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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*, *HSPE1*, *HSP90B1*, 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*, *EZR*, *IGF2*, *SLC2A1*, *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

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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.[1] The pathogenesis of the disease might be related to factors such as advanced age, caucasian ethnicity, heredity, and a history of smoking,[2] 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.[3] Epigenetics has been found to play a certain role in AMD.[4] Hunter et al found *stm1* 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.[5] 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.[6] 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.[7] 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 prophase 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 prophase 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. Immunocomplexes 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, $\Delta Ct_{\text{normalized ChIP}} = Ct_{\text{ChIP}} - [Ct_{\text{Input}} - \log_2(\text{input dilution factor})]$; and ChIP/input

ratio was calculated as $2^{(-\Delta Ct[\text{normalized ChIP}])}$. This study was approved by the ethics statement of Affiliated Eye Hospital of Nanchang University

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 hypermethylated 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 hypomethylatioin-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 ($P=.0037$), dentate gyrus development ($P=.0055$), 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*, *HSPA1L*, *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

Gene ontology analysis result for aberrantly methylated-differentially expressed genes.

Category	Term	Count	%	*P* Value
Hypermethylation-low expression genes				
GOTERM_BP_DIRECT	GO:0061337~cardiac conduction	4	2.919708029	0.003602
GOTERM_BP_DIRECT	GO:0007043~cell-cell junction assembly	3	2.189781022	0.006622
GOTERM_BP_DIRECT	GO:0022400~regulation of rhodopsin mediated signaling pathway	3	2.189781022	0.016733
GOTERM_BP_DIRECT	GO:0008277~regulation of G-protein coupled receptor protein signaling pathway	3	2.189781022	0.029219
GOTERM_BP_DIRECT	GO:0006986~response to unfolded protein	3	2.189781022	0.033505
GOTERM_BP_DIRECT	GO:0032508~DNA duplex unwinding	3	2.189781022	0.036490
GOTERM_CC_DIRECT	GO:0097381~photoreceptor disc membrane	3	2.189781022	
GOTERM_CC_DIRECT	GO:0030496~midbody	5	3.649635036	
GOTERM_MF_DIRECT	GO:0005524~ATP binding	21	15.32846715	
GOTERM_MF_DIRECT	GO:0008094~DNA-dependent ATPase activity	3	2.189781022	
Hypomethylation-high expression genes				
GOTERM_BP_DIRECT	GO:0043410~positive regulation of MAPK cascade	5	4.132231	0.002035
GOTERM_BP_DIRECT	GO:0031532~actin cytoskeleton reorganization	4	3.305785	0.00369
GOTERM_BP_DIRECT	GO:0021542~dentate gyrus development	3	2.479339	0.00552
GOTERM_BP_DIRECT	GO:0016477~cell migration	6	4.958678	0.005696
GOTERM_BP_DIRECT	GO:0030325~adrenal gland development	3	2.479339	0.006881
GOTERM_BP_DIRECT	GO:0000902~cell morphogenesis	4	3.305785	0.008012
GOTERM_BP_DIRECT	GO:0001934~positive regulation of protein phosphorylation	5	4.132231	0.010034
GOTERM_BP_DIRECT	GO:0007422~peripheral nervous system development	3	2.479339	0.01087
GOTERM_BP_DIRECT	GO:0030041~actin filament polymerization	3	2.479339	0.01269
GOTERM_BP_DIRECT	GO:0002543~activation of blood coagulation via clotting cascade	2	1.652893	0.013177

GO = gene ontology.

Table 2

KEGG pathway s analysis result for aberrantly methylated-differentially expressed genes.

Category	Term	Count	%	*P* value
Hypermethylation-low expression genes				
KEGG_PATHWAY	hsa04744:Phototransduction	3	2.189781022	.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:Lysosome	6	4.958678	.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	2.479339	.032356
KEGG_PATHWAY	hsa04066:HIF-1 signaling pathway	4	3.305785	.044665

KEGG = Kyoto Encyclopedia of Genes and Genomes.

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

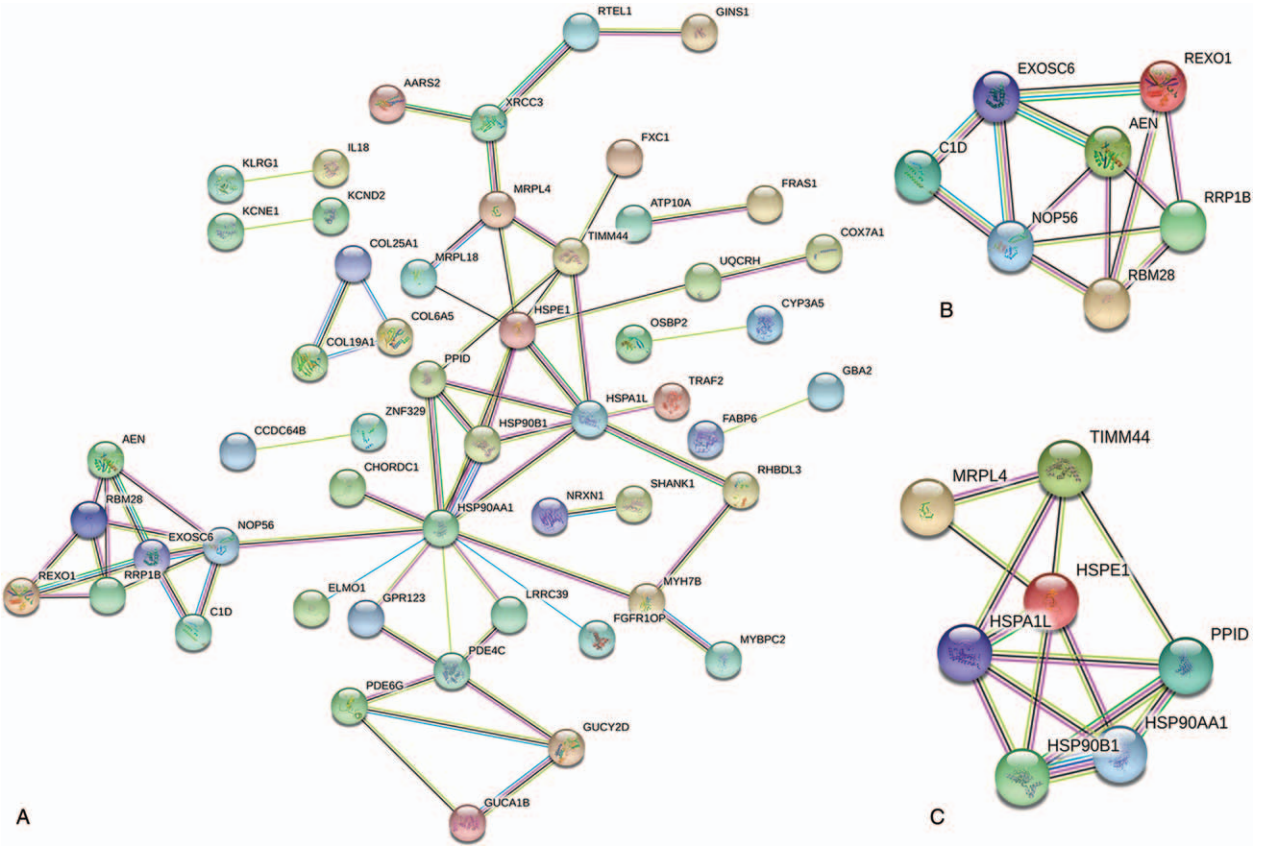

Figure 1. PPI network and top 2 modules of hypermethylation-low expression genes. PPI = protein–protein interaction.

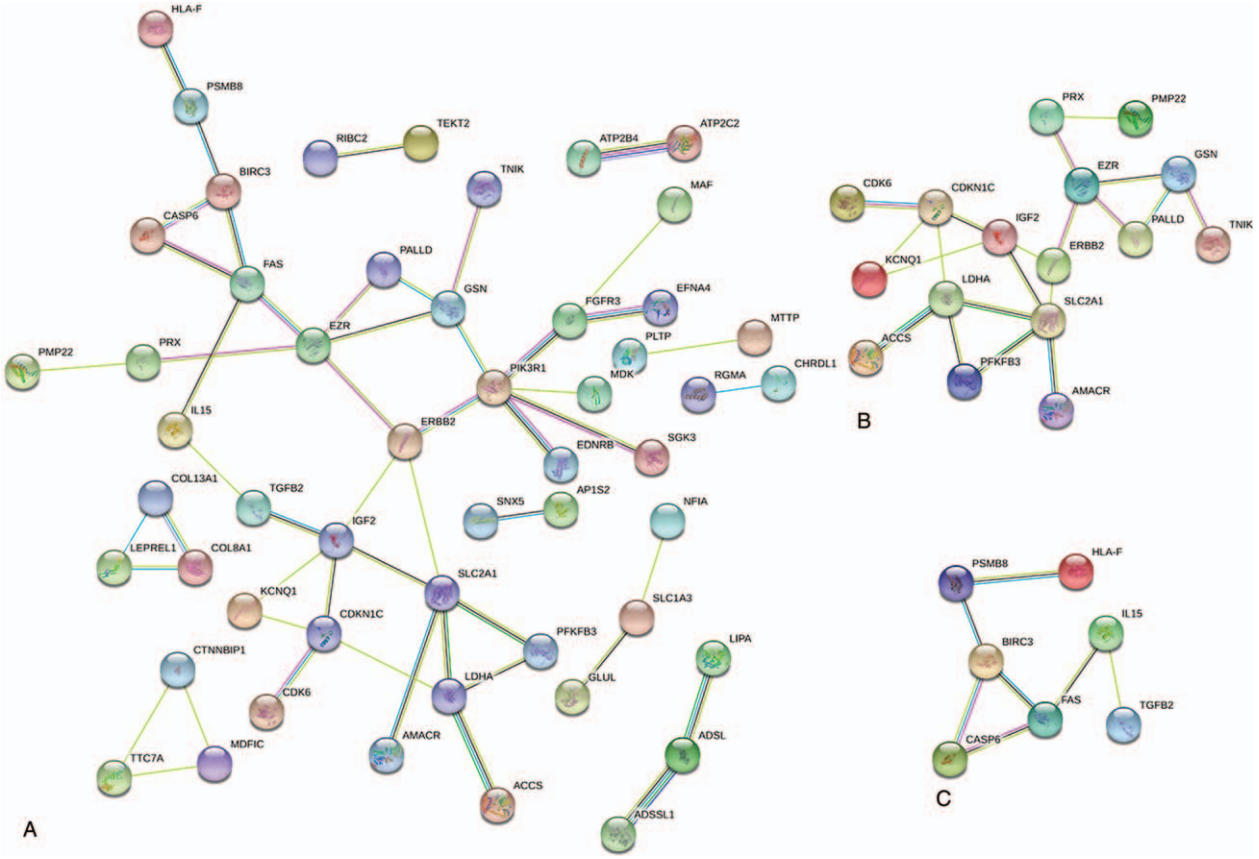

Figure 2. PPI network and top 2 modules of hypomethylation-high expression genes. PPI = protein–protein interaction.

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.[8] 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.[9] 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.[10] 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.[11] *HSP90B1* encodes HSP108, which belongs to a group of proteins induced under stress situations and is closely related with the retina. Infection by *ToxoplasmaE gondii* leads to a lower expression of the *HSP90B1*, indicating that HSP108 protein

Table 3

Modules analysis of PPI networks.

Module	Size	*P* value	Genes
Hypermethylation-low expression genes			
Module.1	13	.000618	*AEN RBM28 RRP1B REXO1 NOP56 C1D EXOSC6*
Module.2	14	.021168	*HSP90AA1 HSPE1 TIMM44 MRPL4 HSP90B1 HSPA1L PPID*
Hypomethylation-high expression genes			
Module.1	16	1.99E-06	*PRX PMP22 SLC2A1 ERBB2 IGF2 AMACR PFKFB3 LDHA CDKN1C ACCS TNIK GSN PALLD EZR KCNQ1 CDK6*
Module.2	7	.001718	*PSMB8 BIRC3 HLA-F TGFB2 IL15 CASP6 FAS*

PPI = protein–protein interaction.

Table 4

Gene ontology analysis for the modules of PPI network.

Category	Term	Count	%	*P* value
Hypermethylation-low expression genes				
Module.1				
GOTERM_BP_DIRECT	GO:0006364~rRNA processing	4	57.14286	3.97E-05
GOTERM_BP_DIRECT	GO:0090305~nucleic acid phosphodiester bond hydrolysis	2	28.57143	.026505
GOTERM_CC_DIRECT	GO:0005730~nucleolus	6	85.71429	1.31E-06
GOTERM_CC_DIRECT	GO:0000176~nuclear exosome (RNase complex)	2	28.57143	.004929
GOTERM_CC_DIRECT	GO:0005654~nucleoplasm	4	57.14286	.049634
GOTERM_MF_DIRECT	GO:0044822~poly (A) RNA binding	4	57.14286	.005119
GOTERM_MF_DIRECT	GO:0004527~exonuclease activity	2	28.57143	.006735
GOTERM_MF_DIRECT	GO:0003676~nucleic acid binding	3	42.85714	.0436
Module.2				
GOTERM_BP_DIRECT	GO:0006457~protein folding	4	57.14286	2.37E-05
GOTERM_BP_DIRECT	GO:0006986~response to unfolded protein	3	42.85714	9.1E-05
GOTERM_BP_DIRECT	GO:0042026~protein refolding	2	28.57143	.005349
GOTERM_BP_DIRECT	GO:0006950~response to stress	2	28.57143	.021602
GOTERM_BP_DIRECT	GO:1900034~regulation of cellular response to heat	2	28.57143	.026505
GOTERM_CC_DIRECT	GO:0005759~mitochondrial matrix	3	42.85714	.00459
GOTERM_CC_DIRECT	GO:0071682~endocytic vesicle lumen	2	28.57143	.005257
GOTERM_CC_DIRECT	GO:0042470~melanosome	2	28.57143	.0328
GOTERM_MF_DIRECT	GO:0051082~unfolded protein binding	4	57.14286	5.31E-06
GOTERM_MF_DIRECT	GO:0005524~ATP binding	5	71.42857	.000794
Hypomethylation-high expression genes				
Module.1				
GOTERM_BP_DIRECT	GO:0010628~positive regulation of gene expression	4	25	.001487
GOTERM_BP_DIRECT	GO:0001934~positive regulation of protein phosphorylation	3	18.75	.005587
GOTERM_BP_DIRECT	GO:0006349~regulation of gene expression by genetic imprinting	2	12.5	.014203
GOTERM_BP_DIRECT	GO:0030033~microvillus assembly	2	12.5	.015085
GOTERM_BP_DIRECT	GO:0007422~peripheral nervous system development	2	12.5	.021234
GOTERM_BP_DIRECT	GO:2000145~regulation of cell motility	2	12.5	.024732
GOTERM_BP_DIRECT	GO:0042552~myelination	2	12.5	.040329
GOTERM_BP_DIRECT	GO:0031532~actin cytoskeleton reorganization	2	12.5	.041188
GOTERM_BP_DIRECT	GO:0071320~cellular response to cAMP	2	12.5	.045476
GOTERM_BP_DIRECT	GO:0050680~negative regulation of epithelial cell proliferation	2	12.5	.048893
Module.2				
GOTERM_BP_DIRECT	GO:0033209~tumor necrosis factor-mediated signaling pathway	3	42.85714	.000721
GOTERM_BP_DIRECT	GO:0042981~regulation of apoptotic process	3	42.85714	.002323
GOTERM_BP_DIRECT	GO:0050778~positive regulation of immune response	2	28.57143	.003212
GOTERM_BP_DIRECT	GO:2001241~positive regulation of extrinsic apoptotic signaling pathway in absence of ligand	2	28.57143	.004993
GOTERM_BP_DIRECT	GO:0006955~immune response	3	42.85714	.008798
GOTERM_BP_DIRECT	GO:0097191~extrinsic apoptotic signaling pathway	2	28.57143	.014916
GOTERM_BP_DIRECT	GO:0006915~apoptotic process	3	42.85714	.015598
GOTERM_BP_DIRECT	GO:0002479~antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	2	28.57143	.022304
GOTERM_BP_DIRECT	GO:0060337~type I interferon signaling pathway	2	28.57143	.022655
GOTERM_BP_DIRECT	GO:0038061~NIK/NF-kappaB signaling	2	28.57143	.023356

AMP = adenosine mono phosphate, GO = gene ontology, PPI = protein–protein interaction.

provides 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.[13] However, there is no reference report the methylation of our selected hub genes in AMD; therefore, we further investigated the methylation statuses of *HSP90AA1*, *HSPA1L*, *HSPE1*, *HSP90B1* and *NOP56* in AMD patients' blood samples. In 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 processing in the endoplasmic reticulum (ER) and the estrogen signaling pathway might be involved in the development of AMD pathogenesis. The ER and oxidative stress can also activate systemic and local cascades directly implicated in AMD pathogenesis. For instance, C-reactive protein and serum amyloid P were 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,

Table 5

KEGG pathways results for the modules of PPI network.

Category	Term	Count	%	*P* value
Hypermethylation and low expression				
Module.1				
KEGG_PATHWAY	hsa03008:Ribosome biogenesis in eukaryotes	3	42.85714	9.25E-04
KEGG_PATHWAY	hsa03018:RNA degradation	2	28.57143	.043843
Module.2				
KEGG_PATHWAY	hsa04915:Estrogen signaling pathway	3	42.85714	6.04E-04
KEGG_PATHWAY	hsa04141:Protein processing in endoplasmic reticulum	3	42.85714	.001755
KEGG_PATHWAY	hsa04621:NOD-like receptor signaling pathway	2	28.57143	.023692
KEGG_PATHWAY	hsa04612:Antigen processing and presentation	2	28.57143	.032639
KEGG_PATHWAY	hsa05215:Prostate cancer	2	28.57143	.037726
Hypomethylation-high expression genes				
Module.1				
KEGG_PATHWAY	hsa04066:HIF-1 signaling pathway	3	18.75	.00832
KEGG_PATHWAY	hsa05205:Proteoglycans in cancer	3	18.75	.03219
Module.2				
KEGG_PATHWAY	hsa04210:Apoptosis	3	42.85714	.001161
KEGG_PATHWAY	hsa04668:TNF signaling pathway	3	42.85714	.003359
KEGG_PATHWAY	hsa05168:Herpes simplex infection	3	42.85714	.009755
KEGG_PATHWAY	hsa04060:Cytokine-cytokine receptor interaction	3	42.85714	.015145
KEGG_PATHWAY	hsa05166:HTLV-I infection	3	42.85714	.018581
KEGG_PATHWAY	hsa05332:Graft-versus-host disease	2	28.57143	.028324
KEGG_PATHWAY	hsa05330:Allograft rejection	2	28.57143	.031712
KEGG_PATHWAY	hsa04940:Type I diabetes mellitus	2	28.57143	.035932
KEGG_PATHWAY	hsa05200:Pathways in cancer	3	42.85714	.041548
KEGG_PATHWAY	hsa05320:Autoimmune thyroid disease	2	28.57143	.044327

HIF = hypoxia inducible factor, HTLV = human T lymphotrophic virus, KEGG = Kyoto Encyclopedia of Genes and Genomes, PPI = protein–protein interaction, TNF = tumor necrosis factor.

and proteins, including eicosapentaenoic acid, triamcinolone, 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.[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.[16] In mammalian cells, activated ERK1/2 enters the nuclei to regulate the activity of transcription factors to promote gene expression.[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.[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,[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.[20] According to Dunaief et al RPE, photoreceptors, and the inner nuclear layer cells in the retina undergo apoptotic cell death in human AMD,[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.[22] After the PPI network was constructed for hypomethylatioin-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, phosphoinositide-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.[23] Meanwhile, *SLC2A1* encodes a major glucose transporter at the blood–brain barrier in mammals.[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.[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

Table 6

Primers for MeDIP assay.

Gene name	Forward primer	Reverse primer
CDKN1C	ATGGTAGGTGGGCCAAAAGG	CAGCCCTTCCTGGTACAGAC
EZR	TCAGGTCTCTCCCGAAGGAA	CACAACCGTCAAGCCTTTGAG
IGF2	CCCTTGGAGAAAGGAGCTGG	GTGGGCCTTTCTCATTCCCA
PI3KR1	CTTTCACCCCTCCTTCACCC	CAGGCTTTTTCGAGCCCCTA
SLC2A1	GGAGAAGTCAATCCCTGGGC	GACTGGAGTTCTCACTCGGC
HSP90AA1	GGGTCTAGTTGACCGTTCCG	GCACCCTCAAGTTCACCTCA
HSP90B1	GCGAAAGTCGTGCTGGAATC	TATTCCGTGACCGGGGAAAC
HSPA1L	CTCAGGCTAGCCGTTATCCG	AATATTCCCGACCTGGCAGC
HSPE1	TCAAGGGTCAAATCGCGTCA	GACTCGGAGGCGGAAGAAAA
NOP56	GTTACGGGTGGGAGGAAAGG	CGTCCATCCCAGACAGAGAC

MeDIP = Methylated DNA immunoprecipitation.

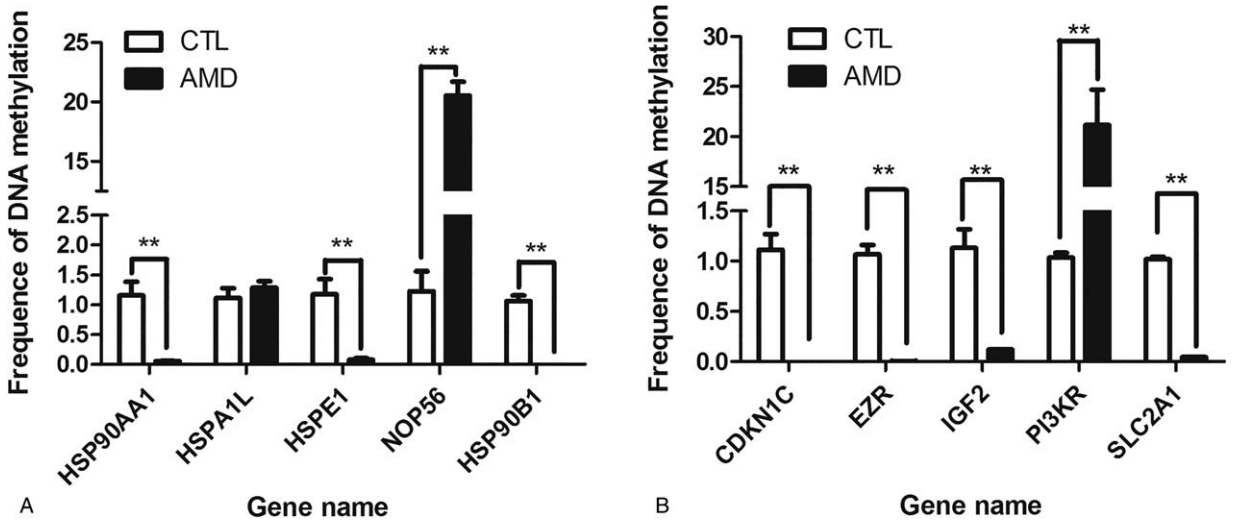

Figure 3. DNA methylation in promoter regions of hub genes in AMD. A. MeDIP-qPCR analysis of *HSP90AA1, HSPA1L, HSPE1, HSP90B1, NOP56*. Frequency of DNA methylation was calculated by the comparative threshold cycle (CT) method, $^{**}P < .05$. B. MeDIP-qPCR analysis of *PIK3R1, EZR, IGF2, SLC2A1, CDKN1C*. Frequency of DNA methylation was calculated by the comparative threshold cycle (CT) method, $^{**}P < .05$. AMD = age-related macular degeneration.

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*) activates the kinase PI3K of the downstream signaling pathway. *EZR*, 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.[28] 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 fits our prediction except *PI3KR1*. 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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