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· Basic Research ·

Expression of lens-related microRNAs in transparent infant lenses and congenital cataract

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Abstract

● AIM: To identify the expression of lens-related microRNAs (miRNAs) in the central epithelium of transparent infant lenses and congenital cataract.

● METHODS: Lens-related miRNAs were retrieved from PubMed database. The expression levels of these miRNAs in transparent infant lenses and congenital cataract were determined by stem-loop reverse transcription-polymerase chain reaction (RT-PCR). miRanda algorithm was used to predict the target genes of these differentially expressed miRNAs. The target mRNA was validated.

● RESULTS: Six lens-related miRNAs were retrieved from screening PubMed database. The most abundant miRNA in transparent infant lenses according to stem-loop RT-PCR was miR-184. miR-182 was up-regulated in congenital cataract. Contrarily, miR-204 and miR-124 was down-regulated. miR-204 exhibited a more significant decrease in expression than miR-124. In addition, Meis2 was predicted to be the target of miR-204 using miRanda algorithm. miR-204 mimic/antagomir transfection experiments suggested the negative correlation between the expression of miR-204 and Meis2.

● CONCLUSION: The expression levels of miR-182, miR-204 and miR-124 differ between the central epithelium of transparent infant lens and congenital cataract, suggesting their involvement in the pathogenesis of congenital cataract. miR-204 may act *via* silencing Meis2 to regulate lens development and congenital cataract formation.

● KEYWORDS: lens-related miRNAs; congenital cataract; miR-204; Meis2

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INTRODUCTION

Congenital cataract is a clinical disorder of opacity of the crystalline lens, usually presenting at birth or during infancy, childhood or adolescence[1]. This is one of the most common causes of treatable visual impairment and blindness during infancy, with an estimated prevalence of 1 to 6 cases per 10 000 live births. Approximately one-quarter to one-third of congenital cataract cases are inherited. To date, at least 60 genes are involved in genetic congenital cataracts and this number continues to increase[2].

MicroRNAs (miRNAs) are short non-coding RNAs that modulate gene expression through translational repression and mRNA decay[3]. They have been detected in a variety of tissues from eyes. miRNA expression is associated with the pathogenesis of various eye disorders, such as pterygium, retinoblastoma and glaucoma[4-6]. However, few studies have focused on the identification and expression of miRNAs in congenital cataract. Therefore, we examined the expression of some miRNAs in transparent infant lenses and congenital cataract to determine the miRNAs involved in the pathogenesis of congenital cataract.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB) of the First Affiliated Hospital of Xi'an Jiaotong University. The guardians of all participants included in this study provided written informed consents, and the consent forms were approved by the IRB. All procedures used in this study conformed to the guidelines mentioned in the Declaration of Helsinki.

Inclusion Criteria, Classification, and Tissue Grouping

Twelve lens were collected from postmortem eyes (6 donors, donor age range was 1-4y, free of ocular diseases) and congenital cataract infants (6 patients, patient age range was 1-4y, free of other ocular diseases). Lenses from postmortem eyes were obtained from donors of corneal transplantation in the

Table 1 Primer sequences used for stem-loop RT-PCR

miRNA name	miRNA sequence (5'-3')	Stem-loop RT-PCR primer (5'-3')	Forward primer (5'-3')
miR-184	UGGACGGAGAACUGAUAAGGGU	[a]-ACCCTT	[b]-AACATTCAACGCTGT
miR-204	UUCCCUUUGUCAUCCUAUGCCU	[a]-AGGCAT	[b]-TTCCCTTTGTCATC
miR-182	UUUGGCAAUGGUAGAACUCACACU	[a]-AGTGTG	[b]-TTTGGCAATGGTAGA
miR-125b	UCCCUGAGACCCUAACUUGUGA	[a]-TCACAA	[b]-TCCCTGAGACCCTA
miR-124	UAAGGCACGCGGUGAAUGCC	[a]-GGCATT	[b]-TAAGGCACGCGG
let-7b	UGAGGUAGUAGGUUGUGUGGUU	[a]-AACCAC	[b]-TGAGGTAGTAGGTT

[a] Additional sequence (GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC) before the stem-loop RT-PCR primer;
[b] Additional sequence (CGGCG) before the forward primer. Universal reverse primer GTGCAGGGTCCGAGGT (5'-3').

First Affiliated Hospital of Xi'an Jiaotong University, 8 to 24h after death. These samples were used as the transparent lens samples. The congenital cataractous lens presented with pulverulent cataracts. These lenses were acquired during surgeries performed on congenital cataract infants. There were no statistically significant differences between the transparent infant lenses and congenital cataract with respect to the age of the donors (P=0.625).

Tissue Preparation and RNA Extraction All lens were centered anterior capsules with a diameter of 5 to 5.5 mm obtained during anterior continuous curvilinear capsulorhexis. Three samples were randomly pooled into one lens sample to insure the quality and quantity of extracted total RNA for subsequent stem-loop reverse transcription-polymerase chain reaction (RT-PCR) experiments. Therefore, the authors pooled the central epithelia of three lenses (transparent or cataractous) to obtain two sets of samples for both transparent infant lenses and congenital cataract. All tissue samples were cooled with liquid nitrogen and homogenized in TRIzol reagent (Invitrogen, CA, USA). Total RNA was then extracted according to the manufacturer's instructions. RNA quality was confirmed by calculating the OD260/280 ratio using absorbances measured by a spectrophotometer (Bio-Rad, CA, USA), and the integrity of the RNA was verified by agarose electrophoresis.

Primary Analysis and Screening of microRNAs A comprehensive search of PubMed database was conducted to identify relevant literatures. We screened for lens-related miRNAs using criteria as follows: 1) the miRNA had been identified in both human lens and lens from other species by different researchers; 2) the miRNA had been identified in lens using different molecular biology techniques.

Real-time Quantification of microRNAs by Stem-loop Reverse Transcription-polymerase Chain Reaction Stem-loop RT-PCR[7] was used to detect miRNAs in lens. Primers used in stem-loop RT-PCR experiments were shown in Table 1. Biefly, total RNA was extracted from the samples using TRIzol reagent according to the manufacturer's instructions. Total RNA (1000 ng) was reverse transcribed for 45min at 37℃, and for 5s at 85℃ using the Prime Script RT reagent Kit (TaKaRa, Dalian, China). Stem-loop RT-PCR was performed using the SYBR Green I assay (TaKaRa, Dalian, China). The amplification conditions used were as follows: 3min at 95℃, 39 cycles of 15s at 95℃, 30s at 60℃, and ramp up from 65℃ to 95℃ at 0.5℃ intervals for 5s each. The results were analyzed using the comparative threshold cycle method and were normalized to β-actin as an internal control. All PCRs were performed in triplicates. The stem-loop RT-PCR was run two times for each sample (pooled central epithelium of two individual transparent and cataractous lenses) and calculated the mean values were shown.

Bioinformatics Analysis to Identify Possible microRNA Target Genes The target sites predicted by miRanda were scored for the likelihood of mRNA-induced down-regulation using mirSVR, a regression model that's based on sequence and contextual features of the predicted miRNA to mRNA duplex. This prediction algorithm was previously cited in the literature[8].

Cell Culture and Treatment with microRNA Mimics/Antagomir Human lens epithelium-B3 (HLE-B3) was obtained from American Type Culture Collection (ATCC; MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, MO, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich, MO, USA) in a humidified atmosphere of 5% CO$_2$ at 37℃.

The miR-204 mimic/antagomir and negative control miRNAs were commercially available(RiboBio, Guangzhou, China), and the experiments were performed according to the manufacturer's instructions. In brief, 5×10^5 cells were seeded per well in 6-well plates. The miR-204 mimics/antagomir (or control miRNAs) and Lipofectamine 2000 (Invitrogen, CA, USA) were diluted and mixed gently in DMEM separately, and then were added to the culture plates. The final concentration of mimic was 50 nmol/L, and the final concentration of antagomir was 100 nmol/L. After a 48h incubation at 37℃, the cells were collected for additional experiments.

Western Blotting SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed according to standard procedures. The cell pellets were collected at 48h post-transfection for protein extraction. The cells were extracted with lysis buffer containing 150 mmol/L

NaCl, 1% NP-40, 0.1% SDS, 2 mg/mL aprotinin and 1 mmol/L PMSF for 30min at 4℃. The protein extracts were separated on 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. After subsequent blocking in Tris-buffered saline with Tween-20 (TBST) containing 25 mmol/L Tris-HCl, pH 7.5, 137 mmol/L NaCl, 2.7 mmol/L KCl and 0.05% Tween-20 with 5% nonfat milk for 1h at 37℃, the membranes were incubated with the primary antibody against Meis2 (Santa Cruz Biotechnology, TX, USA) or GAPDH (Abcam, MA, USA) in TBST with 5% nonfat milk at 4℃ overnight. The membranes were extensively washed three times with TBST and incubated with the goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Abcam, MA, USA) at room temperature for 1h. After additional washes with TBST, the proteins were visualized with an ECL kit (Beyotime Institute of Biotechnology, Shanghai, China).

Statistical Analysis Differences between the two groups were evaluated with an independent samples *t*-test and a statistical computer program (SPSS 18.0; SPSS, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Primary microRNA Screening and Analysis More than 50 miRNAs had been identified in lens using distinct molecular biology techniques in different studies. As described above in the methods section, we retrieved six miRNAs meeting the criteria as lens-related miRNAs (Table 2) for our study.

Expression of Lens-related microRNAs in the Central Epithelium of Transparent Infant Lenses All six miRNAs were detected by stem-loop RT-PCR in the central epithelium of transparent infant lenses. miRNAs were ranked according to average expression levels. The average expression levels of miRNAs were represented by Ct (Cycle threshold) values in stem-loop RT-PCR. Ct levels are inversely proportional to the amount of target miRNA in the sample. The lower the Ct values, the greater the average expression levels of the miRNA. We found the Ct value of miR-184 was 18 and it was significantly lower than the other miRNAs ($P<0.05$) (Figure 1). This indicated that miR-184 was the most abundant miRNA in transparent infant lenses.

Differential Expression of microRNAs between Transparent and Cataractous Samples Stem-loop RT-PCR analysis demonstrated that 3 of the 6 miRNAs were differentially expressed between the transparent and cataractous samples ($P<0.05$) (Figure 2). miR-182 was up-regulated, while miR-204 and miR-124 were down-regulated in cataractous samples. miR-204 exhibited the most significant decrease in expression in cataractous samples (approximately 4.76-fold decrease).

Identification of Candidate Target Genes for microR-204 To determine the potential targets of miR-204, the miRNA target prediction tool miRanda was used. We identified hundreds of putative target mRNAs of miR-204. Among these candidates, Meis2 was selected due to its possible association with cataractogenesis[13].

Table 2 Lens-related miRNAs and corresponding molecular biology technique

Molecular biology technique	Lens-related miRNAs
In situ hybridization[9]	miR-184, miR-204, miR-124, miR-182, miR-125, let-7b
Microarray[10]	
Real-time PCR[11]	
Northern blot[12]	

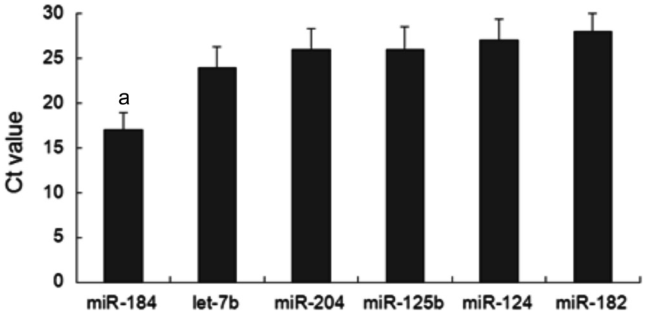

Figure 1 Ct value of lens-related miRNAs The Ct level of miR-184 was significantly lower than the others in transparent infant lenses ($^aP<0.05$ *vs* other miRNAs).

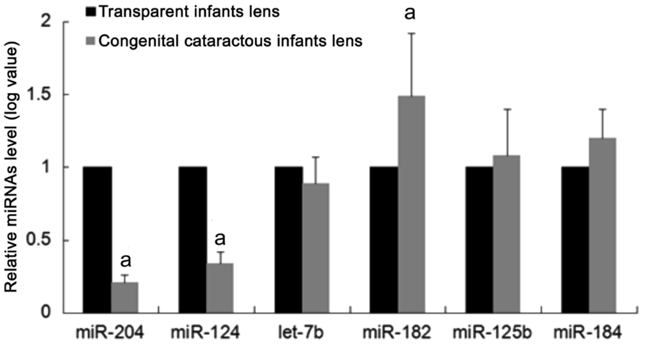

Figure 2 Analysis of the six lens-related miRNAs miR-182 was significantly up-regulated and miR-204, miR-124 were significantly down-regulated in congenital cataractous lenses. $^aP<0.05$.

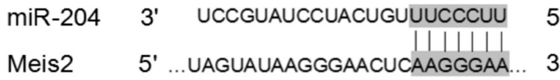

Figure 3 Heteroduplexes formed between miR-204 and Meis2.

Using miRanda to analyze the 3'-UTR of Meis2 to identify potential binding sites for miR-204, a single recognition sequence containing a conserved 7-mer exact seed match at positions 339-345 bp (Figure 3) was identified in the Meis2 3'-UTR. This indicated that miR-204 may directly bind to Meis2 3'-UTR to regulate Meis2 expression.

microR-204 Regulate Meis2 Expression in Human Lens Epithelium-B3 Cells To validate Meis2 as a target of miR-204, HLE-B3 cells were transfected with miR-204 mimics to over express miR-204 (Figure 4A). Cells transfected with miR-204 mimics exhibited lower level of Meis2 compared to the control ($P<0.05$) (Figure 4B, 4C). On the contrary, transfection of miR-204 antagomir in HLE-B3 cells suppressed

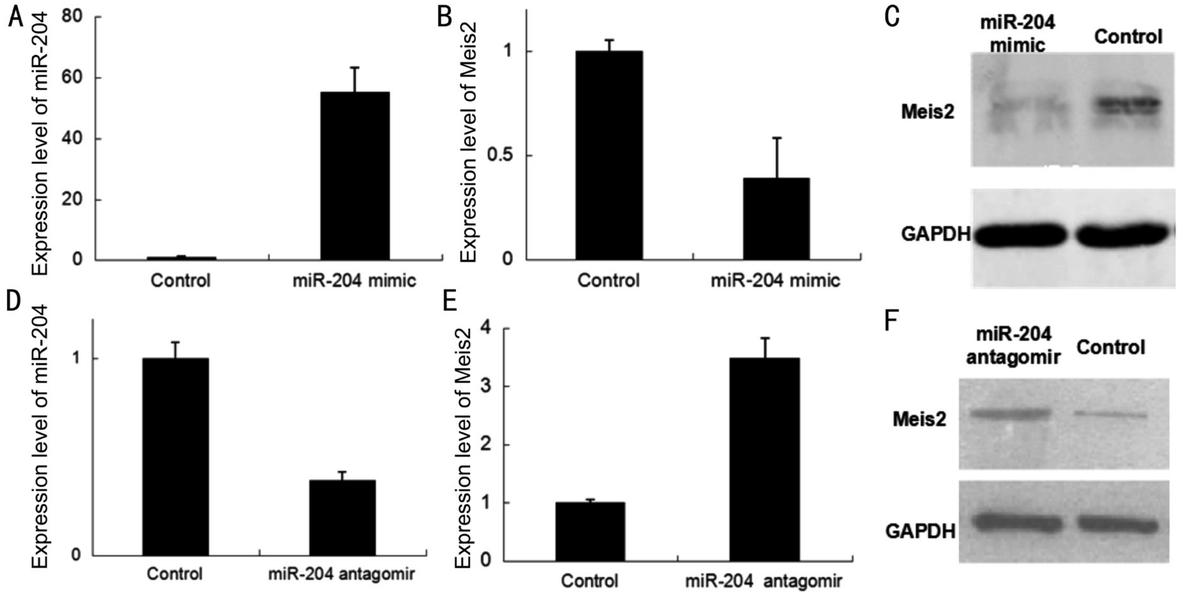

Figure 4 The expression of Meis2 was negative correlation with expression of miR-204.

the expression of miR-204 (Figure 4D), and the level of Meis2 was elevated ($P<0.05$) (Figure 4E, 4F).

DISCUSSION

miRNAs have emerged as prominent gene regulators. Although miRNAs have been identified in human lens, their expression in infant lens remains unknown. This article is the first description of the identification of lens-related miRNAs in the central epithelium of both transparent infant lenses and congenital cataract. Results of the present study showed that the expression of miR-184 was the highest among the lens-related miRNAs in transparent infant lens. miR-182 was up-regulated, while miR-204, miR-124 were down-regulated in congenital cataract, suggesting their involvement in the pathogenesis of congenital cataract.

It showed that miR-184 was the most abundantly expressed miRNA in our study. According to the previous studies, miR-184 was reported in distinct mammalian eyes, particularly in the cornea[10]. In our previous results, we also detected miR-184 both in human transparent lenses and cataract[14]. Subsequent work indicated that miR-184 was related to the avascularity of eye tissue[15]. As far as our current research, we hypothesize that high expression of miR-184 in samples contributes to the maintenance of avascularity both in transparent infant lenses and cataract. miR-184 might be essential for the avascular maintenancein lenses.

The fact that different miRNAs expression in central epithelium of transparent infant lens and congenital cataract suggested miRNAs may play an important role in lenticular development and cataractogenesis. It was found that miR-182 was up-regulated in congenital cataract. Contrarily, miR-204 and miR-124 were down-regulated. miR-204 exhibited the most significant decrease. A few studies have suggested that miR-182 is involved in the tumorigenicity. miRNA-182 shows increased expression in various tumors and is thought to be a tumor promoter[16-17]. miR-182 was up-regulated in congenital cataract in this study, suggesting its involvement in the pathogenesis of congenital cataract.

The prediction of target genes is a key step toward understanding the function of specific miRNAs. Several prediction algorithms have been developed based on the principle that the 5' region of the miRNA pairs with the 3'-UTR of target mRNAs to achieve post-transcription silencing. In the current study, miRanda was used to predict the target genes of miR-204, which exhibited a statistically significant change in expression between transparent infant lenses and congenital cataract. Hundreds of genes were predicted as the target mRNAs of miR-204. Among these genes, Meis2 was involved in lens development and cataract[13,18]. Subsequently, we transfected miR-204 mimic/antagomir and its corresponding controls into HLE-B3 respectively, then the expression of Meis2 was detected. A negative correlation was found between the expression of miR-204 and Meis2. These indicated that miR-204 may act *via* Meis2 to regulate lens development and cataract formation. The results were in agreement with previous work by Conte *et al*[19].

In conclusion, this study was conducted to investigate lens-related miRNAs expression in infant lens, and is the first to detect miRNAs expression in the central epithelium of infant lenses. Among the lens-related miRNAs, miR-182 was up-regulated in congenital cataractwhile miR-204, miR-124 was down-regulated. Our study also suggested Meis2 was the target of miR-204 in HLE-B3. These results may aid the development of novel therapeutic strategies towards congenital cataract.

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Conflicts of Interest: Wu CR, None; **Ye M,** None; **Qin L,** None; **Yin Y,** None; **Pei C,** None.

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