amino acid catabolic process” in BP; “phospholipase activity”, “bioactive lipid receptor activity”, and “transforming growth factor-beta binding” in MF; and “sperm part”, “centriole”, and “fibrillar center” in CC (Figure 7A). The result of KEGG analysis is shown in Supplementary Table 6. The top three pathways were “sphingolipid metabolism”, “ether lipid metabolism”, and “VEGF signaling pathway” (Figure 7B). The result of enrichment analysis showed that the main functions of related target genes of hsa_circRNA_000957 and hsa_circRNA_101798 were enriched in thyroid hormone response, steroid hormone response, signaling pathway, sphingolipid metabolism, and essential amino acid biosynthesis, all of which were involved in the pathogenesis of vitiligo. Thus, hsa_circRNA_000957 and hsa_circRNA_101798 might be candidate biomarkers for vitiligo. Further studies are needed to confirm their role in vitiligo.

Figure 6 CircRNA-miRNA-mRNA network in vitiligo. Hsa_circRNA_000957 with 29 MREs and 144 related target genes and hsa_circRNA_101798 with 4 MREs and 18 related target genes were involved in the network. Nodes with red color are miRNAs. Nodes with light-blue color are protein-coding RNAs. Nodes with brown color are circRNAs. Edges with T-shape arrow represent directed relationships. Edges without arrow represent undirected relationships (ceRNA relationship).
Vitiligo is an idiopathic systemic autoimmune disease that affects skin, hair, and oral mucosa. It is a hereditary acquired disease characterized by the destruction of melanocytes, associated with other autoimmune diseases, including autoimmune thyroid disease, rheumatoid arthritis, psoriasis, alopecia areata, chronic urticaria, pernicious anemia, Addison’s disease, adult-onset type 1 diabetes, inflammatory bowel disease, and lupus erythematosus. The cause of vitiligo remains unknown; the autoimmune mechanism is the focus of vitiligo. Despite a few other physical symptoms, vitiligo seriously affects the physical and mental health of patients because of the impact of appearance.

To date, different drug therapies, surgery, and phototherapy have been used in the treatment of vitiligo, which are not curative; the pathogenesis of vitiligo is under exploration. The involvement of inflammation and autoimmunity is important in vitiligo, especially in the progression of the disease.

Studies on the role of miRNAs in vitiligo have been reported. As an miRNA sponge, circRNAs can regulate gene transcription, modulate the expression of parental genes, and participate in the occurrence and development of various diseases. Some circRNAs regulate inflammation, cell proliferation, and immunity of various cells involved in the pathogenesis of vitiligo, and ceRNA regulation mechanism may be involved in the oxidative stress-mediated melanocyte injury. Therefore, circRNA may be a crucial regulatory factor in the pathogenesis of vitiligo and may become a new target for the treatment of vitiligo. However, the expression profile and potential biological functions of circRNAs in vitiligo have not been reported.

In this study, circRNA microarray and qRT-PCR were used to detect the circRNA expression profile and verify the DEcircRNAs in the lesional or nonlesional skins of patients with vitiligo compared with healthy skin tissues. A total of 817 and 508 DEcircRNAs were identified in lesional and nonlesional skins of patients with vitiligo, respectively. Most of the circRNAs in the microarray expression profile were derived from exons. Three major types of circRNA have been identified: exonic circRNA (ecircRNA), circular intronic RNA (ciRNA), and exon-intron circRNA (EIciRNA). CircRNA acts as an miRNA sponge in the cytoplasm, which is the major role of...
ecircRNAs. However, ciRNAs and ElciRNAs are located in the nucleus and regulate gene transcription. DEcircRNAs were mainly derived from exons in the present study, which was consistent with the function of identified circRNAs. Meanwhile, the DEcircRNAs were widely distributed on all chromosomes, including X chromosome. After verification, hsa_circRNA_000957 was downregulated and hsa_circRNA_101798 was upregulated with statistical significance in the lesional or nonlesional skins of patients with vitiligo compared with healthy skin tissues, but with no significant difference between lesional and nonlesional skins of patients with vitiligo. The qRT-PCR results of the other three circRNAs were not completely consistent with circRNA microarray findings, with minimal difference between lesional and nonlesional skins of vitiligo. It was speculated that the differences in circRNAs might be related to the genetic background of patients with vitiligo or small sample size. Hsa_circRNA_000957 and hsa_circRNA_101798 were located on chromosome 19 and chromosome 16, respectively, which were related to autoimmune diseases such as SLE, psoriasis, and diabetes. Therefore, they might be involved in the pathogenesis of vitiligo.

The MREs and their target genes were predicted to further examine the potential role of hsa_circRNA_000957 and hsa_circRNA_101798 in vitiligo. As a result, 32 MREs and their related target genes were identified, and the circRNA–miRNA–mRNA network was constructed according to the ceRNA theory. The GO analysis was conducted to explore biological functions. The predicted miRNAs were mainly associated with cancer (colorectal cancer, prostate cancer, lung cancer, etc.), diabetes, Alzheimer’s disease, cell growth, apoptosis, immune response, and inflammation, such as miRNA-608 and miRNA-148b-3p. The present study found that the main functions of related target genes of hsa_circRNA_000957 and hsa_circRNA_101798 were enriched in growth hormone receptor signaling pathway, thyroid hormone response, steroid hormone response, signaling pathway, sphingolipid metabolism, and essential amino acid biosynthesis, all of which were involved in the pathogenesis of vitiligo. Above all, hsa_circRNA_000957 and hsa_circRNA_101798 might be candidate biomarkers for vitiligo. However, this is a preliminary study and needs future in-depth studies. Further investigation needs to be performed to explore the interaction between DEcircRNAs and their target miRNAs. In addition, the data of this paper is based on a small number of samples. We will expand the sample size to confirm our findings.

Conclusion
We identified the DEcircRNAs in the lesional or nonlesional skins of patients with vitiligo compared with healthy skin tissues. Furthermore, MREs and target genes of DEcircRNAs were predicted, and the circRNA–miRNA–mRNA network was constructed by bioinformatics analysis. Then, the GO and KEGG pathway analyses were carried out to explore the underlying mechanism of DEcircRNAs in vitiligo, which needs further exploration.

Ethical Approval
All procedures involving human participants were approved by the Ethics Committee of the Second Hospital of Dalian Medical University and performed in agreement with the Declaration of Helsinki Guidelines. Informed consent was obtained from all individual participants.

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Disclosure
The authors report no conflicts of interest in this work.

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