RNA sequencing and bioinformatics analysis of human lens epithelial cells in age-related cataract

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Abstract

Background: Age-related cataract (ARC) is the main cause of blindness in older individuals but its specific pathogenic mechanism is unclear. This study aimed to identify differentially expressed genes (DEGs) associated with ARC and to improve our understanding of the disease mechanism.

Methods: Anterior capsule samples of the human lens were collected from ARC patients and healthy controls and used for RNA sequencing to detect DEGs. Identified DEGs underwent bioinformatics analyses, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Subsequently, reverse transcription quantitative RT-qPCR was used to validate the different expression levels of selected genes.

Results: A total of 698 up-regulated DEGs and 414 down-regulated DEGs were identified in ARC patients compared with controls by transcriptome analysis. Through GO and KEGG bioinformatics analysis, the functions of significantly DEGs and their possible molecular mechanisms were determined. Sequencing results were verified by RT-qPCR as being accurate and reliable.

Conclusions: This study identified several genes associated with ARC, which improves our knowledge of the disease mechanism.

Keywords: Age-related cataract, Differentially expressed genes, Bioinformatics analysis, Pathogenic genes

Background

Age-related cataract (ARC) is a common visual disorder caused by lens opacity, and the main cause of blindness in individuals aged over 50 years [1]. With an increasingly aging society, the incidence of ARC is rising sharply [2]. Currently, surgery is the only effective treatment for ARC, but this is associated with an economic burden for patients and may cause complications [3]. Therefore, alternative effective methods are necessary to prevent and treat the occurrence and development of ARC.

Risk factors such as genetics, oxidative stress, ultraviolet radiation, and drug use were previously shown to be closely related to the pathogenesis of ARC [4]. Of these, changes in the expression of multiple genes under oxidative stress damage seem to be key events [5]. Lens epithelial cells are the only nucleated cells in the lens, and as such contain most of the metabolic, protective, permeable, and other regulatory systems [6]. They communicate with the underlying lens fiber cells and respond to cataract-related damage by altering gene expression [7]. Although several genes associated with the pathogenesis of ARC have been identified, many have not been
explored. Therefore, the identification of candidate genes for ARC progression in lens epithelial cells will help further understand the disease mechanism and provide an important basis for reducing disease occurrence and for targeted therapy.

In the present study, we performed RNA sequencing on human lens anterior capsule tissue and compared transcriptome data between ARC patients and healthy controls. Differentially expressed genes (DEGs) were analyzed using bioinformatics such as Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. The accuracy of RNA sequencing was verified using reverse transcription quantitative RTqPCR for 10 selected DEGs. Several genes were identified as being associated with ARC, which furthers our knowledge of the disease mechanism.

**Methods**

**Patient and tissue sample collection**

Twelve cases of age-related cataract anterior capsule tissue samples were collected from Hongqi Hospital affiliated to Mudanjiang Medical College from January 2019 to March 2019. All the patients were cortical cataracts, including 6 males and 6 females, aged 55 to 64 years. The diagnostic criteria for ARC are non-congenital cataract, non-posterior cataract, and non-metabolic cataract, no hypertension, no diabetes, no glaucoma, no fundus lesions, no uveitis, no eye trauma, and no intraocular surgery history. Twelve cases of normal anterior capsule tissue samples were collected from corneal donors and patients with lens trauma. The information of the samples was displayed in Table 1. Human anterior lens capsule tissue samples were obtained by an ophthalmologist through a continuous annular capsulorhexis of the lens and randomly divided 4 samples into a group. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C for RNA sequencing. This study followed the principles of the Declaration of Helsinki and all participants signed informed consent.

**RNA extraction**

Since the miRNA isolation kit can isolate total RNA containing small RNAs from cultured cells or tissues for better extraction, we selected the mirVana™ miRNA ISOLation Kit (Ambion-1561) to isolate total RNA from tissue samples according to the manufacturer’s protocol. The RNA quality was determined by OD 260/280 absorbance assessment using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, UK). RNA integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. All RNA samples were stored at −80 °C for further application.

**RNA library preparation and transcriptome sequencing**

The 12 healthy controls samples were randomly divided into 3 groups, with 4 samples in each group. Similarly, the 12 samples from the ARC patients were also randomly divided into 3 groups of 4 samples per group. Since the sample tissue of the anterior lens capsule we used was very small, and less RNA was extracted from a single sample, we combined the samples for RNA sequencing analysis. The total RNA extracted from each group was about 0.8 μg, and the RIN value was above 7. Therefore, we used a low start volume library construction method (NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina) for library construction, which includes mRNA enrichment analysis. Briefly, RNA Library Preparation mainly has the following steps: RNA

**Table 1 The information of our patients and normal samples**

| Characteristics of the age related cataract patients | Characteristics of the Normal person |
|------------------------------------------------------|-------------------------------------|
| Patient | Age | Eye | Gender | Patient | Age | Eye | Gender |
| 1       | 55  | Right | Male   | 1       | 43  | Right | Male   |
| 2       | 56  | Right | Male   | 2       | 44  | Left  | Male   |
| 3       | 56  | Left  | Female | 3       | 44  | Right | Female |
| 4       | 58  | Left  | Male   | 4       | 45  | Left  | Female |
| 5       | 59  | Right | Female | 5       | 45  | Right | Male   |
| 6       | 60  | Left  | Female | 6       | 46  | Left  | Female |
| 7       | 61  | Left  | Male   | 7       | 47  | Left  | Male   |
| 8       | 62  | Right | Female | 8       | 47  | Right | Male   |
| 9       | 62  | Right | Female | 9       | 48  | Left  | Male   |
| 10      | 62  | Left  | Male   | 10      | 48  | Left  | Female |
| 11      | 63  | Left  | Female | 11      | 49  | Right | Female |
| 12      | 64  | Right | Male   | 12      | 49  | Right | Female |

Note: There were 12 patients (12 eyes) with ARC in this study, with an average age of 59.83 ± 3.01 years old. And there were 12 normal samples (12 eyes) in this study, with an average age of 46.25 ± 2.05 years old.
purification and fragmentation, Synthesize First Strand cDNA, Synthesize Second Strand cDNA, Adenylate 3’ Ends, Ligate Adapters, Enrich DNA Fragments. The Agilent bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) was used to check the size and purity of the library. Sequencing was performed on Illumina HiSeq X Ten to generate 125 bp/150 bp paired-end reads.

Sequence data analysis
The raw data in the fastq format (raw reads) was first processed using Trimmomatic. Then, clean data (clean reads) were obtained by removing low-quality reads, reads containing adapter, and poly-N from raw data. Q30 content represents the percentage of bases with a Qphred value greater than 30 to the total bases. All subsequent analyses were performed using clean data with high quality. HISAT2 was used to compare the sequence of Clean Reads with the specified reference genome [8]. Fragments Per kb Per Million Reads (FPKM) were calculated to quantify gene expression levels. Differentially expressed genes (DEGs) analysis was performed using the DESeq R package, which based DEGs on the negative binomial distribution. The screening conditions for DEGs were \( P < 0.05 \) with fold change (FC) > 2.

GO and KEGG enrichment analysis of DEGs
Gene Ontology (GO) analysis (http://geneontology.org/) was used to categorize the function of the DEGs. And the functional description was mainly divided into biological process, cellular component, and molecular function. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis [9] (http://www.genome.jp/kegg/) was used to predict the signaling pathways in which these DEGs may be involved, KEGG software from the Kanehisa laboratory was used with prior permission. The significantly enriched GO and KEGG terms met the criterion of corrected \( P < 0.05 \).

Quantitative real-time PCR validation
The remaining RNA after transcriptome was further used for validation studies by qPCR. Total RNA was extracted from the anterior capsule of the normal lens and cataract patients for RNA transcriptome sequencing. Detected the concentration of RNA with a NanoDrop spectrophotometer. Agarose gel electrophoresis detected the integrity of RNA. Quantification was carried out through a two-step reaction process of reverse transcription (RT) and PCR. Each RT reaction consisted of a total of 10 µl of 0.5 µg RNA, 2 µl of 5x Transx all-in-one SuperMix for qPCR, and 0.5 µl of gDNA remover. The reaction was performed on GeneAmp® PCR System 9700 (Applied Biosystems, USA) at 42 °C, 5 s, and 85 °C for 15 min. Next, 10 µl of the RT reaction mixture was diluted 10-fold with nuclease-free water and kept at −20 °C. Use LightCycler®480II Real-Time PCR instrument (Roche, Switzerland) with 1 µl cDNA, 5 µl 2xPerfectStartTM Green qPCR SuperMix, 0.2 µl forward primer, 0.2 µl PCR reverse primer, and 3.6 µl nuclease-free water. The reaction was incubated in a 384-well optical plate (Roche, Switzerland) at 94 °C for 30 s, and then at 94 °C for 5 s and 60 °C for 30 s for 45 cycles. The primer sequence is synthesized according to the mRNA sequence obtained from the NCBI database (Table 2). Each sample was analyzed in triplicate. The \( 2^{-\Delta\Delta C_{T}} \) method was used for data quantification [10].

Statistical analysis
The statistical significance of the experimental data was conducted by a Student’s t-test. The acceptable significance was set as *\( p < 0.05 \); **\( p < 0.01 \).

Results
Descriptive analysis of sequencing data quality assessment
Anterior capsule tissue samples were collected from ARC patients (Disease Group, JBZ) and healthy controls...
We used the Illumina platform to obtain paired-end sequencing data. Because data error can have a major impact on the results, we employed Trimmomatic software to perform quality preprocessing of the original data, and statistically summarize the number of reads throughout the quality control process (Table 3). A total of 55.59 million raw reads were generated from the JBZ1 group, 48.28 million from JBZ2, 48.25 million from JBZ3, 58.89 million from ZCR1, 47.17 million from ZCR2, and 47.97 million from ZCR3. A total of 52.73, 44.64, and 44.59 million clean reads were obtained for JBZ1–3, respectively, and 55.68, 42.94, and 42.95 million for ZCR1–3, respectively. Clean reads ratios were more than 89% for both patients and controls, and over 77% of clean reads could be mapped to human genome sequences. The highest proportion of sequences with only one alignment position on the reference sequence was 89.10% in the JBZ group and 90.47% in the ZCR group. Both JBZ and ZCR groups had approximately 3% multiple alignment positions, and both groups exceeded 90% for Q30 content, representing the percentage of bases with a Qphred value greater than 30. These data indicate that high-quality sequencing was achieved in this study, so subsequent transcriptional analysis could continue.

Analysis of differentially expressed genes (DEGs)
According to the criteria of \( P < 0.05 \) and FC > 2, a total of 1112 DEGs were identified between patients and controls. Of these 698 were up-regulated and 414 were down-regulated in the JBZ group compared with the ZCR group (Fig. 2a). Volcano plots, MA plots, and heat-maps of hierarchical clustering showed that the gene expression levels were distinguished (Fig. 2b–d). The top 20 up-regulated genes and top 20 down-regulated genes are listed in Table 4.

Analysis of GO enrichment
GO is used to annotate gene function and standardize the description of gene products according to biological process, cellular component, and molecular function categories. We performed GO enrichment analysis by screening GO terms with more than two DEGs in the three gene product classifications, then sorting based on \( P < 0.05 \) from the largest to the smallest \(-\log_{10} p\) value, and selecting the top 10 in each category. A total of 848 DEGs (76.3% of all identified DEGs) were mapped to 3646 GO terms, and the top 30 GO terms are shown in Fig. 3. Of these, the most significantly enriched in biological process and molecular function categories were trigeminal nerve structural organization (GO: 0021637, \( P = 9.01\times10^{-6} \)) and the structural constituent of the eye lens (GO: 0005212, \( P = 5.51\times10^{-5} \)), respectively, while the most enriched in the cellular component category were the plasma membrane (GO: 0005886, \( P = 4.65\times10^{-7} \)) and the integral component of the plasma membrane (GO: 0005887, \( P = 3.96\times10^{-6} \)). These findings confirmed the accuracy of our sequencing results.

Analysis of the KEGG pathway
KEGG is a database for the pathway analysis of DEGs to understand the biological functions of identified genes. In this study, KEGG was divided into the following six classifications: Cellular processing (four categories), Environmental information processing (three categories), Genetic information processing (four categories), Human diseases (eight categories), Metabolism (11 categories), and Organismal systems (10 categories). A total of 409 DEGs (36.8% of all identified DEGs) were assigned to 286 KEGG pathways. The top 20 KEGG enrichment pathways are shown in Fig. 4, and include transforming growth factor (TGF)-β, p53, mitogen-activated protein kinase (MAPK), tumor necrosis factor, and other common signaling pathways. A cancer-related pathway had the highest number of DEGs, suggesting that it might be...
### Table 3  Statistics of the sequencing data quality results from all samples

| Sample Name | Raw reads | Clean reads | Clean ratio | Mapped reads | Unique mapped | Multi mapped | Q30 (%) |
|-------------|-----------|-------------|-------------|--------------|---------------|--------------|---------|
| JBZ1        | 55.59 M   | 52.73 M     | 94.86%      | 92.35%       | 89.10%        | 3.25%        | 94.26%  |
| JBZ2        | 48.28 M   | 44.64 M     | 92.46%      | 80.42%       | 77.29%        | 3.13%        | 91.21%  |
| JBZ3        | 48.25 M   | 44.59 M     | 92.41%      | 79.14%       | 75.74%        | 3.41%        | 91.15%  |
| ZCR1        | 58.89 M   | 55.68 M     | 94.55%      | 93.52%       | 90.47%        | 3.05%        | 94.07%  |
| ZCR2        | 47.17 M   | 42.94 M     | 91.03%      | 79.03%       | 75.56%        | 3.47%        | 90.42%  |
| ZCR3        | 47.97 M   | 42.95 M     | 89.54%      | 77.13%       | 73.34%        | 3.38%        | 89.43%  |

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**Fig. 2** Differentially expressed genes (DEGs) in anterior capsule tissues between the two groups.  

- **a** The number of differentially expressed genes (DEGs) in the JBZ group and ZCR group (P < 0.05, Fold change (FC) > 2). The red histogram represented upregulated genes and a blue histogram represented downregulated genes.  
- **b** The MA plots visualizing the DEGs between the two groups. The X-axis represented the average expression of all samples for comparison after normalization; the Y-axis represented log2FoldChange. The red dots above the red line represented the upregulated DEGs, and the red dots below the red line represented the down-regulated DEGs.  
- **c** The volcano plot of the DEGs between two groups. Red dots represented significantly up-regulated differential genes, green dots represented significantly down-regulated differential genes, and gray dots represented non-significantly differential genes.  
- **d** Heat-maps of hierarchical clustering analysis of DEGs between two groups. The horizontal ordinate represents the clustering of samples from the JBZ and ZCR groups. The longitudinal coordinates represent the DEGs and the clustering of genes. The red color indicates up-regulated genes, while the blue color indicates down-regulated genes.
Table 4  Top 20 upregulated and downregulated differentially expressed genes (DEGs) in the JBZ group compared to the ZCR group

| Gene-id | log2FC | Pval | Up-down | Gene-id | log2FC | Pval | Up-down |
|---------|-------|-----|---------|---------|-------|-----|---------|
| NR4A2   | 10.210411523 | 2.66E-60 | Up | DDX3Y   | -9.523209533 | 1.56E-72 | Down |
| NR4A3   | 8.218022790 | 1.39E-22 | Up | EIF1AY  | -8.379329539 | 4.86E-20 | Down |
| GRA3    | 7.601604199 | 4.05E-47 | Up | PVALB   | -8.200440025 | 1.76E-45 | Down |
| ALDH1A2 | 6.166698445 | 8.92E-20 | Up | CLDN5   | -8.150297932 | 3.56E-32 | Down |
| RGD1    | 5.914488712 | 1.17E-05 | Up | RPS4Y1  | -7.891783293 | 1.91E-32 | Down |
| BDNF    | 5.490073820 | 0.008935371 | Up | LUM     | -6.852847001 | 4.09E-10 | Down |
| UNC5A   | 5.263108777 | 0.025651215 | Up | C3      | -6.44725368 | 5.17E-41 | Down |
| CAPN8   | 5.084626101 | 4.50E-07 | Up | SAA1    | -6.256915413 | 0.001039753 | Down |
| SLC16A7 | 4.861755978 | 0.002948834 | Up | APOD    | -5.958632573 | 2.67E-17 | Down |
| ADAM33  | 4.843155381 | 7.86E-60 | Up | CHL1    | -5.886371002 | 4.57E-21 | Down |
| MPZ     | 4.686357999 | 0.00744644 | Up | ABCC3   | -5.584424022 | 4.19E-10 | Down |
| TMEM74B | 4.645467019 | 0.00108655 | Up | PRF1    | -5.297684334 | 0.04650321 | Down |
| RRR     | 4.607044318 | 0.008796089 | Up | KRT78   | -5.152554180 | 0.026495957 | Down |
| NKX3–2  | 4.33196849 | 0.029753521 | Up | OLAH    | -5.13907536 | 0.017935704 | Down |
| UROC1   | 4.315361957 | 0.027292075 | Up | FGR     | -5.11004644 | 0.004889699 | Down |
| PRSS55  | 4.281664428 | 0.01155766 | Up | ISM1    | -5.076786718 | 0.021961678 | Down |
| ATP1A3  | 4.278127228 | 0.03309551 | Up | GPR160  | -5.054228043 | 0.007982878 | Down |
| SLC7A5  | 4.231647204 | 2.05E-63 | Up | SERPINA3 | -4.93814790 | 1.01E-118 | Down |
| RGS2    | 4.230358381 | 6.09E-08 | Up | SGPP2   | -4.916332934 | 0.00138125 | Down |
| ATOH7   | 4.228904441 | 0.01977059 | Up | FABP9   | -4.767243914 | 0.04901258 | Down |

Fig. 3  GO enrichment analysis of differentially expressed genes (DEGs). The GO enrichment analysis of the top 30 GO terms. The X-axis represented the different GO terms, the Y-axis represented -log10Pvalue
associated with cell proliferation and apoptosis. KEGG analysis indicated that ARC involves multiple processes and complex signaling pathways, which require further exploration.

RT-qPCR validation
To confirm RNA sequencing results, 10 DEG transcripts were selected for validation using RT-qPCR. DEG expression levels in the ARC group compared with the control group were consistent with RNA sequencing data, indicating the accuracy of our findings (Fig. 5).

Discussion
ARC is the leading cause of blindness worldwide, and its incidence is increasing annually. Till date, ARC pathogenesis has mainly been studied at the molecular and cellular levels, with particular focus on the epigenetic changes occurring for multiple genes [11]. Although many genes associated with ARC have been identified, several are undiscovered. In the present study, we conducted in-depth research into ARC-related genes using the bioinformatics technology of transcriptome sequencing.

Here, our study showed that the clean ratio in both groups exceeds 89%, and the highest mapping ratio is 93.5%. In all samples, we identified a total of 17,348 genes, including 1112 genes whose expression level was 2 times or more different than that of the transparent lens. A comparative transcriptome analysis between normal and ARC samples showed that 698 DEGs in ARC were up-regulated and 414 DEGs were down-regulated. These data can provide necessary tissue-specific insights for the ARC transcriptome, such as the number, the
expression level, and the distribution of DEGs. It can be used to examine the expression of target genes in ARC, which helps explain genetic-related research. Besides, it can also be used to select candidate genes that are more functionally related to ARC development.

As the only transparent, avascular, and nerveless organ in the human body, the lens is composed of three parts: the lens capsule, lens epithelial cells, and lens fibers [12]. Lens epithelial cells are the only cells that contain a nucleus capable of aerobic metabolism and are closely connected to the anterior lens capsule. Because the stability of lens epithelial cells is important for maintaining lens transparency and metabolic homeostasis [13], we used these cells for sequencing analysis. We obtained a clean reads ratio in both ARC patients and controls of more than 89%, with a highest mapping ratio of 93.5%. From all samples, we identified a total of 17,348 genes, including 1112 whose expression differed by more than 2-fold than that of the transparent lens. Comparative transcriptome analysis between ARC and control samples showed that 698 DEGs were up-regulated and 414 were down-regulated in ARC. These data provide important tissue-specific insights for the ARC transcriptome, and can be used to examine the expression of target genes that are more functionally related to ARC development.

GO term analysis showed that structural constituents of the eye lens were highly enriched in the present study, which undoubtedly verifies the reliability of our results. DEGs in this GO term included BFSP1, LIM2, CRYBA4, CRYBA2, MIP, TMOD1, GJA1, LIM2, HSF4, and WNT2B, several of which are closely associated with cataract development. Additionally, some genes that we identified were previously reported to be related to the pathogenesis of ARC, such as CRYA2 and HSF4 [14, 15], and we have also verified these genes used RT-qPCR that are consistent with the results. Our transcriptome results also allowed us to identify and verify some genes rarely associated with ARC. Yan et al. found that the increased expression of laminin α4 in the anterior lens capsule is the main mechanism leading to the occurrence of ARC [16], which is consistent with our transcriptome sequencing results. Moreover, the inhibition of NLRP3 was shown to reduce oxidative stress-induced damage to human lens epithelial cells, while NLRP3 protein expression was significantly increased in lens epithelial cells under oxidative stress [17], which was also consistent with our verification results. However, more than 80% of the genes identified by transcriptome sequencing in this study have not previously been associated with ARC, so should be explored in future in-depth studies.

Some genes of interest identified here could be studied as future candidate genes of ARC. As a member of the cell adhesion molecule (CAM) family, the NrCAM gene plays a key role in the development of the nervous system [18, 19]. NrCAM expression was reported to be essential for maintaining contact between lens cells, and a lack of NrCAM caused lens fiber disorder which ultimately led to the formation of mouse cataracts [20]. However, NrCAM expression changes have not been investigated in ARC. We showed by transcriptome sequencing that NrCAM was down-regulated in ARC patients compared with controls, but RT-qPCR found it to be elevated in patients, which we speculate might reflect its low expression level. With this in mind, we aim to explore its function in ARC.
in future studies. We also identified potential ARC candidate genes previously shown to be involved in the occurrence and development of a variety of eye diseases, but not ARC, such as ANGPTL4. Aberrant ANGPTL4 expression levels are associated with branch retinal vein occlusion, macular edema, and age-related macular degeneration diseases, and are considered to be a biomarker and therapeutic target for retinopathy caused by ischemia [21, 22]. However, no studies have directly linked ANGPTL4 with ARC, so we will investigate this in our future research.

The apoptosis of lens epithelial cells is a common cellular basis for the occurrence and development of ARC [23]. A number of common apoptosis pathways such as p53, MAPK, and TGF-β signaling pathways are closely related to the occurrence of ARC, and several appear in our top 20 KEGG pathways [24–26]. Some genes of interest in these pathways include DUSP1, ID1, and CAV2, which all function in multiple signaling pathways [27–29]. We also verified their expression by RT-qPCR and obtained results consistent with transcriptome sequencing analysis.

Conclusions
The present study aimed to explore the transcription of genes expressed in the human anterior lens capsule of ARC patients to aid future research into the pathogenesis of ARC and novel therapies. We identified 112 DEGs between ARC patients and controls using RNA sequencing technology of which many were novel as reported from this study. Although the functions of most identified DEGs have not been explored in ARC, we selected several potential ARC candidate genes for future studies.

Abbreviations
ARC: Age-related cataract; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: Differentially expressed genes; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; CAMs: Cell adhesion molecules; FC: Fold change; BRVO: Branch retinal vein occlusion; ME: Macular edema; AMD: Age-related macular degeneration

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Authors’ contributions
SH, ZS, and XM designed this study. ZW wrote the manuscript. ZW, DS, SL, GQ Z, GB Z, and TC collected the samples and performed data analyses. ZW and SL performed the experiments. All authors have read and approved the final manuscript.

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Availability of data and materials
All data files are available from the NCBI database (accession number PRJNA678236), https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA678236.

Declarations

Ethics approval and consent to participate
The present study complied with the Declaration of Helsinki for research involving human subjects and was approved by the research ethics committee of Heilongjiang Provincial People’s Hospital. Written informed consent was obtained from each patient.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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