• Basic Research •

# Characterization of N$^6$-methyladenosine long non-coding RNAs in sporadic congenital cataract and age-related cataract

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Received: 2023-11-15 Accepted: 2024-07-09

## Abstract

• **AIM:** To characterize the N$^6$-methyladenosine (m$^6$A) modification patterns in long non-coding RNAs (lncRNAs) in sporadic congenital cataract (CC) and age-related cataract (ARC).

• **METHODS:** Anterior capsule of the lens were collected from patients with CC and ARC. Methylated RNA immunoprecipitation with next-generation sequencing and RNA sequencing were performed to identify m$^6$A-tagged lncRNAs and lncRNAs expression. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses and Gene Ontology annotation were used to predict potential functions of the m$^6$A-lncRNAs.

• **RESULTS:** Large amount of m$^6$A peaks within lncRNA were identified for both CC and ARC, while the level was much higher in ARC (49 870 peaks) than that in CC (18 688 peaks), yet those difference between ARC in younger age group (ARC-1) and ARC in elder age group (ARC-2) was quite slight. A total of 1305 hypermethylated and 1178 hypomethylated lncRNAs, as well as 182 differential expressed lncRNAs were exhibited in ARC compared with CC. On the other hand, 5893 hypermethylated and 5213 hypomethylated lncRNAs, as well as 155 significantly altered lncRNA were identified in ARC-2 compared with ARC-1. Altered lncRNAs in ARC were mainly associated with the organization and biogenesis of intracellular organelles, as well as nucleotide excision repair.

• **CONCLUSION:** Our results for the first time present an overview of the m$^6$A methylomes of lncRNA in CC and ARC, providing a solid basis and uncovering a new insight to reveal the potential pathogenic mechanism of CC and ARC.

• **KEYWORDS:** congenital cataract; age-related cataract; N$^6$-methyladenosine; RNA modification; long non-coding RNA; epigenetics

**DOI:10.18240/ijo.2024.11.02**

**Citation:** Ye HF, Zhang X, Zhao ZN, Zheng C, Fei P, Xu Y, Lyu J, Chen JL, Guo XX, Zhu H, Zhao PQ. Characterization of N$^6$-methyladenosine long non-coding RNAs in sporadic congenital cataract and age-related cataract. *Int J Ophthalmol* 2024;17(11):1973-1986

## INTRODUCTION

Epigenetics is defined as the heritable alteration in genome function in absence of changing DNA sequence. It explains the long-term intersection of genetics and environments on the susceptibility in human diseases[1]. With the rapid development and prevalence of second-generation sequencing technology and bioinformatics, wide range of studies have demonstrated the role of epigenetics in aging as well as a variety of multifactorial diseases, including vascular, neurodegenerative, autoimmune diseases, and cancer[2-3]. In the

field of ophthalmology, limited but growing evidence suggested an epigenetic foundation in several ocular disorders[4-5].

Age-related cataract (ARC) is one of the major causes of vision impairment that accounts for approximately 50% of the blindness among elderly population worldwide[6]. Currently, there were studies demonstrated that the pathogenesis of ARC was controlled by epigenetic regulation[7]. Some crucial genes, which encode major structural proteins, antioxidant enzymes, or chaperones of crystalline lens, are altered in expression levels caused by DNA methylation or histone modification, the two principal patterns of epigenetic mechanisms[8]. Meanwhile, chromatin-remodeling participates in abnormal differentiation of lens cells, resulting in cataract. In addition, certain non-coding RNAs suppress epithelial-to-mesenchymal transition (EMT) and lens fibrosis, preventing cataractogenesis[9]. Repression of long non-coding RNA (lncRNA)-myocardial infarction-associated transcript (MIAT) resulted in abnormal growth and migration of lens epithelial cells (LECs)[10], and the inhibition of lncRNA-HOX antisense intergenic RNA (HOTAIR) repressed cell viability, proliferation, and EMT of LECs[11], both of which participate in the pathogenesis of ARC and posterior capsular opacification.

On the other hand, congenital cataract (CC), characterized by its onset of lens opacification at the birth and ranked as the leading cause of medicable childhood blindness, is considered primarily to arise from genetic mutations. While Liu *et al*[12] for the first time explained the pathogenesis of CC from the epigenetic perspective, whose study revealed that the methylation level of five core genes changed in idiopathic CC patients, leading to disfunction of cytoskeleton and intercellular junctions, eventually causing sporadic CC. Based on the above conclusions, the potential epigenetic mechanisms on cataract need to be further verified.

As a critical layer of epigenetic regulations of gene expression, post-transcriptional modifications of RNA have drawn great attention in recent years due to the advances of the sequencing technology of methylated RNA immunoprecipitation with next-generation sequencing (MeRIP-seq)[13]. N$^6$-methyladenosine (m$^6$A) is the most prevalent RNA modification in messenger RNAs (mRNAs) and lncRNAs in higher eukaryotes, including methylation at the 6$^{th}$ position nitrogen atom of adenine (A) of RNA. Clinical relevance of m$^6$A RNA modification has been reported in some age-related diseases[14]. However for cataract, only three studies have involved the role of m$^6$A modification[15-17], and only one study revealed the profile of m$^6$A modifications to circular RNAs (circRNAs) in ARC[15]. Little is known about the m$^6$A modified mRNAs and lncRNAs in ARC, as well as the uncovered m$^6$A modification pattern in CC.

Increasing evidence indicates that lncRNAs participate in cataract development and the progression of EMT in LECs[18]. Certain lncRNAs, including *MIAT*, *TUG1* and *KCNQ1OT1*, act as cataract-specific biomarkers and their silencing can impact on the proliferation of LECs and EMT. Using microarray analysis and high-throughput sequencing, differentially expressed profiles of lncRNAs were identified between normal LECs and EMT.

Since mounting studies support the notion that the interaction between m$^6$A methylation and lncRNAs is involved in EMT[19] and functions in the pathogenesis of both ARC and CC[8,12], figuring out the m$^6$A profiles of lncRNAs would be valuable to further reveal the potential pathogenic mechanism of ARC and CC. Herein, we for the first time comprehensively analyzed the profile of lncRNAs m$^6$A methylation of anterior lens capsule in ARC and CC patients, as well as ARC in different age groups, in order to acquire their potential functional implications. Using MeRIP-seq and RNA Sequencing (RNA-seq), we order to identify the m$^6$A modification patterns, the m$^6$A-modified lncRNAs, as well as the differentially expressed lncRNAs among LEC samples between ARC and CC, and among different age groups. Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) enrichment analyses and Gene Ontology (GO) annotation were also performed to predict potential functions of the m$^6$A-lncRNAs.

## MATERIALS AND METHODS

**Ethical Approval** This study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-XHYY-2020-016) and carried out in accordance with the principles of the Helsinki Declaration.

**Acquisition of Biological Samples** Seventeen anterior lens capsule samples were collected and divided into three groups: CC group, ARC in younger age group (ARC-1), and ARC in elder age group (ARC-2). For CC group, all the patients suffered from sporadic CC without familial history. Patients with any other congenital ocular diseases or trauma, a family history of hereditary CC or viral infection during gestation, or long-term medication, or radiation exposure that increases the risk of cataract were all excluded. After continuous circular capsulorhexis (CCC), routine lensectomy, posterior vitrectorhexis and anterior vitrectomy were conducted with or without intraocular lens (IOL) implantation. For ARC samples, this study focused on ARC with cortical type because it is the major form of ARC. Patients with complicated cataracts due to high myopia, trauma, uveitis or glaucoma, and patients with systematic diseases, such as hypertension and diabetes, were all excluded. In ARC-1 group, patients were under the age of 70y, and in ARC-2 group were above the age of 80y. Each ARC

patient underwent conventional cataract phacoemulsification and IOL implantation. For all the samples, the anterior capsule sample was collected into cryopreservation tube and stored in -80°C immediately after the procedure of CCC. All the patients' information is demonstrated in Table 1.

**RNA Extraction and Quality Control** Total RNA was isolated from each anterior lens capsule sample by TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA concentration was identified by NanoDrop ND-1000 at 260/280 nm, and the OD260/OD280 ratio of RNA in all the samples ranged from 1.8 to 2.1. Total RNA quality was estimated by the ratio of the 18S/28S ribosomal band intensities in an ethidium bromide-containing 1% agarose gel after electrophoresis.

**High-Throughput m$^6$A and RNA Sequencing** MeRIP-seq and RNA-seq service were conducted by Cloudseq Biotech Inc. (Shanghai, China) in accordance with a previously reported procedure[20]. In brief, m$^6$A RNA immunoprecipitation was performed using the GenSeq™ m6A-MeRIP Kit (GenSeq Inc., China) by following the manufacturer's instructions. Both the m$^6$A immunoprecipitation samples and the input samples without immunoprecipitation were applied for RNA-seq library generation with NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs, Inc., USA). Bioanalyzer 2100 system (Agilent Technologies, Inc., USA) was used to evaluate the library quality. Library sequencing was conducted using an Illumina Hiseq instrument with 150 bp paired-end reads. In final, raw data of MeRIP-seq and RNA-seq have been uploaded to Gene Expression Omnibus (GEO) database (accession number GSE221970). Figure 1 presents an graphical flowchart of the key steps for peak annotation processes.

**Data Analysis** Briefly, paired-end reads were harvested from Illumina NovaSeq 6000 sequencer and quality control were done by Q30. Low quality reads were abandoned by cutadapt software (v1.9.3) after 3' adaptor-trimming. Then, clean reads of all libraries were aligned to the reference genome by Hisat2 software (v2.0.4). Methylated sites on RNAs (peaks) and differentially methylated sites were respectively identified by MACS software and by diffReps. These peaks recognized by both softwares overlapping with lncRNA were figured out and chosen by home-made scripts. Identified m$^6$A peaks were subjected to motif enrichment analysis using HOMER[21]. Differentially expressed lncRNAs were identified according to *P*-value and fold change (FC). When the *P*-value was less than 0.05 and the FC was larger than 2 between two groups, lncRNAs were defined as significantly differentially expressed.

**Functional Enrichment Analysis** The GO project provides a controlled, species-independent vocabulary to describe gene and gene products attributes in any organism, which covers three associated domains: biological process (BP), cellular component and molecular function (MF). KEGG pathway analysis further investigates the biological functions and molecular interactions of gene products. In this study, functional enrichment analysis was presented for the differentially methylated lncRNAs. $P \leq 0.05$ was considered statistically significant.

**Table 1 Clinical characteristics of CC and ARC patients**

| Samples | Sex | Age (y) |
|---|---|---|
| CC | | |
| No.1 | Male | 3 |
| No.2 | Male | 9 |
| No.3 | Female | 5 |
| No.4 | Female | 8 |
| No.5 | Female | 7 |
| ARC-1 | | |
| No.1 | Male | 60 |
| No.2 | Male | 65 |
| No.3 | Male | 61 |
| No.4 | Female | 63 |
| No.5 | Female | 55 |
| No.6 | Female | 67 |
| ARC-2 | | |
| No.1 | Male | 83 |
| No.2 | Male | 90 |
| No.3 | Female | 83 |
| No.4 | Female | 81 |
| No.5 | Female | 86 |
| No.6 | Female | 83 |

CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

**Statistical Analysis** Data from three or more independent experiments were presented as the mean±standard deviation (SD). The negative binomial distribution were performed by diffReps software for analyzing differentially methylated sites on lncRNAs peaks between ARC and CC groups, as well as bewteen ARC-1 and ARC-2 groups. For differentially expressed lncRNAs and functional enrichment analysis, statistical analysis was conducted by SPSS software (Version 22.0, NY, USA) and GraphPad Prism 5.0 software (Graph-Pad Software, CA, USA). Paired Student's *t*-tests were performed between between ARC and CC groups, as well as bewteen ARC-1 and ARC-2 groups to compare differentially expressed lncRNAs. $P<0.05$ were defined as the threshold for significant differences.

## RESULTS

**Landscape of m$^6$A Modification Patterns in ARC and CC** MeRIP-seq and RNA-seq were performed in LECs from biological replicates from the CC ($n$=5) and ARC ($n$=12) groups, and the ARC group was subdivided into younger age group (ARC-1, $n$=6) and elder age group (ARC-2, $n$=6) as

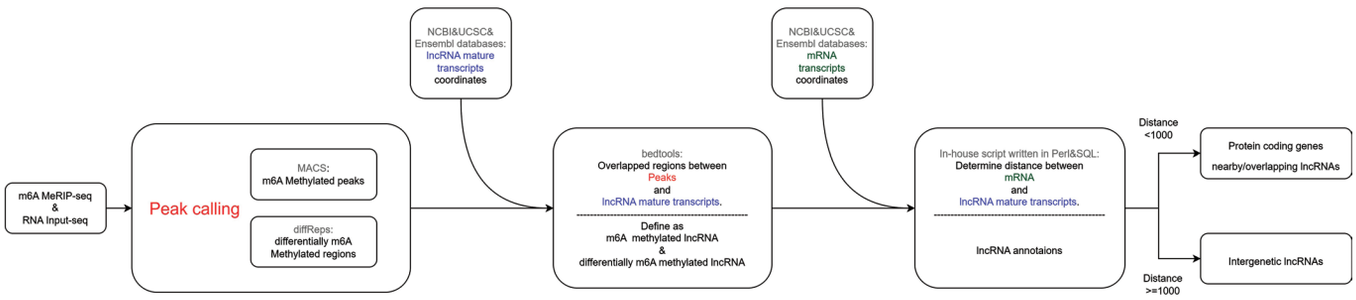


**Figure 1 The bioinformatics pipeline for m$^6$A methylated lncRNAs** 1) Paired-end reads were harvested from DNBSEQ-T7 sequencer, and were quality controlled by Q30. After 3' adaptor-trimming and low quality reads removing by cutadapt software. The reads were aligned to the reference genome with Hisat2 software. Methylated m$^6$A peaks were identified with MACS software, and differentially methylated m$^6$A regions were defined with DiffReps software. 2) The coordinates of lncRNA mature transcripts were fully collected from three well-known transcriptome databases (NCBI, UCSC, Ensembl). The overlapped regions between differntially methylated m$^6$A regions and lncRNA transcripts were identified with Bedtools, while these lncRNAs with overlapped regions were defined as "differentially m$^6$A methylated lncRNA". 3) The differentially m$^6$A lncRNAs were annotated as 5 types (intronic, antisense, sense-overlapping, bidirectional and intergenic) according to the genomic location relative to nearby protein-coding genes with in-house scripts written in Perl&SQL. lncRNA: Long non-coding RNA; m$^6$A: N$^6$-methyladenosine.

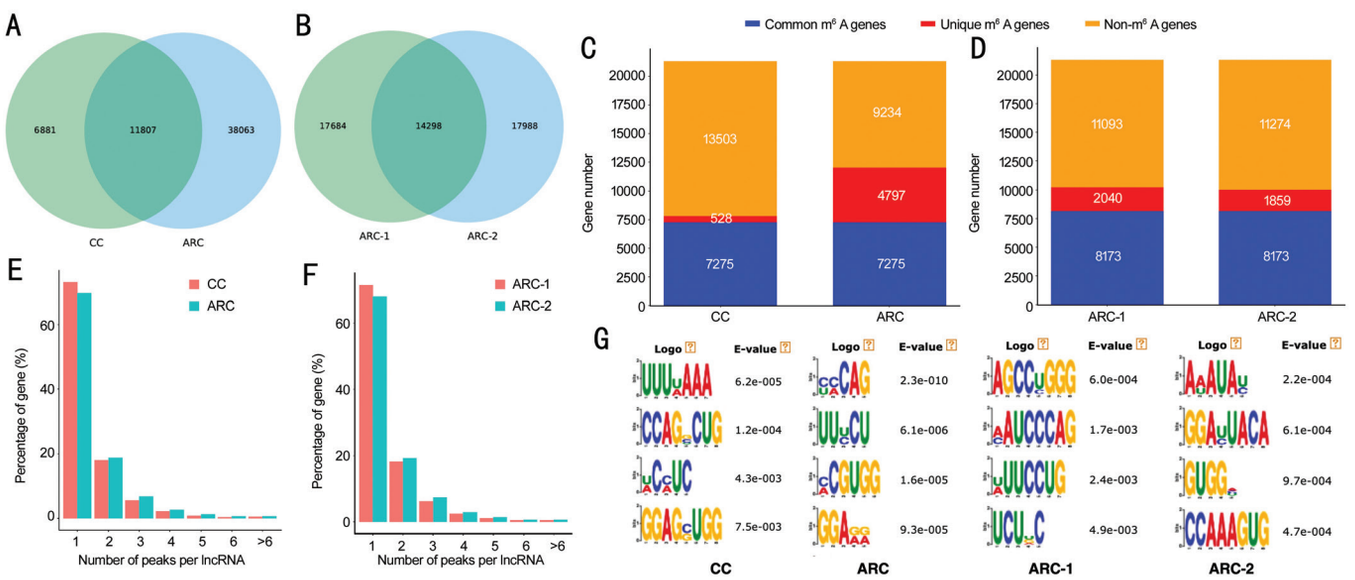


**Figure 2 Overview of m$^6$A modification patterns within lncRNAs** A, B: Venn diagram showing the overlap of m$^6$A peaks within lncRNAs in CC and ARC groups, and in ARC-1 and ARC-2 groups. C, D: The overlap of gene transcripts within lncRNAs in CC and ARC groups, and in ARC-1 and ARC-2 groups. E, F: The number of m$^6$A peaks per lncRNA between CC and ARC groups, and between ARC-1 and ARC-2 groups. G: Consensus sequences in CC and ARC, ARC-1 and ARC-2 respectively. m$^6$A: N$^6$-methyladenosine; lncRNA: Long non-coding RNA; CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

mentioned in methods part. As the Venn diagrams indicated, the m$^6$A peaks in CC (6881+11 807) were markedly less than those in ARC (38 063+11 807). A total of 11 807 m$^6$A peaks within lncRNAs were overlapped between CC and ARC tissues, representing 7275 gene transcripts. Whereas 6881 peaks were identified in CC but absent in ARC, and 38 063 peaks in ARC but absent in CC, corresponding to 528 and 4797 gene transcripts respectively in CC and ARC (Figure 2A, 2C). By contrast, difference of m$^6$A peaks between ARC-1 (17 684+14 298) and ARC-2 (17 988+14 298) were slight, with 17 684 m$^6$A peaks found in ARC-1 but absent in ARC-2, and 17 988 peaks found in ARC-2 but absent in ARC-1 (Figure 2B). A total 14 298 overlapping peaks were confirmed, representing 8173 gene transcripts (Figure 2B, 2D). The high nonoverlapping percentages of m$^6$A peaks and their gene transcripts indicate that the overall m$^6$A modification patterns between CC and ARC were quite different. In the contrary, those between ARC-1 and ARC-2 were not remarkable.

Furthermore, the numbers of m$^6$A peaks varied among the modified lncRNAs. Most of them contained a single peak, and approximately 20% of the methylated transcripts contained two m$^6$A peaks per lncRNA, whereas a small number of them contained three or more. There were no significant differences when compared either bewteen CC and ARC or bewteen

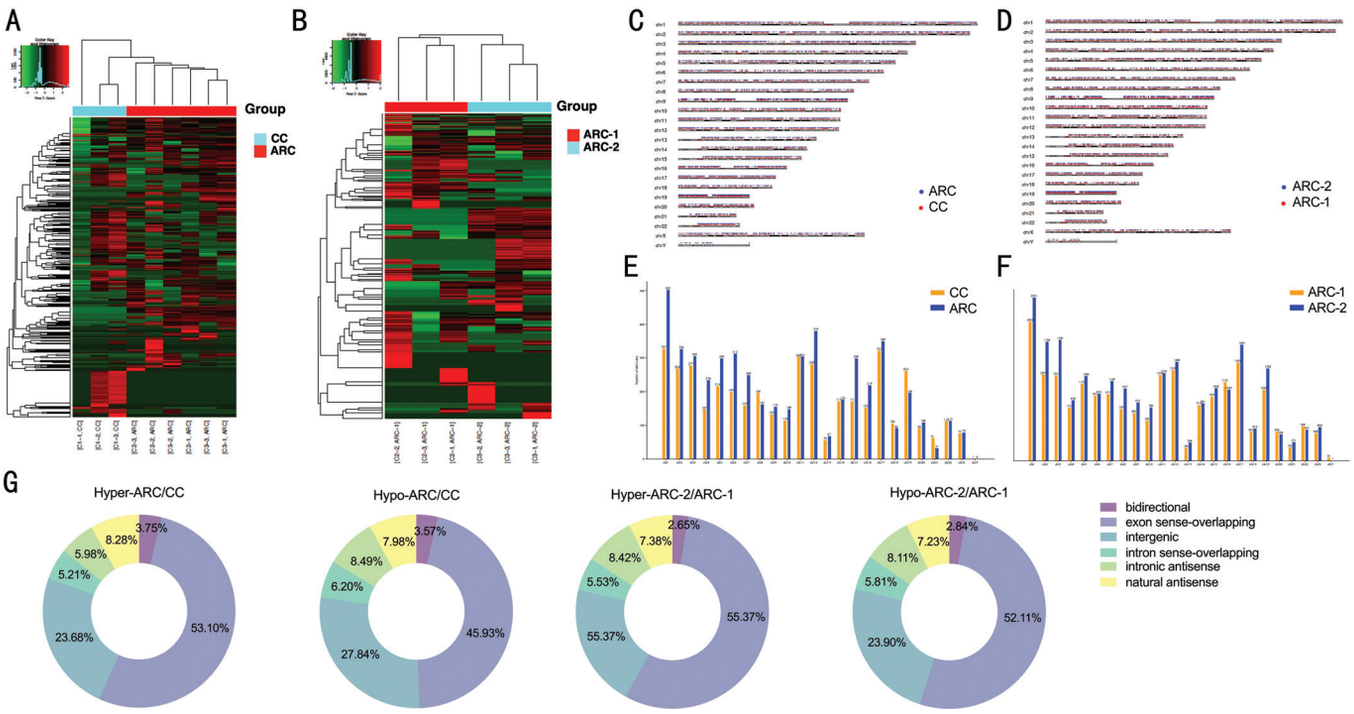


**Figure 3 Landscape of m$^6$A-modified lncRNAs in lens epithelial cells** A, B: Hierarchical clustering analysis showed that there were significant differences in the m$^6$A methylation patterns within lncRNAs between CC and ARC and not so dramatical between ARC-1 and ARC-2. C, D: Distribution profiles on chromosomes of m$^6$A sites within lncRNAs between CC and ARC and between ARC-1 and ARC-2. E, F: The number of m$^6$A peaks within lncRNA on each chromosome between CC and ARC groups and between ARC-1 and ARC-2. G: Distributions of genomic origins of differentially distributed m$^6$A lncRNAs. m$^6$A: N$^6$-methyladenosine; lncRNA: Long non-coding RNA; CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

ARC-1 and ARC-2 (Figure 2E, 2F). Motif analysis was conducted by DREME software (version 5.3.0). A total of 2000 peaks within lncRNAs with the highest scores (-10×log10, *P*) obtained from three biological replicates (1000 peaks per replicate) revealed consensus sequences (RRACH) in CC and ARC respectively (Figure 2G). Compared to CC samples, the top consensus motifs in m$^6$A peaks within lncRNAs were CCCAG and UUUCU in ARC samples.

**Abnormal m$^6$A-Modified lncRNAs and m$^6$A Peak Distribution Pattern in LECs of CC and ARC** Abnormal m$^6$A-modified lncRNAs were identified in LEC samples. Totally 1305 hypermethylated and 1178 hypomethylated lncRNAs were identified with the threshold of $|\log_2 FC|>1$ and $P<0.05$ in ARC group compared with CC group, and 5893 hypermethylated and 5213 hypomethylated lncRNAs in ARC-2 compared with ARC-1.

Hierarchical clustering analysis was done to present the m$^6$A methylation patterns within lncRNAs between CC and ARC, as well as between ARC-1 and ARC-2 (Figure 3A, 3B). To acquire the distribution profiles, all differentially methylated m$^6$A sites within lncRNAs were mapped to chromosomes (Figure 3C, 3D). As Figure 3E indicated, the dysregulated m$^6$A peaks were transcribed from nearly all chromosomes, while chr1 (829), chr12 (659), chr11 (605), chr2 (594), and chr3 (582) are the top five chromosomes harboring the most differentially methylated m$^6$A sites. Compared to that in CC samples, the number of altered m$^6$A peaks was much more in ARC samples (Figure 3E), and same tendency was also found in ARC-2 group when compared with ARC-1 group (Figure 3F).

To be more detailedly, we found 5098 significantly hypermethylated m$^6$A peaks and 4195 significantly hypomethylated m$^6$A peaks in ARC, compared with those in CC. On the other hand, 23 930 significantly hypermethylated m$^6$A peaks and 20 570 significantly hypomethylated m$^6$A peaks were identified in ARC-2 compared with those in ARC-1 (FC ≥2 and $P$≤0.05). Table 2 presented the top ten up and down methylated m$^6$A sites within lncRNAs with the highest fold change values between CC and ARC, as well as between ARC-1 and ARC-2. Distributions of genomic origins of differentially distributed m$^6$A lncRNAs were examined, which demonstrated that most significantly m$^6$A peaks were encoded by exon sense-overlapping, followed by intergenic as shown in Figure 3G.

**Analysis of Differentially Expressed lncRNAs Among LEC Samples** In the RNA-seq data set (m$^6$A-seq input library), we found significant differences in the overall lncRNA expression patterns between CC and ARC samples. The results of the lncRNA expression profile analysis showed that compared with CC group, 182 lncRNA expressions were significantly altered in the ARC group, of which 134 lncRNA expressions were upregulated and 48 lncRNA expressions were downregulated

**Table 2 The top 20 differently methylated m$^6$A peaks**

| Gene name | Regulation | Fold change | Chromosome | Peak length | Peak start | Peak end | *P* |
|---|---|---|---|---|---|---|---|
| CC *vs* ARC | | | | | | | |
| *RP11-342M1.3* | Up | 2949.5 | chr1 | 29 | 43300351 | 43300380 | <0.001 |
| *WDR27* | Up | 2717.5 | chr6 | 242 | 170066512 | 170066754 | <0.001 |
| *IGFL1P1* | Up | 2212.3 | chr19 | 110 | 46700470 | 46700580 | <0.001 |
| *PSMD14* | Up | 1949 | chr2 | 141 | 162241941 | 162242082 | <0.001 |
| *RP11-44N11.2* | Up | 1935.6 | chr8 | 651 | 123792209 | 123792860 | <0.001 |
| *MVP* | Up | 1842.2 | chr16 | 165 | 29845255 | 29845420 | <0.001 |
| *ZNF496* | Up | 1746.9 | chr1 | 339 | 247461641 | 247461980 | <0.001 |
| *FAXDC2* | Up | 1626.8 | chr5 | 339 | 154199701 | 154200040 | <0.001 |
| *CECR7* | Up | 1561.7 | chr22 | 56 | 17541484 | 17541540 | <0.001 |
| *LOC102723927* | Up | 1559.9 | chr2 | 46 | 242914661 | 242914707 | <0.001 |
| *MIP* | Down | 1643964.5 | chr12 | 355 | 56846581 | 56846936 | <0.001 |
| *CTAGE3P* | Down | 4057.8 | chr13 | 479 | 52483021 | 52483500 | <0.001 |
| *ANKIB1* | Down | 2984.1 | chr7 | 58 | 92025182 | 92025240 | <0.001 |
| *AP3S1* | Down | 2895.4 | chr5 | 204 | 115239581 | 115239785 | <0.001 |
| *RP11-690C23.4* | Down | 2863 | chr1 | 146 | 246674738 | 246674884 | <0.001 |
| *SCPEP1* | Down | 2846.9 | chr17 | 350 | 55064450 | 55064800 | <0.001 |
| *CTD-2319I12.3* | Down | 2754 | chr17 | 152 | 58193160 | 58193312 | <0.001 |
| *HSPA8P9* | Down | 2693.8 | chr3 | 118 | 137600961 | 137601079 | <0.001 |
| *TSC22D1* | Down | 2614.1 | chr13 | 319 | 45112461 | 45112780 | <0.001 |
| *OR7E100P* | Down | 2601.5 | chr3 | 467 | 112243581 | 112244048 | <0.001 |
| ARC-1 *vs* ARC-2 | | | | | | | |
| *PRICKLE2-AS2* | Up | 3659.6 | chr3 | 177 | 64091394 | 64091571 | <0.001 |
| *SH3BP5-AS1* | Up | 2840.6 | chr3 | 399 | 15297861 | 15298260 | <0.001 |
| *LOC100133461* | Up | 2804.4 | chr4 | 81 | 3679501 | 3679582 | <0.001 |
| *AC105760.3* | Up | 2671.7 | chr2 | 268 | 237957341 | 237957609 | <0.001 |
| *LYST* | Up | 2272.8 | chr1 | 619 | 235991121 | 235991740 | <0.001 |
| *C3orf52* | Up | 2220.9 | chr3 | 639 | 111847701 | 111848340 | <0.001 |
| *RP11-83A24.2* | Up | 2164.7 | chr4 | 117 | 140335061 | 140335178 | <0.001 |
| *IRF7* | Up | 2143.4 | chr11 | 198 | 614782 | 614980 | <0.001 |
| *RP11-173G21.1* | Up | 2127 | chr9 | 419 | 98056021 | 98056440 | <0.001 |
| *RP11-90L20.2* | Up | 2064.3 | chr1 | 122 | 201693921 | 201694043 | <0.001 |
| *FAM90A24P* | Down | 7924.8 | chr8 | 359 | 7877261 | 7877620 | <0.001 |
| *RP11-61F12.1* | Down | 5473.7 | chr16 | 399 | 84628501 | 84628900 | <0.001 |
| *AC025811.1* | Down | 4800.6 | chr19 | 33 | 22640607 | 22640640 | <0.001 |
| *BAI2* | Down | 4037.9 | chr1 | 379 | 32192861 | 32193240 | <0.001 |
| *L3HYPDH* | Down | 3091.4 | chr14 | 395 | 59941445 | 59941840 | <0.001 |
| *TRAPPC9* | Down | 3054.4 | chr8 | 519 | 140913781 | 140914300 | <0.001 |
| *WDR60* | Down | 2945.9 | chr7 | 93 | 158697881 | 158697974 | <0.001 |
| *LINC01105* | Down | 2889.2 | chr2 | 579 | 6118541 | 6119120 | <0.001 |
| *BCYRN1* | Down | 2870.9 | chr2 | 399 | 47560081 | 47560480 | <0.001 |
| *RP11-276H1.2* | Down | 2672.4 | chr16 | 37 | 12187483 | 12187520 | <0.001 |

CC: Congenital cataract; ARC: Age-related cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80; m$^6$A: N$^6$-methyladenosine.

(FC>2, *P*<0.05). On the other hand, between ARC-1 and ARC-2 groups, 155 lncRNA expressions were significantly altered in the ARC-2 group, of which 92 lncRNA expressions were upregulated and 63 lncRNA expressions were downregulated. The top 20 altered genes were listed in Table 3. Figure 4A, 4C, 4E were scatter plots, hierarchical clustering and volcano of the RNA-seq data between CC and ARC, and Figure 4B, 4D, 4F are those between ARC-1 and ARC-2.

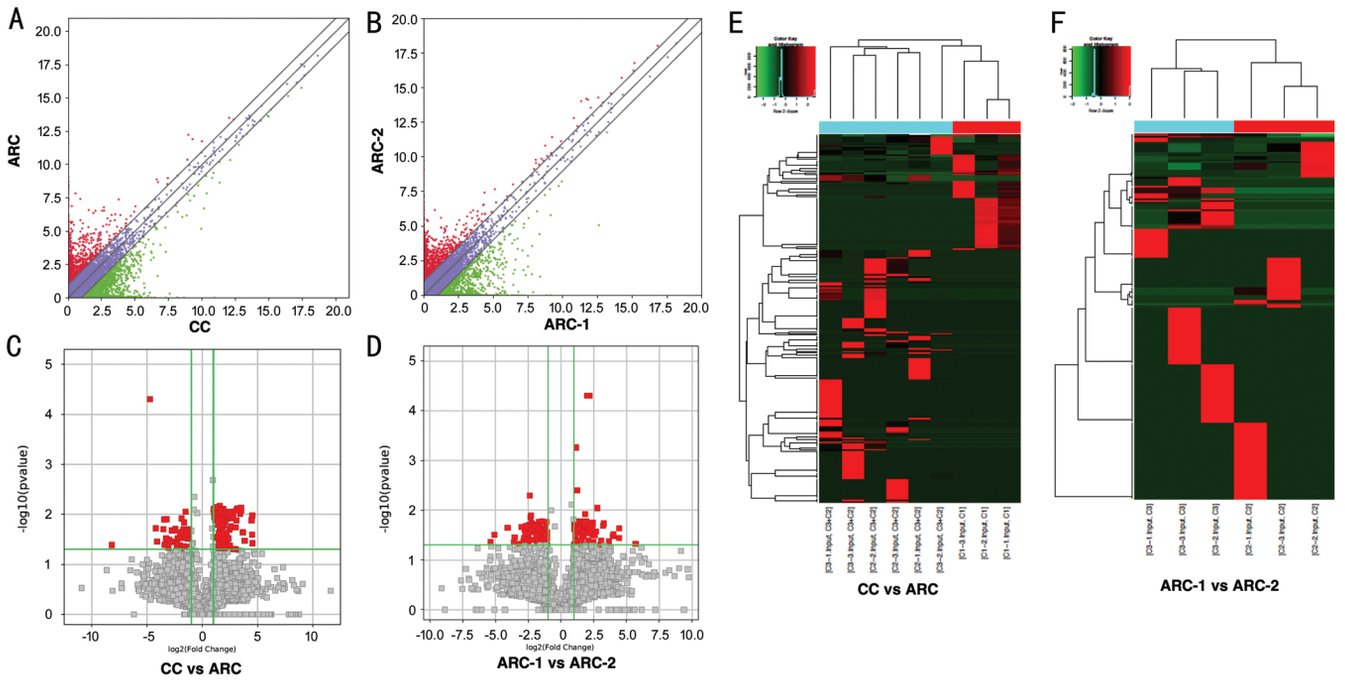


**Figure 4 Overall lncRNA expression patterns between groups** Scatter plots (A, B), hierarchical clustering (C, D), and volcano (E, F) of the RNA-seq data between CC and ARC and between ARC-1 and ARC-2. lncRNA: Long non-coding RNA; CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

**Functional Annotation of the Distinctly Distributed m$^6$A lncRNAs** To explore the physiological and pathological significance of protein coding genes harboring altered methylated m$^6$A sites in CC and ARC, functional enrichment analyses were performed to select differentially methylated m$^6$A sites-contained lncRNA-associated genes. As GO analysis demonstrated, hypermethylated lncRNA-associated genes in ARC were significantly associated with organelle organization, cellular component organization or biogenesis and cellular component organization (BP category), with intracellular, organelle and intracellular organelle (cellular component category), and with protein binding, binding and heterocyclic compound binding (MF category) compared with those in CC (Figure 5A-5C); while the hypomethylated lncRNA-associated genes in ARC were significantly associated with cellular localization, macromolecule localization and protein localization (BP category), with interacellular, organelle and cytoplasm (cellular component category), and with protein binding, ion binding and protein-membrane adaptor activity (MF category) compared with those in CC (Figure 5D-5F). On the other hand between ARC-1 and ARC-2 samples (Figure 5G-5I), upregulated peaks were in a significant correlation with cellular macromolecule metabolic process, cellular metabolic process and organelle organization (BP category), with intracellular, cytoplasm and organelle (cellular component category), and with protein binding, enzyme binding and binding (MF category) in ARC-2 rather than ARC-1, while downregulated peaks in ARC-2 were in a significant correlation with cellular metabolic process, cellular macromolecular metabolic process and cellular component organization or biogenesis (BP category), with intracellular, cytoplasm and cytosol (cellular component category), and with protein binding, binding and enzyme binding (MF category) compared with ARC-1 (Figure 5J-5L).

Markedly, KEGG analysis revealed the result of unique CC and ARC. Hypermethylated genes in ARC/CC were significantly associated with nucleotide excision repair and base excision repair (Figure 6A) and hypomethylated genes were associated with central carbon metabolism in cancer and FoxO signaling pathway (Figure 6B). While in ARC-2/ARC-1, hypermethylated genes were significantly associated with RNA transport and spliceosome (Figure 6C) and hypomethylated genes were associated with spliceosome and lysosome (Figure 6D).

**Conjoint Analysis of MeRIP-Seq & RNA-Seq in CC and ARC Tissues** We comprehensively analyzed MeRIP-seq and RNA-seq data in CC and ARC to explore the significance of m$^6$A modification. The conjoint analysis revealed 1 hypermethylated m$^6$A peak in lncRNAs that was dramatically enriched (1 hyper-up) or subdued (0 hyper-down), and 5 differentially lncRNAs in ARC group that were significantly upregulated (5 hypo-up) or subdued (0 hypo-down).

## DISCUSSION

Epigenetic modifications, especially for DNA methylation, have been demonstrated to involve in the pathogenesis of cataract in recent years, not only for ARC[22], but also for

**Table 3 The top 20 differently expressed lncRNAs**

| Gene ID | Regulation | Fold change | Locus | lncRNA source | lncRNA length | Relationship | *P* |
|---|---|---|---|---|---|---|---|
| CC *vs* ARC | | | | | | | |
| *XLOC_009474* | Up | 155.927 | chr11:65265232-65278498 | TCONS | 3579 | Intergenic | 0.03385 |
| *ENSG00000231971* | Up | 7.58548 | chr6:134749377-134846048 | Ensembl | 1360 | Intergenic | 0.0377 |
| *XLOC_l2_001324* | Up | 5.09094 | chr1:222645270-222652914 | TUCP | 5492 | Intergenic | 0.042 |
| *ENSG00000250303* | Up | 4.50709 | chr11:112141471-112233257 | Ensembl | 2749 | Intergenic | 0.0499 |
| *ENSG00000273117* | Up | 4.28096 | chr7:155087627-155089251 | Ensembl | 1624 | Bidirectional | 0.03035 |
| *ENSG00000273117* | Up | 4.28096 | chr7:155087627-155089251 | Ensembl | 1624 | Bidirectional | 0.03035 |
| *ENSG00000273117* | Up | 4.28096 | chr7:155087627-155089251 | Ensembl | 1624 | Bidirectional | 0.03035 |
| *ENSG00000273117* | Up | 4.28096 | chr7:155087627-155089251 | Ensembl | 1624 | Bidirectional | 0.03035 |
| *XLOC_013852* | Up | 1.88636 | chr21:9825743-9826389 | TCONS | 557 | Intergenic | 0.03025 |
| *RNF141* | Up | 1.84059 | chr11:10533224-10562777 | UCSC_knowngene | 4064 | Exon sense-overlapping | 0.0462 |
| *ENSG00000214691* | Down | -9.44823 | chr2:42104213-42121179 | Ensembl | 1803 | Intergenic | 0.035 |
| *XLOC_008434* | Down | -9.40672 | chr10:30248093-30249155 | TCONS | 702 | Intergenic | 0.01895 |
| *XLOC_001642* | Down | -9.34008 | chr2:121334544-121362263 | TCONS | 788 | Intergenic | 0.03485 |
| *AX747631* | Down | -9.17522 | chr1:18434239-18704977 | UCSC_knowngene | 1111 | Intron sense-overlapping | 0.0403 |
| *AX747631* | Down | -9.17522 | chr1:18434239-18704977 | UCSC_knowngene | 1111 | Intron sense-overlapping | 0.0403 |
| *XLOC_007279* | Down | -9.12916 | chr9:7924793-7961234 | TCONS | 5199 | Intergenic | 0.02045 |
| *ENSG00000244567* | Down | -5.04468 | chr2:208686013-208687493 | Ensembl | 1262 | Exon sense-overlapping | 0.0125 |
| *ENSG00000261449* | Down | -4.61585 | chr8:42009289-42010281 | Ensembl | 992 | Bidirectional | 0.0322 |
| *ENSG00000261449* | Down | -4.61585 | chr8:42009289-42010281 | Ensembl | 992 | Bidirectional | 0.0322 |
| *ENSG00000261449* | Down | -4.6158 | chr8:42009289-42010281 | Ensembl | 992 | Bidirectional | 0.0322 |
| ARC-1 *vs* ARC-2 | | | | | | | |
| *XLOC_l2_002994_2* | Up | inf | chr2:20314685-20317929 | TUCP | 1161 | Intergenic | 0.0489 |
| *XLOC_l2_007237* | Up | inf | chr4:9569104-9648636 | TUCP | 487 | Intergenic | 0.0171 |
| *XLOC_l2_011048* | Up | inf | chr1:38495003-38495216 | TUCP | 1369 | Intergenic | 0.01525 |
| *uc.14* | Up | inf | chr16:29262828-30215631 | UCR | 213 | Intergenic | 0.0301 |
| *AF072097* | Up | inf | chr16:29262828-30215631 | UCSC_knowngene | 739 | Natural antisense | 0.0287 |
| *AF072097* | Up | inf | chr1:176432306-176814735 | UCSC_knowngene | 739 | Natural antisense | 0.0287 |
| *PAPPA2* | Up | 15.8823 | chr1:176432306-176814735 | UCSC_knowngene | 7092 | Exon sense-overlapping | 0.0283 |
| *PAPPA2* | Up | 15.8823 | chrX:73012039-73072588 | UCSC_knowngene | 7092 | Exon sense-overlapping | 0.0283 |
| *ENSG00000229807* | Up | 2.27408 | chrX:139863223-139866829 | Ensembl | 19275 | Intergenic | <0.001 |
| *ciRS-7* | Up | 1.13598 | chr6:134749377-134846048 | lncRNAdb | 1502 | Natural antisense | <0.001 |
| *ENSG00000231971* | Down | -6.66335 | chr4:128702975-128765195 | Ensembl | 1360 | Intergenic | 0.0315 |
| *ENSG00000261668* | Down | -6.3444 | chr1:234507931-234519795 | Ensembl | 3843 | Intergenic | 0.0441 |
| *LOC101927765* | Down | -5.82173 | chr1:234507931-234519795 | RefSeq | 614 | Bidirectional | 0.0438 |
| *LOC101927765* | Down | -5.82173 | chr1:234507931-234519795 | RefSeq | 614 | Bidirectional | 0.0438 |
| *LOC101927765* | Down | -5.82173 | chr17:3710041-3712148 | RefSeq | 614 | Bidirectional | 0.0438 |
| *ENSG00000262758* | Down | -2.61997 | chr6:81176674-81178797 | Ensembl | 2107 | Exon sense-overlapping | 0.00505 |
| *ENSG00000260645* | Down | -2.53679 | chr9:127115744-127177723 | Ensembl | 2123 | Intergenic | 0.014 |
| *LOC100129034* | Down | -2.52632 | chr9:127115744-127177723 | RefSeq | 5714 | Natural antisense | 0.01265 |
| *LOC100129034* | Down | -2.52632 | chr2:20314685-20317929 | RefSeq | 5714 | Natural antisense | 0.01265 |
| *CD24* | Down | -1.98 | chrY:21034386-21239433 | RefSeq | 2336 | Exon sense-overlapping | 0.0474 |

CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80; lncRNA: Long non-coding RNA.

cataract secondary to pars plana vitrectomy[23], diabetic cataract[24], high myopia induced cataract[25], or cataract complicated with peudoexfoliation syndrome[26]. For ARC, DNA hypermethylation of lens structural protein (CRYAA), antioxidant genes (OGG1), anti-aging gene (Klotho), DNA repair gene (MGMT, ERCC6, WRN), are all concerned with the etiology of ARC[27-30]. On the other hand, although CC is considered to derived from high-risk genetic mutations,

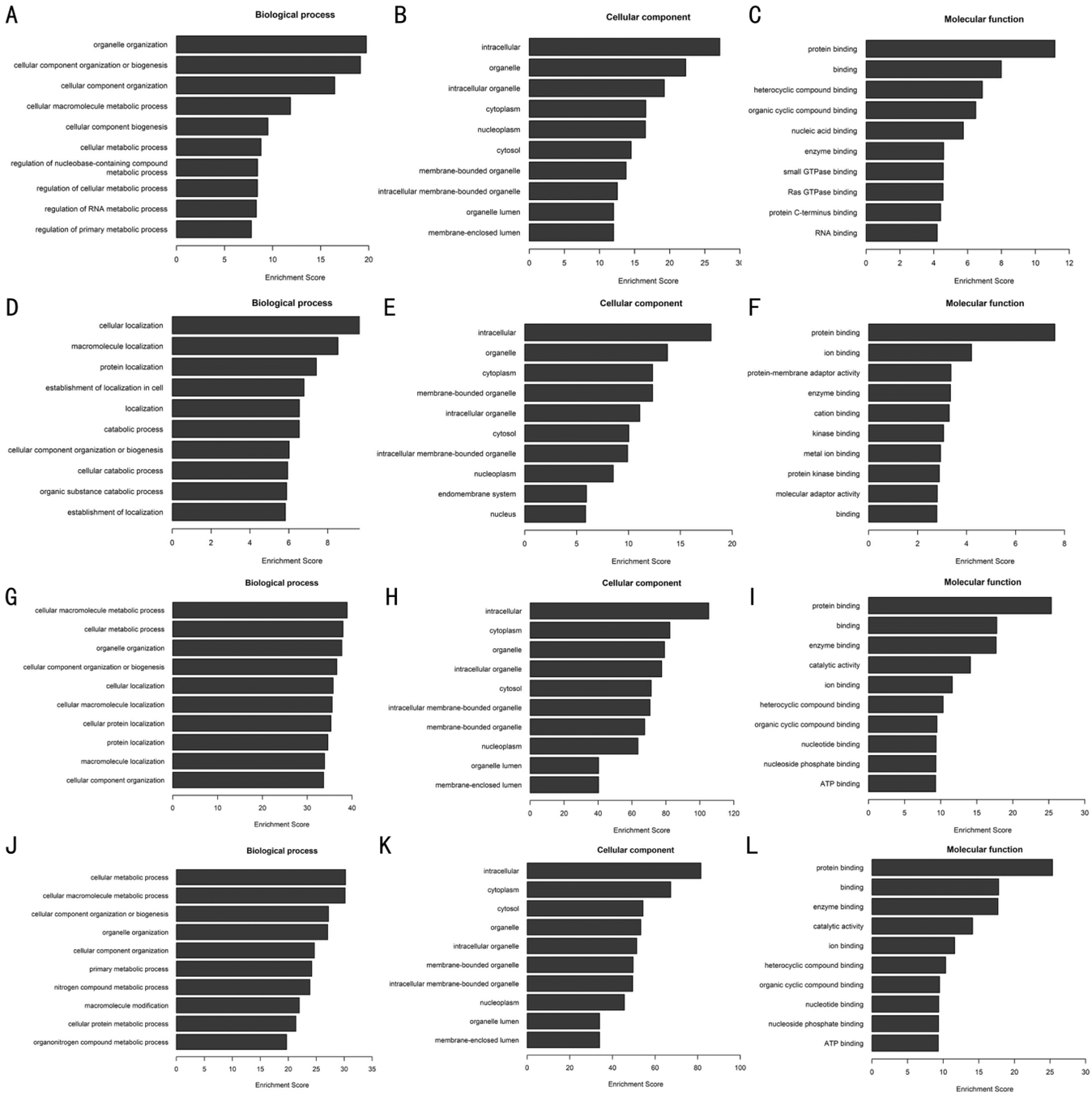


**Figure 5 The GO project to describe gene and gene product attributes** A-C: Major GO terms significantly enriched for hypermethylated lncRNA-associated genes in ARC compared with those in CC; D-F: For hypomethylated lncRNA-associated genes in ARC compared with those in CC; G-I: Hypermethylated lncRNA-associated genes in ARC-2 compared with those in ARC-1; J-L: Hypomethylated lncRNA-associated genes in ARC-2 compared with those in ARC-1. GO: Gene ontology; lncRNA: Long non-coding RNA; CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

metabolic and drug-induced disorders, and intrauterine infection[31], Liu *et al*'s[12] research revealed altered DNA methylation levels of five core genes in idiopathic CC patients, which for the first time proved that epigenetics involves in the pathogenesis of CC. Kroeze *et al*[32] demonstrated that methylation suppress promotor activity of γB-crystallin and γD-crystallin, causing defects in lens development, which provides a novel etiology for the lesions of crystalline lens of early stage from epigenetic perspective.

Similar to DNA methylation, there are a variety of reversible modifications on RNA, among which $m^6A$ modification is the most prevalent one and has gradually gained attention as a new epigenetic event recently. High-throughput genomic technologies have revealed $m^6A$ modifications and expression changes in mRNA as well as noncoding RNAs (microRNA, circRNA, lncRNA), and $m^6A$ has been implicated in mRNA stability, translation, splicing and miRNA processing, altogether exerting regulatory control

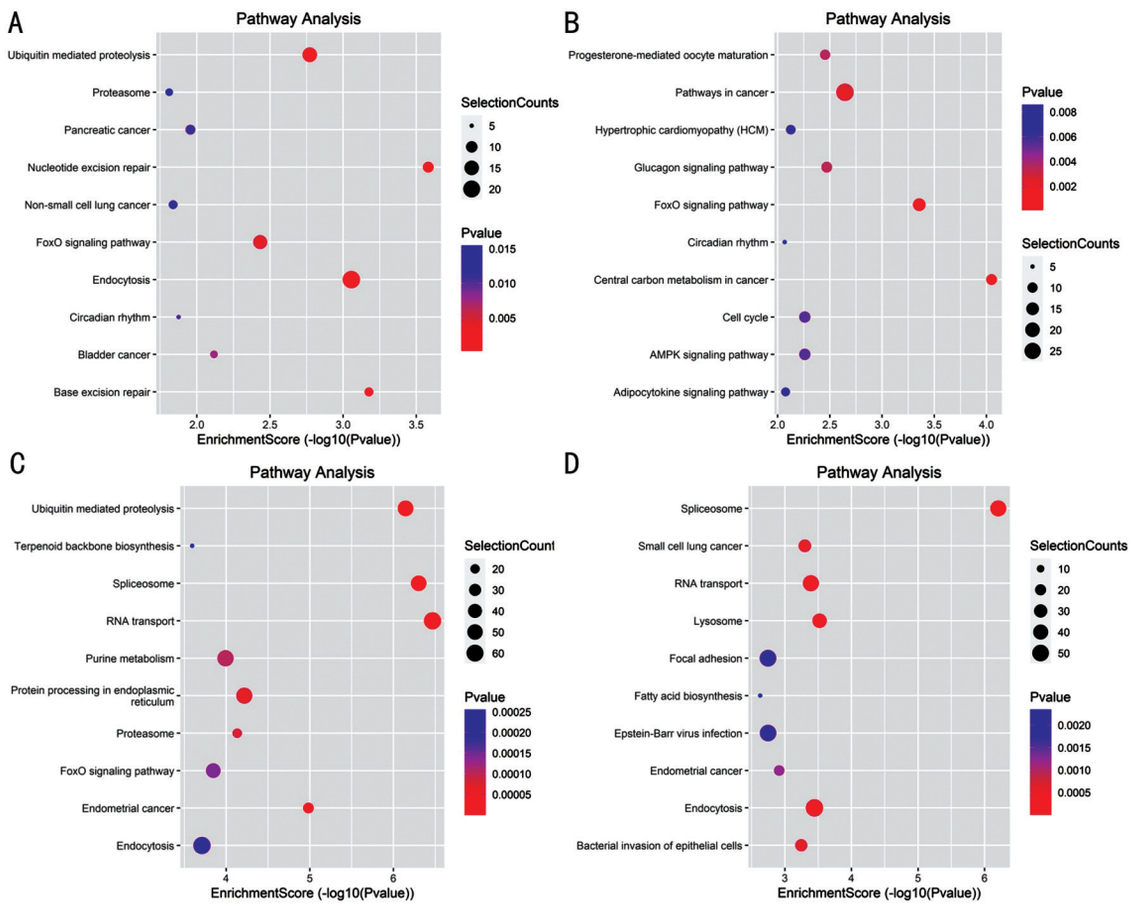


**Figure 6 KEGG pathways for pathway analysis** A: Hypermethylated lncRNA-associated genes in ARC compared with those in CC; B: Hypomethylated lncRNA-associated genes in ARC compared with those in CC; C: Hypermethylated lncRNA-associated genes in ARC-2 compared with those in ARC-1; D: Hypomethylated lncRNA-associated genes in ARC-2 compared with those in ARC-1. KEGG: Kyoto Encyclopedia of Genes and Genomes pathways; lncRNA: Long non-coding RNA; CC: Congenital cataract; ARC: Age-related cataract; ARC-1: ARC patients under the age of 70; ARC-2: ARC patients above the age of 80.

over a biological process[14]. However, the role of this new epigenetic modification in cataract has only been reported in three studies. Li *et al*[15] for the first time investigated the m$^6$A state for circRNA in ARC, indicating that the dynamic characteristic of m$^6$A modification in LECs is associated with ARC pathogenesis. Wen *et al*[16] assessed the m$^6$A methylome and gene expression in the anterior capsule of the lens for high myopia patients, proving that the upregulation of m$^6$A methylation regulates the composition of the extracellular matrix through encoding protein, which may ultimately change fundus anatomy. In addition, Yang *et al*'s[17] research focused on diabetic cataract, demonstrating a higher level of m$^6$A modification in high glucose-induced LECs, which provided a potential pathogenic insight for diabetic cataract. Whereas more researches are still needed to clarify roles of m$^6$A in the pathogenesis of a series of cataract.

In this study, we for the first time showed the m$^6$A modification pattern for lncRNA in CC and ARC samples. We found that the amount m$^6$A lncRNA modification in CC and ARC were quite different, while this difference between ARC with young (ARC-1) and elder age (ARC-2) was slight, though increasing age has been principally associated with lens opacities[33]. A much higher total m$^6$A level, more than 31 182 m$^6$A peaks and 4271 gene transcripts, were identified in ARC samples compared with CC samples. In the contrary, the nonoverlapping percentages of m$^6$A peaks and their gene transcripts between ARC in different age groups (ARC-1 and ARC-2) were significantly lower. As a qualitative observation, hierarchical clustering analysis also indicated these remarkable differences in the m$^6$A methylation patterns within lncRNAs between CC and ARC, while not so dramatical between ARC-1 and ARC-2 (Figure 3A, 3B). In addition, the motif analysis revealed different top consensus motifs in m$^6$A sites within lncRNAs between CC and ARC. Whereas on the level of RNA, the amount of m$^6$A modified lncRNAs was much higher in ARC than that in CC, which is statistically significant either for hypermethylated or hypomethylated lncRNAs. When comparing between different age groups, m$^6$A modified lncRNAs were much more in ARC of elder age than those in ARC of younger age. These all together demonstrated

epigenetic effects on both CC and ARC, while the extent is drastically differed. Compared to CC, these epigenetic effects were much more distinct in ARC samples, which reveals a cumulative effect of environments on the body susceptibility to stress, diseases and injury. Functional experiments are needed to further verify biological relevance of certain lncRNAs and their regulatory roles and specific pathways on the pathogenesis of cataract *in vivo* and *in vitro* models.

Concerning CC, there are a number of well accepted pathogenesis[34-35], including genetic mutations of high-risk, metabolic diseases, drug-induced disorders and intrauterine infections. Nevertheless, most of the unilateral and approximately 25%-30% bilateral CC are idiopathic[36], and for patients of these kinds, the etiologies still remain uncertain. Liu *et al*[12] for the first time reveals changed DNA methylation level of five core genes, namely *TUBA1A*, *TUBA1C*, *TUBB4B*, *ACTN4,* and *ACTG1*, which encode proteins in tight or gap junction pathways. These changes lead to disfunction of cytoskeleton and intercellular junctions. The present research provides a novel epigenetic perspective for the pathogenic mechanism of CC, which can explain the long-term efferts of intrauterine and external environments on the susceptibility of idiopathic CC. Similar to DNA methylation, our present study for the first time proves the existence of RNA methylation in CC patients. Although the extent of $m^6A$ modification is less than that in ARC, it is still reasonable that epigenetics in RNA level also participates in the pathogenesis of CC.

lncRNAs are defined as RNA transcripts longer than 200 nucleotides with limited or no coding potential. According to the modes of action, functions as well as genomic localizations, lncRNAs are divided into enhancer lncRNAs, intronic lncRNAs, intergenic lncRNAs, bidirectional lncRNAs, sense-overlapping lncRNAs and antisense transcripts. Wide range of researches have proved that lncRNAs involve in the regulation of numerous biological processes, namely cell differentiation, stem cell maintenance, chromatin modification, splicing, transcription, translation, transport and degradation of mRNA[37-38]. lncRNAs also participate in regulation of gene expression, respectively at pre-transcriptional, transcriptional and post-transcriptional level[39]. Concerning cataract, studies revealed large amount of differentially expressed lncRNAs in ARC and posterior capsular opacification using high-throughput sequencing and bioinformatics comparing with normal eyes, which indicated potential roles of lncRNAs in the pathogenesis of cataract[40-41]. Specifically, high expression of lncRNA-MIAT was found in both the plasma and aqueous humor of ARC patients, and lncRNA-MIAT repression resulted in abnormal growth and migration of LECs through miR-150-5p/Akt axis[10]. These both demonstrated that lncRNA-MIAT can be identified as a specific biomarker of ARC. Moreover, it is suggested that lncRNAs might shed new light on the treatment and prevention of cataract.

In recent decades, emerging evidence has proved the functions of lncRNAs after $m^6A$ modification. $m^6A$ modification of certain lncRNA changes the structure of the lncRNA and the accessibility for proteins, mediates subsequent gene transcriptional regulation[42], affects mRNA precursor splicing[43-44], as well as regulates lncRNA stability and translation[45-46]. These have been well recognized in human cancers[47], while seldom have been reported in other diseases. Our present study for the first time identified $m^6A$ modification pattern for lncRNA in both CC and ARC samples, revealing a potential impact for $m^6A$-modified lncRNAs in the pathogenic process of CC and ARC. To uncover the functions of m6A lncRNAs in CC and ARC, functional enrichment analysis of differentially methylated lncRNAs was performed. GO enrichment analysis revealed that compared with CC, both hypermethylated and hypomethylated lncRNAs in ARC were mainly linked to intracellular organelles as well as their organization and biogenesis. Recently, a new insight[48-49] has been arisen that crystalline lens achieves optimal transparency by timely degradation of intracellular organelle, hence it is indicated that $m^6A$ methylation may disrupt the homeostasis of intracellular organelle degradation, participating in the process of cataractogenesis. Meanwhile, through the KEGG pathway analysis, highly methylated lncRNAs in ARC were mainly associated with nucleotide excision repair (NER) in our present study. It is well accepted that oxidative stress leads to the damage of mitochondrial DNA and nuclear DNA, which plays an essential role in the pathogenesis of ARC[50]. In physiologic conditions, most oxidative DNA lesions are repaired by base excision repair, NER and double-strand break repair pathways, and NER mainly copes with damages caused by ultraviolet irradiation and chemicals[51]. Studies have demonstrated that polymorphisms of the genes in NER pathway, namely xeroderma pigmentosum complementation group D, *ERCC1*, *XPG*, *ERCC5*, *ERCC6*, were with the high risk of cataract[52-54]. At the epigenetic perspective, the methylation and expression level of *ERCC6*[29], and $O^6$-methylguanine-DNA methyl-transferees[28], both of which are key components of NER pathway, are also found dramatically altered in cataract samples. Based on the essential role of NER on the pathogenesis of cataract, our KEGG outcomes suggest that $m^6A$ methylation may also invloved in the mechanism of cataract.

Limitation of this study should be acknowledged that we only validated the results of CC and ARC samples in absence of non-cataract samples. Although the initial design of this study was to set non-cataract samples as control, while these samples

were hardly qualified primarily for the following reasons. First, non-cataract samples are mainly obtained from body donation in China, but it is relatively rare out of traditional concept among population. Second, a considerable number of non-cataract samples donated may not be suitable for epigenetic research due to the presence of systemic disorders in the donors. Systemic disorders can cause substantial changes in the ocular environments, resulting in variations in the qualities of capsular samples. At all event, further studies are warranted to obtain proper samples of non-cataract anterior lens and overcome this limitation.

In conclusion, this was the first study to present an overview of the m$^6$A methylomes of lncRNA in CC and ARC tissues employing MeRIP-seq and RNA-seq. Our results demonstrated that m$^6$A methylation levels of lncRNAs might in varying degrees play essential roles in the pathogenesis of ARC and CC. Futhermore, we revealed that DNA damage repair, especially NER, was most associated with m$^6$A-lncRNA modification in ARC, which might explain the mechanism of ARC from a novel epigenetic perspective. All in all, this comprehensive m$^6$A profiling provides a solid basis for the determination of potential functional roles for lncRNA m$^6$A modification in pathological processes of ARC and CC patients.

**ACKNOWLEDGEMENTS**

We thank Wei-Hua Xu, Pei-Yan Hua, Ying Zhu, Yan Zheng for collecting biological samples. We thank Cloud-Seq Biotech Ltd. Co. (Shanghai, China) for the MeRIP-Seq service and the subsequent bioinformatics analysis.

**Authors' contributions:** Conceived and designed the work (Ye HF, Zhao PQ); Collected the data (Ye HF, Zhang X, Zhao ZN); Contributed data (Zheng C, Fei P, Xu Y, Lyu J, Chen JL); Performed the analysis (Ye HF, Guo XX, Zhang X); Wrote the paper (Ye HF, Zhao ZN); Final editing and approval (Guo XX, Zhu H, Zhao PQ). All authors agree to be accountable for the content of the work.

**Foundations:** Supported by the National Natural Science Foundation of China (No.82171069; No.82371070); Shanghai Science and Technology Committee (No.22015820200); Shanghai Municipal Health Commission Innovative Medical Device Application Demonstration Project (No.23SHS03500-03); Project of Shanghai Municipal Commission of Health and Family Planning (No.202140224); Grants from Interdisciplinary Program of Shanghai Jiao Tong University (No.YG2021QN52).

**Conflicts of Interest: Ye HF,** None; **Zhang X,** None; **Zhao ZN,** None; **Zheng C,** None; **Fei P,** None; **Xu Y,** None; **Lyu J,** None; **Chen JL,** None; **Guo XX,** None; **Zhu H,** None; **Zhao PQ,** None.

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