Identification of aberrantly methylated differentially expressed genes in age-related macular degeneration

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Abstract

DNA methylation plays a significant role in many diseases. Age-related macular degeneration (AMD) is a leading cause of vision loss for people aged 50 years and above, but the etiology and pathogenesis are largely unknown. This study aimed to identify the aberrantly methylated differentially expressed genes (DEGs) in AMD and predict the related pathways on the basis of public data.

Aberrant methylation can influence the functions of key genes by altering their expression. Here, we found out DEGs by overlapping public microarray data (GSE29801 and GSE102952). Functional and enrichment analyses of selected genes were performed using the DAVID database. Subsequently, protein–protein interaction (PPI) networks were constructed by using STRING and visualized in cytoscape to determine hub genes. Finally, we collected AMD patients’ blood samples to identify the methylation statuses of these hub genes by using methylated DNA immunoprecipitation.

In total, 156 hypermethylation-low expression genes and 127 hypomethylation-high expression genes were predicted. The hypermethylation-low expression genes were enriched in biological processes of response to cardiac conduction, ATP binding, and cell–cell junction assembly. The top 5 hub genes of the PPI network were HSP90AA1, HSPA1L, HSP1, HS60B1, and NOP56. Meanwhile, the hypomethylation-high expression genes were enriched in the biological processes of response to positive regulation of the MAPK cascade, actin cytoskeleton reorganization, dentate gyrus development, and cell migration. The top 5 hub genes of this PPI network were PIK3R1, EZR, IGF2, SLC2A1, and CDKN1C. Moreover, the methylation statuses of NOP56, EZR, IGF2, SLC2A1, CDKN1C were confirmed to be altered in the blood of AMD patients.

This study indicated possible aberrantly methylated DEGs and differentially expressed pathways in AMD by bioinformatics analysis, providing novel insights for unraveling the pathogenesis of AMD. Hub genes, including NOP56, IGF2, SLC2A1, and CDKN1C, might serve as aberrant methylation-based candidate biomarkers for AMD in future applications.

Abbreviations: AMD = age-related macular degeneration, CDKN1C = cyclin-dependent kinase inhibitor 1C, ClusterONE = clustering with overlapping neighborhood expansion, CNV = choroidal neovascularization, DAVID = the database for annotation, visualization and integrated discovery, DEGs = differentially expressed genes, DMGs = differentially methylated genes, ER = endoplasmic reticulum, GEO = gene expression omnibus, GO = gene ontology, HSP = heat shock protein, HSPA1L = HSP 70 kDa protein 1-like, KEGG = Kyoto Encyclopedia of Genes and Genomes, LAMP-1 = Lysosomal-associated membrane protein 1, MeDIP = Methylated DNA immunoprecipitation, PIK3R1 = Phosphoinositide-3-kinase regulatory subunit 1, PPI = protein–protein interaction, RPE = retinal pigmented epithelium, SLC2A1 = Solute carrier family 2 member 1, SNPs = single nucleotide polymorphisms, STRING = search tool for the retrieval of interacting genes, VEGF = vascular endothelial growth factor.

Keywords: age-related macular degeneration, methylation, microarray analysis
1. Introduction

Age-related macular degeneration (AMD) is the most common disease-causing visual impairment and blindness in elderly people worldwide, seriously affecting the quality of life of patients. The pathogenesis of the disease might be related to factors such as advanced age, caucasian ethnicity, heredity, and a history of smoking, but the precise mechanism remains unknown. Therefore, exploring the molecular mechanism underlying the pathogenesis and development of AMD is urgently warranted.

Epigenetics refers to the new field of genetics in which an inheritable phenotype can be altered by environmental changes without involving mutation of the DNA sequence. DNA methylation is an epigenetic event involved in regulating gene transcription and cell differentiation. DNA methylation is generally associated with gene silencing, while demethylation promotes gene transcription. Many studies have demonstrated DNA methylation to be involved in complex biological processes such as aging, oxidative stress, and inflammation. Epigenetics has been found to play a certain role in AMD. Hunter et al. found stmn1 and Gstm5 expression to be downregulated by DNA methylation, which increased the susceptibility of the macula to oxidative stress injury and increased the likelihood of AMD occurrence. The promoter region of IL-17RC was found to be significantly demethylated in AMD cells, corresponding to the promotion of IL-17RC transcription in the macula and the number of IL-17RC-positive monocytes, making AMD patients more sensitive to IL-17-mediated inflammation. This indicates that the demethylation of IL-17RC promoter regions might be related with AMD. Suuronen et al suggested that the decrease in the methylation level of the CpG island in the clusterin gene might be involved in AMD occurrence. However, the mechanism of how DNA methylation causes the pathogenesis and development of AMD is still being studied.

Thus far, no research has been performed to jointly analyze information of both gene expression and methylation profiling microarray analysis in the pathogenesis of AMD. In our present study, data of gene expression and methylation profiling microarray analysis of AMD (GSE29801 and GSE102952) were integrated and analyzed by bioinformatics tools. aberrantly methylated differentially expressed genes (DEGs) and pathways were identified in AMD. The respective protein–protein interaction (PPI) networks were constructed, and hub genes were determined. Finally, we detected the methylation statuses of hub genes in AMD patients using methylated DNA immunoprecipitation (MeDIP). In this way, we expect to identify novel aberrantly methylated genes and predict relative pathways in AMD to provide the basis for further research on the molecular pathogenesis of AMD.

2. Methods

2.1. Microarray data and screening for DEGs or differentially methylated genes

Gene expression datasets (GSE29801), which contained 175 samples from extramacular retinal pigmented epithelium (RPE)-choroid and 118 samples from the retina, were downloaded from Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo). Among them, we chose the data from the retina for further analysis because methylation profiling data sets were from the retina. The series of data contained 55 samples from donors without ocular disease and 63 samples from donors with preclinical AMD or AMD. Then, according to the information in platform GPL4133, the probes were transformed into the corresponding gene symbol. After quality control and other probe phase data preparation, the limma package was used to screen for different genes. logFC (fold change) > 0.1 and P value < .05 were considered statistically significant.

Gene methylation datasets (GSE102952) were also downloaded from GEO, which included altogether nine samples from donors without ocular disease and 8 samples from donors with preclinical AMD. After quality control and other probe phase data preparation, the minfi package was used to screen for different genes. P < .05 was considered statistically significant.

finally, hypomethylation-high expression genes were obtained by hypomethylated and upregulated genes, while hypermethylation-low expression genes were obtained by hypermethylated and downregulated genes.

2.2. GO and KEGG pathway enrichment analysis

The database for annotation, visualization and integrated discovery (DAVID; http://david.ncifcrf.gov; version 6.8) — a bioinformatics tool widely used for functional and pathway enrichment analysis — was used to perform gene ontology (GO) and KEGG pathway analysis in the selected hypomethylation-high expression genes and hypermethylation-low expression genes. P < .05 was considered statistically significant.

2.3. PPI network and hub gene identification

The PPI network of hypomethylation-high expression genes and hypermethylation-low expression genes was constructed by using the search tool for the retrieval of interacting genes (STRING; http://string-db.org). A threshold of combined score ≥0.4 was considered the cut-off criterion. The top five hub genes in each network were screened out by using cytoHubba, an app in Cytoscape, using the Maximal Clique Centrality method.

2.4. Module analysis of PPI

Clustering with overlapping neighborhood expansion (ClusterONE), an app in Cytoscape, was used to screen modules within the PPI network of minimum size = 5 and minimum density = 0.05. The top 2 most rewarding modules in the PPI networks of both hypomethylation-high expression genes and hypermethylation-low expression genes were screened out. DAVID was used to identify the functions and pathways of the DEGs in these modules. P < .05 was considered statistically significant.

2.5. Methylated DNA immunoprecipitation

Genomic DNA was extracted from the blood of AMD patients, and genomic DNA was sonicated on ice 6 times for 30 seconds each. The polyclonal antibody against 5-methylcytosine was added to each sample and incubated overnight at 4°C with gentle mixing. Immuno complexes were recovered by salmon sperm DNA–protein A agarose beads and sequentially and extensively washed. DNA fragments were purified with phenol-chloroform extraction followed by acid ethanol precipitation. Immunoprecipitated DNA was subjected to quantitative real-time PCR using primers specific for about 200 bp segments corresponding to CpG sites within the hub genes’ promoter regions. Primer sets for PCR are shown in Table 6. Amplifications were run in triplicate. The results of PCR were analyzed as following, ∆Ct(normalized) = Ct(CpG) − [Ct(input) − log2(input dilution factor)]; and ChIP/input
3. Results

3.1. Microarray data screening for DEGs and differentially methylated genes

The R package was used to screen DEGs or differentially methylated genes (DMGs). For DMGs in gene methylation datasets (GSE102952), 6537 hypermethylated genes and 3805 hypomethylated genes were found. The results indicate abnormal methylation of large numbers of genes associated with AMD development. For DEGs in gene expression datasets (GSE29801), 954 high-expression genes and 554 low-expression genes were screened out. Subsequently, 156 hypermethylation-low expression genes were found by overlapping low-expression genes and hypomethylated genes. By overlapping high-expression genes and hypomethylated genes, 127 hypomethylation-high expression genes were found.

3.2. GO and KEGG pathway enrichment analysis

DAVID was used for functional annotation in the selected hypomethylation-high expression genes and hypermethylation-low expression genes. The top 10 significant terms of GO enrichment analysis are shown in Table 1, and top 10 significant terms of KEGG pathway enrichment analysis are shown in Table 2. Our results indicated that hypermethylation-low expression genes were related to 15 functions, including cardiac conduction (P = .0036), ATP binding (P = .0039), cell–cell junction assembly (P = .0066), and photoreceptor disc membrane (P = .0076). Hypomethylation-high expression genes were related to 40 functions, including positive regulation of the MAPK cascade (P = .0020), actin cytoskeleton reorganization (P = .0037), actin filament polymerization (P = .0036), cardiac conduction (P = .0126), and cell migration (P = .0057). The top 10 results are shown in Table 1. For the result of KEGG pathway enrichment analysis, hypermethylation-low expression genes were enriched in phototransduction (P = .0179), hypomethylation-high expression genes were enriched in 8 pathways, including pathways in cancer (P = .0011), pancreatic cancer (P = .0018), lysosome (P = .0028), and proteoglycans in cancer (P = .0053) (Table 2).

3.3. PPI network and hub gene identification

Interactions among proteins play a major part in many biological processes. Hence, we used STRING to construct the PPI network of hypomethylation-high expression genes and hypermethylation-low expression genes (Figs. 1A and 2A). Among them, the top 5 hub genes that might have a significant position were screened out. For the PPI network of hypermethylation-low expression genes, the top 5 hub genes were HSP90AA1, HSPA1L1, HSPE1, HSP90B1, and NOP56. For the PPI network of hypomethylation-high expression genes, the top 5 hub genes were PIK3R1, EZR, IGF2, SLC2A1, and CDKN1C.

3.4. Module analysis of PPI

By using ClusterONE, the top 2 most rewarding modules in the PPI networks of both hypomethylation-high expression genes and hypermethylation-low expression genes were screened out (Table 3). Finally, DAVID was used for GO and KEGG pathway enrichment analysis in the genes in the selected modules. For the modules in the PPI network of hypomethylation-high expression genes, 26 functions and 2 pathways were found to be related to module 1. Twelve functions and 11 pathways were identified to be related to module 2. The top 5 results are shown in Tables 4 and 5, or the modules in the PPI network of hypermethylation-low expression genes, 8 functions and 2 pathways were identified.

### Table 1

| Category Term Count Value | % | P Value |
|---------------------------|---|---------|
| Hypermethylation-low expression genes | 4 | 2.919708029 | 0.003602 |
| GOTERM_BP_DIRECT GO:0001934 → cardiac conduction | 3 | 2.189781022 | 0.006622 |
| GOTERM_BP_DIRECT GO:0007043 → cell–cell junction assembly | 3 | 2.189781022 | 0.016733 |
| GOTERM_BP_DIRECT GO:0022400 → regulation of rhoGTPase mediated signalling pathway | 3 | 2.189781022 | 0.020291 |
| GOTERM_BP_DIRECT GO:0008277 → regulation of G-protein coupled receptor protein signaling pathway | 3 | 2.189781022 | 0.035005 |
| GOTERM_BP_DIRECT GO:0002508 → DNA duplex unwinding | 3 | 2.189781022 | 0.036090 |
| GOTERM_BP_DIRECT GO:0097381 → photoreceptor disc membrane | 3 | 2.189781022 |
| Hypomethylation-high expression genes | 2 | 15.32847615 | 0.0179 |
| GOTERM_BP_DIRECT GO:00030496 → midbody | 21 | 15.32847615 | 0.0011 |
| GTPM_BP_DIRECT GO:0005524 → ATP binding | 2 | 15.32847615 |
| GTPM_BP_DIRECT GO:0008094 → DNA-dependent ATPase activity | 3 | 2.189781022 |

**GOTERM_BP_DIRECT** → gene ontology.
to be related to module 1, and 18 functions and 5 pathways were found to be related to module 2.

3.5. Altered DNA methylation of hub genes in promoter regions is associated with AMD

By contrast, MeDIP-qPCR showed that the DNA methylation levels of these hub genes were altered in AMD patients (Fig. 3). Compare with normal samples, HSP90AA1, HSPE1, HSP90B1, CDKN1C, EZR, IGF2, SLC2A1 were hypomethylated significantly in AMD patients’ blood samples. NOP56 and PI3KR were hypermethylated significantly. Meanwhile, the methylation levels of HSPA1L was detect to increase slightly in AMD patients.

4. Discussion

Here, we identified 156 hypermethylation-low expression genes and 127 hypomethylation-high expression genes by analyzing the data of gene expression (GSE29801) and methylation (GSE102952) in AMD by bioinformatics tools. Enrichment of these genes suggested that certain related pathways and hub genes were affected by aberrant methylation, which may provide the basis for pathogenesis research in AMD.

As suggested by DAVID analysis, hypermethylation-low expression genes were enriched in biological processes of response to ATP binding, cardiac conduction, DNA duplex unwinding, cell–cell junction assembly, the rhodopsin-mediated

| Category                              | Term                                      | Count | %       | P value  |
|---------------------------------------|-------------------------------------------|-------|---------|----------|
| Hypermethylation-low expression genes | KEGG_PATHWAY hsa04744:Phototransduction   | 3     | 2.189731022 | .017924875 |
| Hypomethylation-high expression genes | KEGG_PATHWAY hsa05200:Pathways in cancer | 11    | 9.090909 | .001066 |
|                                       | KEGG_PATHWAY hsa05212:Pancreatic cancer   | 5     | 4.132231 | .001815 |
|                                       | KEGG_PATHWAY hsa04142:LYSoome             | 6     | 4.956786 | .002838 |
|                                       | KEGG_PATHWAY hsa05205:Proteoglycans in cancer | 7    | 5.785124 | .00531  |
|                                       | KEGG_PATHWAY hsa04210:Apoptosis           | 4     | 3.305785 | .013607 |
|                                       | KEGG_PATHWAY hsa05230:Central carbon metabolism in cancer | 4 | 3.305785 | .014821 |
|                                       | KEGG_PATHWAY hsa00250:Alanine, aspartate and glutamate metabolism | 3 | 2.470339 | .032356 |
|                                       | KEGG_PATHWAY hsa04066:HF-1 signaling pathway | 4 | 3.305785 | .044665 |

KEGG = Kyoto Encyclopedia of Genes and Genomes.
signaling pathway, and the G-protein coupled receptor protein signaling pathway. Molecular function of GO analysis showed enrichment in ATP binding, cardiac conduction, the midbody, DNA-dependent ATPase activity, and unfolded protein. These findings are reasonable because ATP binding, cardiac conduction affect energy metabolism, which is a crucial factor in AMD development. For instance, glycolysis inhibition may promote the onset and progression of AMD. One of the proteins, ABCG1, belongs to the ATP-binding cassette transporter family. Its dysregulation causes defects in the retina, leading to the deposition of oxidized lipids, inflammation, and abnormal vascular growth, which are the risk factors in AMD. KEGG pathway enrichment analysis suggested significant enrichment in pathways such as phototransduction. Light transmission pathways are susceptible to oxidative stress in the retina, and the gradual loss of photoreceptors could lead to AMD. These changes in the phototransduction pathway might have an impact on the development of AMD. The PPI network of hypermethylation-low expression genes illustrated the overview of their functional connections, of which the top 5 hub genes were also selected: HSP90AA1, HSPA1L, HSPE1, HSP90B1, and NOP56.

Hypermethylation of heat shock protein (HSP) genes has been found to protect against some diseases. Studies have shown that promoting HSP transcription factors to reduce the high expression of HSP25 and HSP70 might prevent the risk of AMD. HSP90AA1 was found to be more highly expressed in the foveomacular region compared with other regions of the retina in healthy donor eyes, implying that certain retinal regions are susceptible to different forms of metabolic and oxidative stress. HSP90B1 encodes HSP108, which belongs to a group of proteins induced under stress situations and is closely related with the retina. Infection by Toxoplasma gondii leads to a lower expression of the HSP90B1, indicating that HSP108 protein

### Table 3

| Module                        | Size | P value     | Genes                                                                 |
|-------------------------------|------|-------------|-----------------------------------------------------------------------|
| Hypermethylation-low expression genes |      |             | AEN RRM28 PRP18 REXO1 NOP56 C1D EXOSO6                                |
| Module.1                      | 13   | 0.00618     | HSP90AA1 HSPE1 TIMM44 MRPL4 HSP90B1 HSPA1L PPID                        |
| Module.2                      | 14   | 0.021168    |                                                                       |
| Hypomethylation-high expression genes |      |             | PRX PMP22 SLC2A1 ERBB2 IGF2 AMACR PFKFB3 LDHA CDNN1C ACC5 TNF GSN PALD EZR KCNQ1 CDX6 |
| Module.1                      | 16   | 1.99E-06    | PSMB8 HAX1 FAS                                                        |
| Module.2                      | 7    | 0.001718    |                                                                       |

PPI = protein–protein interaction.
Hypomethylation-high expression genes

possibility of targeting this process to prevent illness onset.\[13\] indicates that differential methylation patterns might in it decreased in older bipolar patients compared to controls. This inhibition in cells from young patients with bipolar disorder, but receptor signaling pathway, increased after DNA methylation

HSPA1L

partial unfolding. Fries et al reported that the expression level of

has been shown to inactivate the glucocorticoid receptor through

protein 1-like (HSPA1L) is a member of the HSP70 family and

amino mono phosphate, GO

AMP

, 1 of the 4 risk genes assigned to the glucocorticoid

signaling pathway. For instance, C-reactive protein and serum amyloid

systemic and local cascades directly implicated in AMD pathogenesis. The ER and oxidative stress can also activate

signaling pathway might be involved in the development of AMD pathogenesis. For instance, C-reactive protein and serum amyloid

upregulation of vascular endothelial growth factor (VEGF) was

upregulated in drusen by ER stress. STAT3-dependent

upregulation of vascular endothelial growth factor (VEGF) was upregulated to trigger the progression to choroidal neovascularization (CNV), which is a high-risk factor for AMD.\[14\]

Kaarniranta et al reported that many genes, lipids, steroids,

provide a protective effect during infections.\[12\] HSP 70 kDa

protein 1-like (HSPA1L) is a member of the HSP70 family and has been shown to inactivate the glucocorticoid receptor through partial unfolding. Fries et al reported that the expression level of

HSPA1L, 1 of the 4 risk genes assigned to the glucocorticoid receptor signaling pathway, increased after DNA methylation inhibition in cells from young patients with bipolar disorder, but it decreased in older bipolar patients compared to controls. This indicates that differential methylation patterns might influence the alterations seen in high-risk genotypes and suggests the possibility of targeting this process to prevent illness onset.\[11\] However, there is no reference report the methylation of our

study, we found NOP56 was significantly hypermethylated

in AMD compared with normal controls by the MeDIP-qPCR assay, but methylation level of HSPA1L slightly increased. The results are different from our prediction. However, it might be a novel finding and the detailed mechanism needs our further investigation. Module analysis of the PPI network for hypermethylation-low expression genes suggested that protein proc-

Table 4

| Category Term Count % P value |
|--------------------------------|
| **Hypermethylation-low expression genes** |
| Module 1 | G00063604–RNA processing | 4 | 57.14286 | 3.97E-05 |
| | G00093035–nucleic acid phosphodiester bond hydrolysis | 2 | 28.57143 | 0.26505 |
| | G0005730–nucleolus | 6 | 85.71429 | 1.31E-06 |
| | G0000176–nucleolar exosome (RNase complex) | 2 | 28.57143 | 0.04929 |
| | G0005954–nucleoplasm | 4 | 57.14286 | 0.49634 |
| | G0004822–poly (A) RNA binding | 4 | 57.14286 | 0.05119 |
| | G0004527–exonuclease activity | 2 | 28.57143 | 0.06735 |
| | G0003676–nucleic acid binding | 3 | 42.85714 | 0.0436 |
| Module 2 | G0006457–protein folding | 4 | 57.14286 | 2.37E-05 |
| | G0006966–response to unfolded protein | 3 | 42.85714 | 9.1E-05 |
| | G00042026–protein refolding | 2 | 28.57143 | 0.05349 |
| | G0006950–response to stress | 2 | 28.57143 | 0.01602 |
| | G1000934–regulation of cellular response to heat | 2 | 28.57143 | 0.26505 |
| | G0005759–mitochondrial matrix | 3 | 42.85714 | 0.0459 |
| | G00071682–endocytic vesicle lumen | 2 | 28.57143 | 0.05257 |
| | G00042470–melanosome | 2 | 28.57143 | 0.0328 |
| | G00051082–unfolded protein binding | 4 | 57.14286 | 5.31E-06 |
| | G00055294–ATP binding | 5 | 71.42857 | 0.00794 |
| **Hypermethylation-high expression genes** |
| Module 1 | G0001628–positive regulation of gene expression | 4 | 25 | 0.01487 |
| | G0001934–positive regulation of protein phosphorylation | 3 | 18.75 | 0.05587 |
| | G0006349–regulation of gene expression by genetic imprinting | 2 | 12.5 | 0.14203 |
| | G0003033–microtubus assembly | 2 | 12.5 | 0.15085 |
| | G00072442–peripheral nervous system development | 2 | 12.5 | 0.21234 |
| | G00001455–regulation of cell motility | 2 | 12.5 | 0.04132 |
| | G00042552–myelination | 2 | 12.5 | 0.04032 |
| | G0001532–actin cytoskeleton reorganization | 2 | 12.5 | 0.41188 |
| | G0007320–cellular response to cAMP | 2 | 12.5 | 0.45476 |
| | G0005860–negative regulation of epithelial cell proliferation | 2 | 12.5 | 0.48893 |
| Module 2 | G00032209–tumor necrosis factor-mediated signaling pathway | 3 | 42.85714 | 0.00721 |
| | G00042981–regulation of apoptotic process | 3 | 42.85714 | 0.00223 |
| | G0005778–positive regulation of immune response | 3 | 42.85714 | 0.03212 |
| | G00021241–positive regulation of extrinsic apoptotic signaling pathway in absence of ligand | 2 | 28.57143 | 0.04903 |
| | G0006955–immune response | 3 | 42.85714 | 0.08798 |
| | G00097191–extrinsic apoptotic signaling pathway | 2 | 28.57143 | 0.14916 |
| | G00039815–apoptotic process | 2 | 28.57143 | 0.15598 |
| | G00002479–antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent | 2 | 28.57143 | 0.22354 |
| | G0000337–type I interferon signaling pathway | 2 | 28.57143 | 0.24755 |
| | G00038081–NF-kappaB signal transduction | 2 | 28.57143 | 0.23556 |

AMP = adenosine mono phosphate, GO = gene ontology, PPI = protein–protein interaction.
Hypomethylation-high expression genes promote gene expression. The process in RPE cells was the enters the nuclei to regulate the activity of transcription factors to

| Gene Name | Forward Primer | Reverse Primer |
|-----------|---------------|---------------|
| CDKN1C    | ATGTAGGTGCTGCCACAGAG | CACGCTCTTGGATACAGAC |
| EZR       | TCAAGTCTGCTCCAGAGAA | CACAAGCCTCAAGGCTTGAG |
| IGF2      | CTCGCTGAGGAGAGGAGTG | GTGGGCTTCTCATGTCGATA |
| PDKR1     | TCTTATTCTTTCAATCTGCC | CAAGGTCAGTGGCAAGCTTCA |
| SLC2A1    | GTAGCCAGAATAGCCACGAC | TCACGGTCGAGAGGAGAAC |
| HSP90B1   | GCCTGCTCAGGCGGACATC | TATTCGTCGACGGGAAAC |
| HSPA1L    | CTGAGCTGACAGGCTACCT | ATATTCGGGGACAGCTGAG |
| HSPC1     | TAACGGTGAATACGGTCACT | GCAGCGAGGAGGAGAAC |
| NOP56     | GTTCACGGTGGAGGAGAG | CGCTCAGGAGCACGAGAC |

MeDIP = Methylated DNA immunoprecipitation.

and proteins, including eicosapentaenoic acid, triacsinclonone, and cadherin-3e, in the estrogen signaling pathway were found to interact with responses of chronic oxidative stress, inflammation, and impaired proteolysis, which are central factors in the development of AMD.\cite{15} Our findings highlighted the likely importance of the regulation of these key biological behaviors by aberrant hypomethylation in AMD, which warrants further investigation.

For hypomethylation-high expression genes in AMD, GO analysis showed that the enriched biological processes were cell migration, positive regulation of the MAPK cascade, actin cytoskeleton reorganization, and cell morphogenesis. SanGiovanni et al reported that single nucleotide polymorphisms (SNPs) were enriched in Jun N-terminal kinase (JNK)/MAPK signaling pathways in AMD.\cite{16} In mammalian cells, activated ERK1/2 enters the nuclei to regulate the activity of transcription factors to promote gene expression.\cite{17} The process in RPE cells was the main reason for the pathogenesis and development of AMD. Dridi et al also found that the inhibition of ERK1/2 could affect RPE and retinal structures to lead to retinal degeneration by affecting the formation of retinol in retinal pigment epithelium.\cite{18} KEGG analysis displayed enrichment in the pathways of proteoglycans in cancer, lysosome, apoptosis, and the HIF-1 signaling pathway. Rastmanesh et al found that HIF-1 could upregulate VEGF and promote vascular proliferation,\cite{19} which was associated with AMD. Alivand et al suggested HIF-1-mediated hypoxia and IL-17RC methylation to be likely key targets for the pathogenesis of AMD.\cite{20} According to Duniaef et al RPE, photoreceptors, and the inner nuclear layer cells in the retina undergo apoptotic cell death in human AMD,\cite{21} and the TORC1 signaling pathway, which influences lysosomal function, might be a potential target for therapeutic intervention in AMD, where lysosomal function is defective.\cite{22} After the PPI network was constructed for hypomethylation-high expression genes, the top 5 hub genes appeared to be PIK3R1, EZR, IGF2, SLC2A1, and CDKN1C. Two hub genes were found to be related to glucose metabolism and glucose transport, namely, phosphonositide-3-kinase regulatory subunit 1 (PIK3R1) and solute carrier family 2 member 1 (SLC2A1). PIK3R1 plays an important role in insulin metabolism, and mutations in the gene were found to be associated with insulin resistance. Studies have shown that PIK3R1 plays an important role in improving glucose tolerance.\cite{23} Meanwhile, SLC2A1 encodes a major glucose transporter at the blood–brain barrier in mammals.\cite{24} By analyzing the SNPs of SLC2A1 in AMD, Baas et al found that SLC2A1 could regulate glucose bioavailability in RPEs, which might affect the pathological progress of AMD mediated by oxidative stress.\cite{25} It has been found that hyperglycemia could increase the severity of CNV and bone marrow cells, which can differentiate into vascular cells and participate in the formation of
new blood vessels in AMD. It can be recruited to CNV and participate in angiogenesis.[26] Incidentally, PIK3R1 affects the activity of PI3 kinase and regulates cell growth and development, and cyclin-dependent kinase inhibitor 1C (CDKN1C), a target of miR-184 in human RPE, is a membrane cytoskeleton crosslinker binding to lysosomal-associated membrane protein 1 (LAMP-1) during the formation of phagocytic vacuoles. LAMP-1 is known to be involved in the phagocytic digestion of photoreceptor outer segments by RPE, which may result in the dysregulation of RPE function.[27] Barbara et al. reported significantly decreased expression of IGF2 mRNA in AMD patients after intravitreal injections of ranibizumab.[18] However, there is no literature on the methylation of these top 5 hypomethylation-high expression genes in relation with AMD. Here, we further investigated the methylation statuses of PIK3R1, EZR, IGF2, SLC2A1, and CDKN1C in AMD patients’ blood samples. CDKN1C, EZR, IGF2, SLC2A1 were significantly hypomethylated in AMD compared with the normal control by MeDIP-qPCR assay. This might fit our prediction except PIK3R1. Module analysis of the PPI network for hypomethylation-high expression genes suggested that the HIF-1 signaling pathway, apoptosis, and TNF signaling pathway might be involved in AMD pathogenesis development, similar with our KEGG analysis results. According to our analysis, hypermethylation might modulate key genes responsible for cell fate determination, thereby influencing the progression of AMD.

The molecular mechanism of the pathogenesis and development of AMD is not clear yet. Epigenetic changes might partly explain it; however, this aspect has not been researched much. Therefore, we used bioinformatics tools to explore the molecular mechanism of AMD from the perspective of gene methylation. We propose that genes such as NOP56, CDKN1C, EZR, IGF2, and SLC2A1 might be related with AMD. However, additional studies are needed to confirm our results conclusively. Nevertheless, this study provides a valuable basis for further research a larger patient cohort. Obtaining insights into the mechanisms of AMD pathogenesis would greatly benefit diagnosis, treatment, and prognosis evaluation in the clinic.

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