Cmah deficiency may lead to age-related hearing loss by influencing miRNA-PPAR mediated signaling pathway

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ABSTRACT

Background. Previous evidence has indicated CMP-Neu5Ac hydroxylase (Cmah) disruption inducesaging-related hearing loss (AHL). However, its function mechanisms remain unclear. This study was to explore the mechanisms of AHL by using microarray analysis in the Cmah deficiency animal model.

Methods. Microarray dataset GSE70659 was available from the Gene Expression Omnibus database, including cochlear tissues from wild-type and Cmah-null C57BL/6J mice with old age (12 months, $n = 3$). Differentially expressed genes (DEGs) were identified using the Linear Models for Microarray data method and a protein–protein interaction (PPI) network was constructed using data from the Search Tool for the Retrieval of Interacting Genes database followed by module analysis. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery. The upstream miRNAs and potential small-molecule drugs were predicted by miRwalk2.0 and Connectivity Map, respectively.

Results. A total of 799 DEGs (449 upregulated and 350 downregulated) were identified. Upregulated DEGs were involved in Cell adhesion molecules (ICAM1, intercellular adhesion molecule 1) and tumor necrosis factor (TNF) signaling pathway (FOS, FBJ osteosarcoma oncogene; ICAM1), while downregulated DEGs participated in PPAR signaling pathway (PPARG, peroxisome proliferator-activated receptor gamma). A PPI network was constructed, in which FOS, ICAM1 and PPARG were ranked as hub genes and PPARG was a transcription factor to regulate other target genes (ICAM1, FOS). Function analysis of two significant modules further demonstrated PPAR signaling pathway was especially important. Furthermore, mmu-miR-130b-3p, mmu-miR-27a-3p, mmu-miR-27b-3p and mmu-miR-721 were predicted to regulate PPARG. Topiramate were speculated to be a potential small-molecule drug to reverse DEGs in AHL.

Conclusions. PPAR mediated signaling pathway may be an important mechanism for AHL. Downregulation of the above miRNAs and use of topiramate may be potential treatment strategies for ALH by upregulating PPARG.

Subjects Bioinformatics, Geriatrics, Public Health

Keywords Cmah, Aging-related hearing loss, PPAR signaling pathway, Inflammation, miRNAs, Small-molecule drug

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INTRODUCTION

Hearing loss is the most common sensorineural deficit in the elderly, and it is estimated that 700 million persons have moderate to profound hearing loss worldwide in 2015, with approximately 30% of them occurred in their seventies and 50% in their eighties (*Niklaus, Dirk & Rudolf, 2011*; *Hjalte, Brännström & Gerdtham, 2012*; *Quaranta et al., 2015*). Age-related hearing loss (AHL) can lead to communication difficulties and cause social isolation, depression and anxiety, all of which severely influence the quality of life of patients (*Ciorba et al., 2012*). Furthermore, AHL is demonstrated to trigger cognitive function impairment in patients and thus may impose a large economic burden on families and society (*Peelle & Wingfield, 2016*). Thus, how to manage AHL has been an important public health issue.

Increasing evidence has indicated that oxidative stress is a crucial pathogenesis for AHL (*Fujimoto & Yamasoba, 2014*). Plasma reactive oxygen species (ROS) levels (i.e., hydrogen peroxide, hypochlorite and hydroxyl radicals) are observed to be significantly elevated (*Hwang et al., 2012*), while antioxidant retinol and zinc levels are significantly reduced in AHL patients (*Lasisi & Lasisi, 2015*). Linear regression reveals ROS and radical scavenger levels are positively and negatively associated with hearing thresholds of patients, respectively (*Hwang et al., 2012*; *Lasisi & Fehintola, 2011*). Furthermore, AHL animal model experiments also confirmed ROS excessively accumulated (*Riva et al., 2007*), but antioxidant enzymes [such as superoxide dismutase (SOD), reduced glutathione (GSH)/oxidized glutathione (GSSG)] strongly decreased in the cochlear spiral ganglion neurons and hair cells (*Coling et al., 2009*; *Menardo et al., 2012*). Even, AHL phenotype can be directly mimicked by selective knockout of SOD1 gene in mice (*Watanabe et al., 2014*). Oxidative stress may result in mitochondrial DNA mutations (*Markaryan, Nelson & Raul Hinojosa, 2009*; *Yamasoba et al., 2007*) and subsequently initiate BCl-2/Bax and caspase-3 mediated apoptotic pathways in the sensory cells and neurons of the cochlea (*Du et al., 2015*; *Huang et al., 2016*), which ultimately contribute to the development of hearing loss. Accordingly, supplementation of antioxidants (i.e., vitamin C, N-acetyl-cysteine) (*Ding et al., 2016*; *Kang et al., 2014*) or suppression of cell apoptosis of (i.e., caloric restriction, Erlong Zuoci decoction) (*Dong et al., 2016*; *Someya et al., 2010a*) may be underlying strategies to delay the onset of AHL and prevent pathological damages in the cochlea. However, the mechanisms of AHL remain not completely understood and current preventative or therapeutic interventions have not been universally acknowledged. Thereby, there is still a need to investigate the etiology of AHL to develop potential approaches for intervention of AHL.

CMP-Neu5Ac hydroxylase (Cmah) is an enzyme to catalyze the hydroxylation of N-acetylneuraminic acid (Neu5Ac) to N-glycoloylneuraminic acid (Neu5Gc). Neu5Gc is an important sialic acid and thus may play an important role for maintaining the structural and function of auditory system (*Go, Yoshikawa & Inokuchi, 2011*; *Inokuchi et al., 2017*). Cmah-deficient mice are shown to exhibit reduced hearing sensitivity in old age, accompanied with loss of sensory hair cells, spiral ganglion neurons, and/or stria vascularis degeneration throughout the cochlea (*Kwon et al., 2015*; *Hedlund et al., 2007*). These studies indicate mice with Cmah-null can act as a model for studying the mechanisms

of AHL in humans, which had been used in the study of *Kwon et al. (2015)*. Based on a high throughput microarray analysis technology, *Kwon et al. (2015)* demonstrated there were 631 up-regulated and 729 down-regulated genes in Cmah-null mice-derived cochlear tissues compared to control mice-derived cochlear tissues. Function enrichment analysis and PCR validation suggsted downregulated sirtuin deacetylase 3 (Sirt3), a mitochondrial NAD+-dependent deacetylase, may be involved in AHL via decreasing the expression of Fox1 and then promoting the production of ROS (*Kwon et al., 2015*). The key roles of Sirt3 for the development of AHL were also proved in the studies of other scholars (*Someya et al., 2010b*; *Zeng et al., 2014*). Therefore, manipulation of Sirt3 expression might represent a new approach to combat AHL. However, the use of Cmah-null mice to investigate the mechanism of AHL remains rarely reported.

The present study aimed to screen more crucial genes for explaining the mechanisms of AHL by re-analyzing the microarray data of *Kwon et al. (2015)* through addition of network-related bioinformatics algorithms. In addition, small molecule drugs were also predicted in order to find potential treatments for AHL.

MATERIAL AND METHODS

Microarray data

The microarray data under accession number GSE70659 were collected from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) (*Kwon et al., 2015*) (Supplemental Information 1), which contained cochlear tissues from wild-type (WT, $n = 3$) and Cmah-null ($n = 3$) C57BL/6J mice with old age (12 months).

Data normalization and DEGs identification

The raw data (CEL files) downloaded from the Illumina MouseRef-8 v2.0 expression beadchip platform GPL6885 were preprocessed (including background adjustment, log2 transformation and quantile normalization) using the lumiR package in R (*R Core Team, 2017*). The DEGs between WT and Cmah-null mice were identified using the Linear Models for Microarray data (LIMMA) method (*Smyth, 2005*) in the Bioconductor R package (http://www.bioconductor.org/packages/release/bioc/html/limma.html). After the t-test, and the p-value was multiple corrected with the Benjamini–Hochberg (BH) procedure (*Benjamini & Hochberg, 1995*). Genes were considered to be significantly differentially at $p < 0.05$ and |logFC(fold change)| >0.5 due to the poor BH-adjusted p-value.

Protein–protein interaction (PPI) network construction

The PPI pairs were downloaded from acknowledged STRING 10.0 (Search Tool for the Retrieval of Interacting Genes; https://string-db.org/) database (*Szklarczyk et al., 2015*) and then the DEGs were imported into the PPI data to obtain the whole PPI network. The PPIs with combined scores >0.4 were selected to construct the PPI network which was visualized using the Cytoscape software (version 2.8; http://www.cytoscape.org/) (*Kohl, Wiese & Warscheid, 2011*). The crucial nodes within the PPI network were analyzed based on three topological properties using the CytoNCA plugin in Cytoscape software (http://apps.cytoscape.org/apps/cytonca) (*Tang et al., 2015*), including degree [the number

of interactions per node (protein)], betweenness (the number of shortest paths that pass through each node) and closeness centrality (the average length of the shortest paths to access all other proteins in the network). Functionally related and densely interconnected clusters were extracted from the large PPI network using the Molecular Complex Detection (MCODE) plugin of Cytoscape software according to the following parameters: degree cutoff = 5; node score cutoff = 0.4; k-core = 5; and maximum depth = 100 (ftp://ftp.mshri.on.ca/pub/BIND/Tools/MCODE) (*Bader & Hogue, 2003*). Modules were considered significant with MCODE score ≥ 4 and nodes ≥ 6.

Furthermore, whether the DEGs were transcription factors (TFs) and the TF-target gene interactions were predicted by the TRANSFAC database (http://www.gene-regulation.com/pub/databases.html) (*Matys et al., 2006*), and then were integrated into the PPI network to establish a regulatory network.

Function enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed to investigate the underlying functions of all DEGs and DEGs in the network using The Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (version 6.8; http://david.abcc.ncifcrf.gov) (*Huang, Sherman & Lempicki, 2009*). P-value <0.05 was set as the cut-off value.

miRNA-target gene regulatory network construction

The miRNAs that can regulate the DEGs in the PPI network were predicted using the miRWalk database (version 2.0; http://www.zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2) with default significant parameters. Only the interaction relationships can be predicted by nine common algorithms, including miRWalk, Microt4, miRanda, miRDB, miRMap, miRNAMap, RNA22, RNAhybrid and Targetscan, were included to construct the miRNA-target gene regulatory network using the Cytoscape software (*Kohl, Wiese & Warscheid, 2011*).

Screening of small-molecule drugs for treatment of AHL

The name of DEGs identified in the PPI network were converted to HG-U133A probe set IDs and uploaded to the Connectivity Map (CMAP, http://www.broadinstitute.org/cmap/) database which is a collection of genome-wide transcriptional expression data from human cancer cell lines treated with bioactive small molecules. If the enrichment score was close to –1, the corresponding small molecules were the potential drugs to reverse the expression of the query signature. Significant small-molecule drugs were selected according to the threshold value of $p < 0.05$ and |mean| >0.4.

RESULTS

Identification of DEGs

After data normalization, 799 genes were identified as DEGs between WT and Cmah-null mice based on the threshold of $p < 0.05$ and |logFC| >0.5, including 449 upregulated (such as Fos, FBJ osteosarcoma oncogene) and 350 downregulated genes (such as Ucp1, uncoupling

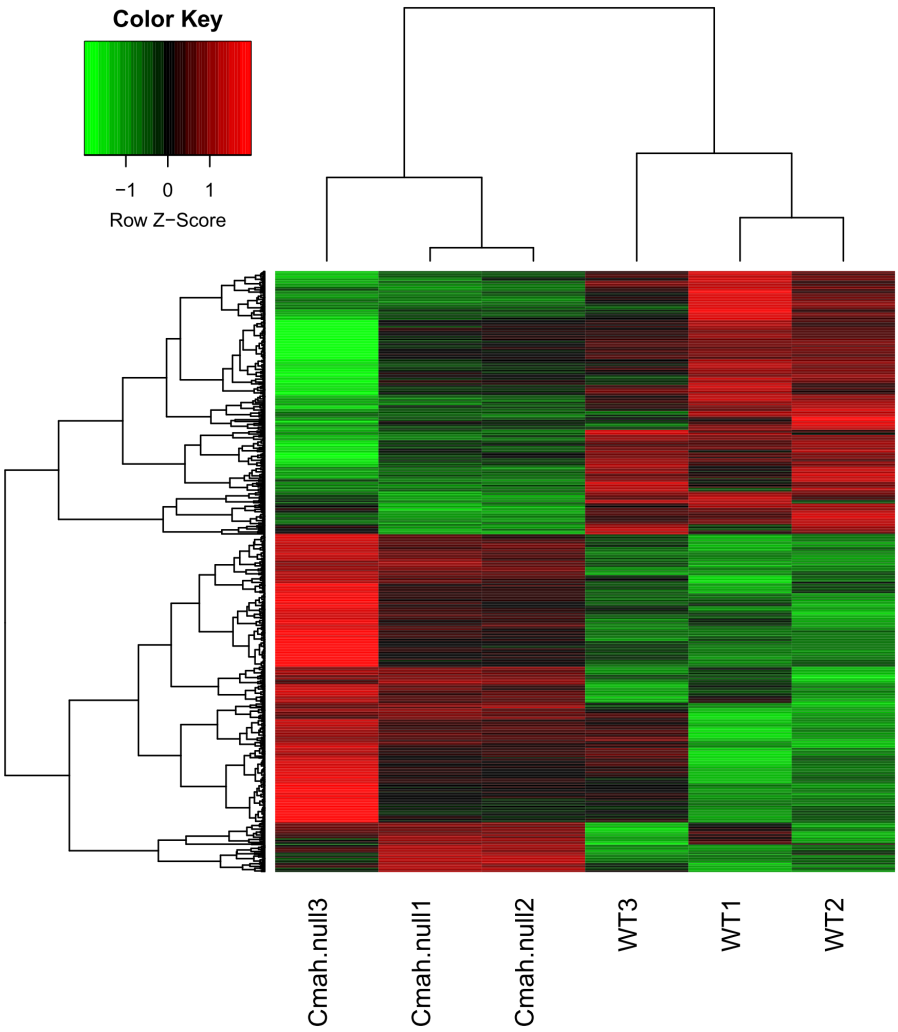

Figure 1 **Heat map of differentially expressed genes between Cmah-null and wild-type mice.**
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protein 1 (mitochondrial, proton carrier); Acadm, acyl-Coenzyme A dehydrogenase, medium chain). All the DEGs are listed in Supplemental Information 2. As shown in Fig. 1, heat map illustrated that the expression patterns of genes were obviously altered in Cmah-knockout mice compared with control.

Function enrichment analysis of DEGs

The above differential genes were subjected to the DAVID for function enrichment analysis. As a result, 16 KEGG pathways were enriched for upregulated DEGs, including ECM-receptor interaction (LAMA1, laminin, alpha 1; ITGA5, integrin subunit alpha 5), Cell adhesion molecules (ICAM1, intercellular adhesion molecule 1; ITGA5), Focal adhesion (LAMA1; ITGA5), Cytokine-cytokine receptor interaction (TNFSF13B, TNF superfamily member 13b) and TNF signaling pathway (FOS; ICAM1); while 20 KEGG pathways were for downregulated DEGs, including Oxidative phosphorylation (UQCRC2,

ubiquinol cytochrome c reductase core protein 2), metabolism related and PPAR signaling pathway (ACADM; PPARG, Peroxisome proliferator-activated receptor gamma; UCP1; SCD1, stearoyl-Coenzyme A desaturase 1) (Table 1).

PPI network construction

After mapping the DEGs into the protein interactions downloaded from the STRING database, a PPI network was constructed (Fig. 2) which included 587 nodes (303 upregulated and 284 downregulated; 52 TFs) and 2,944 edges (interaction relationships) (Supplemental Information 3). UQCRC2, SOD2 (superoxide dismutase 2), FOS, ICAM1 and PPARG were suggested to be hub genes by calculating the degree, betweenness, and closeness centrality of nodes in the PPI network. Among them, FOS and PPARG were TFs (Table 2) and they both interacted with ICAM1. Furthermore, PPARG also could interact with FOS and SOD2.

After cluster analysis according to the given parameters, two significant modules were obtained (Table 3). Function enrichment analysis showed that the genes in module 1 (Fig. 3) were closely related to Oxidative phosphorylation (UQCRC2), while the genes in module 2 (Fig. 4) were significantly enriched in metabolism related and PPAR signaling pathway (ACADM) (Table 4).

miRNA–target gene regulatory network analysis

Using the miRWalk2.0 database, 82 DEGs were predicted to be regulated by 193 miRNAs in 9 databases (Supplemental Information 4). Then, 166 interaction relationships between 43 upregulated DEGs and 117 miRNAs as well as 182 interaction relationships between 39 downregulated DEGs and 76 miRNAs were used for constructing the upregulated (Fig. 5) and downregulated (Fig. 6) miRNA-mRNA regulatory networks, respectively. As shown in Fig. 5, FOS can be regulated by mmu-miR-221-3p or mmu-miR-222-3p, while PPARG can be regulated by mmu-miR-130b-3p, mmu-miR-27a-3p, mmu-miR-27b-3p and mmu-miR-721 in Fig. 6.

Small-molecule drugs

The DEGs in the PPI network were uploaded into CMAP database to obtain the small-molecule drugs. As a result, 69 small-molecule chemicals with negative mean and enrichment scores were predicted, such as adiphenine, DL-PPMP, decitabine and topiramate. This finding indicated their potential ability to inhibit the development of AHL (Table 5).

DISCUSSION

In the present study, Cmah-null mice were used as an animal model to investigate the underlying mechanisms of AHL. In line with the study of Kwon et al. (*Kwon et al., 2015*), oxidative phosphorylation pathway was enriched in the DEGs between Cmah-null mice and WT, and SOD2, SIRT3 were crucial genes in PPI network (Table 3), further demonstrating the oxidative stress pathogenesis of AHL (*Fujimoto & Yamasoba, 2014*). In addition, our current study also found ECM-receptor interaction (LAMA1), adhesion (ICAM1),

Table 1 KEGG pathways for differentially expressed genes in the PPI network.

	Term	*P*-value	Genes
UP	mmu04640:Hematopoietic cell lineage	1.89E−05	CD37, GP5, GP1BB, CD3E, ITGA5, CSF1, H2-EB1, ANPEP, ITGA3, ITGA2B…
	mmu04512:ECM-receptor interaction	2.85E−05	VWF, LAMA1, CD47, LAMB3, GP5, GP1BB, ITGA5, ITGB4, ITGA3, ITGA2B…
	mmu05164:Influenza A	0.001774	ICAM1, MYD88, HSPA2, SOCS3, IRF7, H2-EB1, PML, H2-AB1, OAS2, CCL5…
	mmu05168:Herpes simplex infection	0.002779	FOS, MYD88, SOCS3, IRF7, H2-EB1, PML, PER2, PER1, H2-AB1, OAS2…
	mmu05150:Staphylococcus aureus infection	0.005378	ICAM1, SELP, C4B, H2-EB1, H2-AB1, SELPLG
	mmu04510:Focal adhesion	0.007604	VWF, LAMA1, LAMB3, ITGA5, RASGRF1, PAK4, ITGB4, ITGA3, ZYX, MYL12A…
	mmu05217:Basal cell carcinoma	0.008061	BMP4, WNT10A, WNT7B, WNT4, WNT3A, WNT6
	mmu05200:Pathways in cancer	0.008918	BMP4, WNT10A, RALBP1, WNT3A, PML, FGF10, FOXO1, ITGA3, LAMA1, FOS…
	mmu05323:Rheumatoid arthritis	0.010803	FOS, ICAM1, TNFSF13B, CSF1, H2-EB1, H2-AB1, CCL5
	mmu04060:Cytokine-cytokine receptor interaction	0.024171	OSM, CXCL14, TNFSF13B, PRLR, CSF1, CXCR1, CXCR2, CX3CL1, CCL5, BMP7…
	mmu04151:PI3K-Akt signaling pathway	0.029269	EFNA1, CSF1, ITGB4, FGF10, ITGA3, CHAD, OSM, VWF, LAMA1, LAMB3…
	mmu04611:Platelet activation	0.02988	VWF, ORAI1, GP5, GP1BB, PLCG2, MYL12A, ITPR3, ITGA2B
	mmu04514:Cell adhesion molecules (CAMs)	0.031608	ICAM1, SIGLEC1, SELP, CLDN4, CLDN3, H2-EB1, H2-AB1, SELPLG, CLDN23
	mmu04668:TNF signaling pathway	0.037976	FOS, ICAM1, SOCS3, CSF1, CX3CL1, CCL5, JUNB
	mmu04550:Signaling pathways regulating pluripotency of stem cells	0.038071	BMP4, WNT10A, WNT7B, WNT4, OTX1, WNT3A, WNT6, MEIS1
	mmu05205:Proteoglycans in cancer	0.042265	WNT10A, WNT7B, WNT4, TIAM1, ITGA5, WNT3A, PLCG2, ITPR3, WNT6, TWIST2
Down	mmu00190:Oxidative phosphorylation	6.68E−28	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu05012:Parkinson's disease	1.48E−25	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu05016:Huntington's disease	2.16E−25	UQCRC2, NDUFB3, POLR2G, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, PPARG, COX7B…
	mmu01100:Metabolic pathways	1.11E−23	UQCRC2, ATP5E, GNPDA2, CYC1, PDHB, CMBL, UQCR10, UQCR11, NDUFS4, IDH3G, MCEE…
	mmu05010:Alzheimer's disease	8.60E−22	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu04932:Non-alcoholic fatty liver disease (NAFLD)	2.29E−21	UQCRC2, NDUFB3, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2, UQCR10…
	mmu01200:Carbon metabolism	2.99E−11	ALDH6A1, ACADM, ACO2, SUCLG1, ECHS1, FBP2, ACAT2, PDHB, SDHB, TPI1…
	mmu01130:Biosynthesis of antibiotics	2.59E−10	ACAA2, ACADM, ACO2, SUCLG1, ECHS1, AK2, FBP2, ACAT2, PDHB, CMBL…

(*continued on next page*)

Table 1 (*continued*)

Term	*P*-value	Genes
mmu00071:Fatty acid degradation	1.21E−09	ECI1, ECI2, ACAA2, ACADSB, CPT2, ACADM, ECHS1, ACADL, ACAT2, HADHB…
mmu00020:Citrate cycle (TCA cycle)	2.22E−09	SDHB, IDH3G, ACO2, SUCLG1, DLD, SDHD, IDH2, IDH1, PDHA1, FH1…
mmu00280:Valine, leucine and isoleucine degradation	5.07E−09	ACAA2, ALDH6A1, ACADSB, ACADM, ECHS1, ACAT2, HADHB, DBT, MCEE, DLD…
mmu01212:Fatty acid metabolism	2.57E−08	ACADVL, SCD1, ACAA2, ACADSB, ACSL1, ACADM, CPT2, ECHS1, ACADL, ACAT2…
mmu00640:Propanoate metabolism	2.58E−06	ALDH6A1, ACADM, SUCLG1, MCEE, ECHS1, ACAT2, PCCB, PCCA
mmu04260:Cardiac muscle contraction	1.47E−05	UQCRC2, UQCR10, CACNA2D1, UQCR11, COX8B, COX7A1, UQCRH, COX7B, CYC1, COX6B1…
mmu03320:PPAR signaling pathway	2.07E−05	SCD1, ACSL1, ACADM, CPT2, PPARG, FABP3, AQP7, UCP1, ACADL, ACSL5…
mmu04146:Peroxisome	1.66E−04	ECI2, ACSL1, ECH1, NUDT7, ABCD2, IDH2, IDH1, SCP2, SOD2, ACSL5
mmu00630:Glyoxylate and dicarboxylate metabolism	5.85E−04	ACO2, MCEE, DLD, ACAT2, PCCB, PCCA
mmu01210:2-Oxocarboxylic acid metabolism	0.010183	IDH3G, ACO2, IDH2, IDH1
mmu00620:Pyruvate metabolism	0.014154	DLD, PDHA1, FH1, ACAT2, PDHB
mmu00310:Lysine degradation	0.036609	EHMT1, HYKK, ECHS1, ACAT2, NSD1

Notes.
PPI, protein and protein interaction; KEGG, Kyoto Encyclopedia of Genes and Genomes.

inflammation (FOS, ICAM1, TNFSF13B) and PPAR signaling pathways (PPARG). Among them, PPAR signaling pathway may be especially important because the following causes: (1) this pathway was enriched for the genes in PPI and significant modules; (2) PPARG was a TF; and (3) PPARG could interact with SOD2, FOS and ICAM1. Furthermore, we predicted PPARG can be regulated by mmu-miR-130b-3p, mmu-miR-27a-3p, mmu-miR-27b-3p and mmu-miR-721. Also, adiphenine, DL-PPMP, decitabine and topiramate were speculated to be potential small-molecule drugs to reverse the expression of PPARG in AHL. Accordingly, we hypothesize downregulated PPARG may be involved in AHL by influencing adhesion, inflammation and oxidative stress, while downregulation of the above miRNAs and the use of the above small-molecule drugs may be potential treatment strategies for AHL by upregulating PPARG.

The extracellular matrix (ECM), the non-cellular component throughout all tissues and organs, and adherens junctions between cells and ECM are essential for maintenance of the structural and functional integrity of organs. ECM (i.e., laminin, integrin, fibronectin or collagen) and adherens (i.e., cadherin, syndecan-1, tenascin-C, Connexin or Icam) molecules are suggested to play a vital role for the growth and proliferation of cochlear sensorineural epithelial cells and sensory cell synaptogenesis (*Evans et al., 2007*; *Toyama et al., 2005*; *Wang, Hu & Yang, 2015*). The expression changes in the above molecules may cause hearing loss. For example, *Suzuki et al. (2005)* found type IX collagen knockout mice exhibited abnormal integrity of collagen fibers in the tectorial membrane and showed progressive hearing loss by auditory brainstem response assessment. *Gottesberge et al. (2008)* demonstrated deletion of the discoidin domain receptor 1 (DDR1) in mouse, a tyrosine

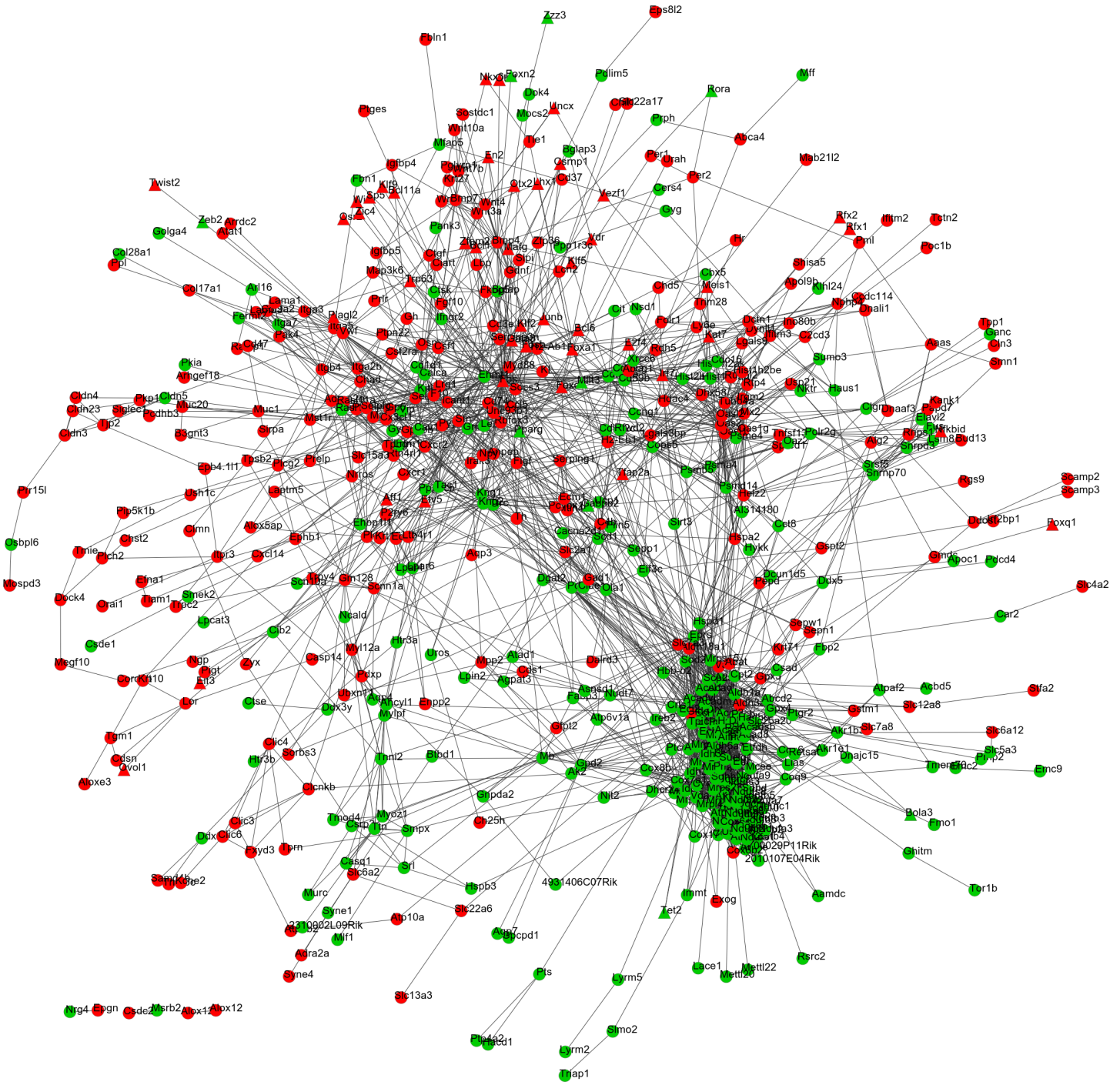

Figure 2 **The protein–protein interaction network.** The red and green nodes represent the upregulated and downregulated genes, respectively. Triangle, transcription factors; circular, mRNA.

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Table 2 Hub genes in the protein–protein interaction network.

Gene_Symbol	Degree	Gene_Symbol	Betweenness	Gene_Symbol	Closeness
Uqcrc2	57	Ehmt1	17581.75493	Sod2	0.000195886
Sdhb	55	Fos	16050.50737	Eprs	0.000194932
Atp5 h	53	Icam1	9986.745004	Fos	0.000194515
Ndufs3	52	Sod2	9941.869934	Icam1	0.000193573
Uqcr11	48	Eprs	9124.185173	Pdk4	0.000192976
Ndufs2	48	Pdk4	7466.1032	Pparg	0.000192827
Ehmt1	47	Cav1	7458.835522	Dld	0.000192641
Dld	47	Helz2	7173.879054	Aldh18a1	0.000192345
Cyc1	47	Cops5	6791.408479	Tpi1	0.000191939
Pmpcb	46	Dld	6500.567441	Foxo1	0.000191608
Ndufa9	46	Gm128	6201.287809	Ehmt1	0.000191168
Fos	46	Tpi1	6150.610424	Socs3	0.000190949
Suclg1	45	Pparg	6092.823564	Hspd1	0.000190767
Ndufv2	44	Aldh18a1	5723.478434	Aldh3a1	0.000190404
Ndufa8	44	Tac1	5073.412043	Aldh1a7	0.000190259
Ndufb5	43	Gldc	4915.109074	Sirt3	0.00019015
Acadvl	43	Acadvl	4870.244131	Helz2	0.000190042
Uqcr10	42	Mb	4867.912647	Ccng1	0.00018997
Ndufa5	42	Bmp4	4710.157376	Acadvl	0.000189934
Uqcrh	41	Foxo1	4510.416535	Dbt	0.000189934

Table 3 Significant modules screened from the protein–protein interaction network.

Cluster	Score (Density*#Nodes)	Nodes	Edges	Node IDs
1	15.692	39	612	Atp5j2, Ndufv2, Ndufs8, Ndufs2, Sdhb, Sdhd, Ndufs4, Ndufs3, Ndufa8, Ndufc2, Uqcrc2, Cyc1, Etfb, Atp5 h, Atp5e, Uqcr10, Uqcr11, Pmpcb, Ndufb5, Ndufb9, Ndufa9, Ndufa5, Suclg1, Ndufb8, Ndufb4, Ndufb2, Uqcrh, Cox6c, Ndufc1, Ndufa1, 1700029P11Rik, Ndufa7, Ndufa3, Ndufb3, Atp5f1, Usmg5, Cycs, Cox6b1, Cox7b
2	6.241	58	362	Cpt2, Slc25a20, Ech1, Acadl, Acsl1, Aldh1a7, Aldh6a1, Aldh3a1, Acsl5, Acat2, Acadsb, Eci1, Echs1, Idh1, Dbt, Acadvl, Hadhb, Eci2, Acaa2, Acadm, Etfdh, Pcca, Pccb, Etfa, Dld, Aco2, Idh3g, Hspe1, Mrps15, Helz2, Irgm2, Rtp4, Mx2, Lgals3bp, Figf, Pcyox1l, Ola1, Ecm1, Sepp1, Serping1, Itih4, Kng2, Kng1, Mrpl20, Mrps28, Ptcd3, Mrpl30, Mrpl42, Mrpl53, Mrpl9, Mrpl27, Mrpl12, Dhx58, Oasl2, Oas2, Oasl1, Irf7, Fh1

kinase receptor activated by native collagen, induced deterioration of the supporting cells and consequently interfere with mechanical properties of the organ of Corti, leading to a severe decrease in auditory function. *Cai et al. (2012)* proved that exposure to an intense noise for 2 h caused site-specific changes in expression levels of genes from adhesion families in the apical (upregulated: Sell, Thbs1, Itgae, Icam1, and Itga5) and the basal (upregulated: Itga3, Itgb2, Selp, Sele, Cdh1, and Cdh2) sections of the sensory epithelium

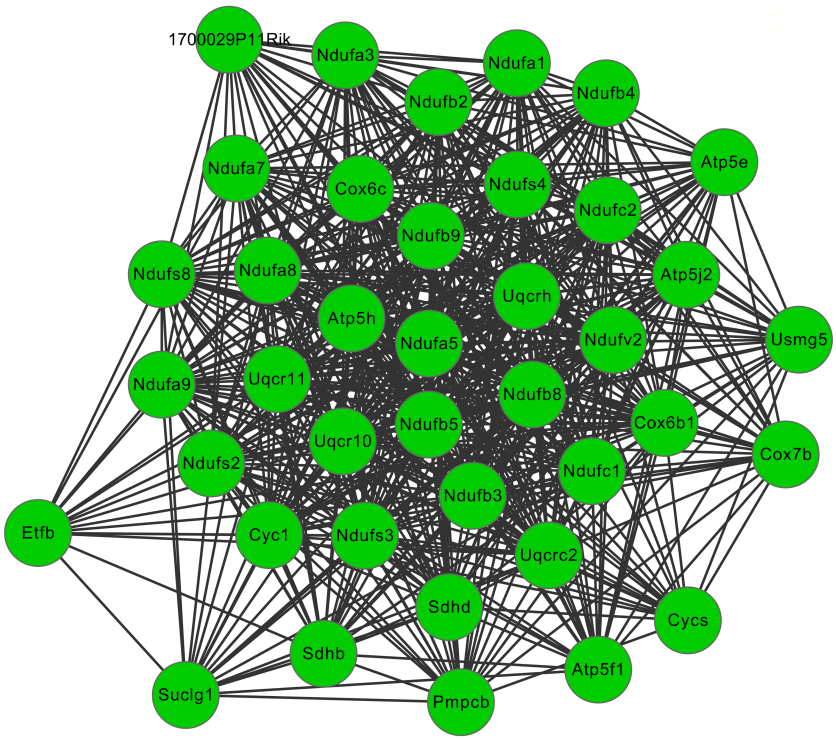

Figure 3 **The significant module 1 extracted from the protein–protein interaction network.** The green nodes represent downregulated genes.

Full-size DOI: 10.7717/peerj.6856/fig-3

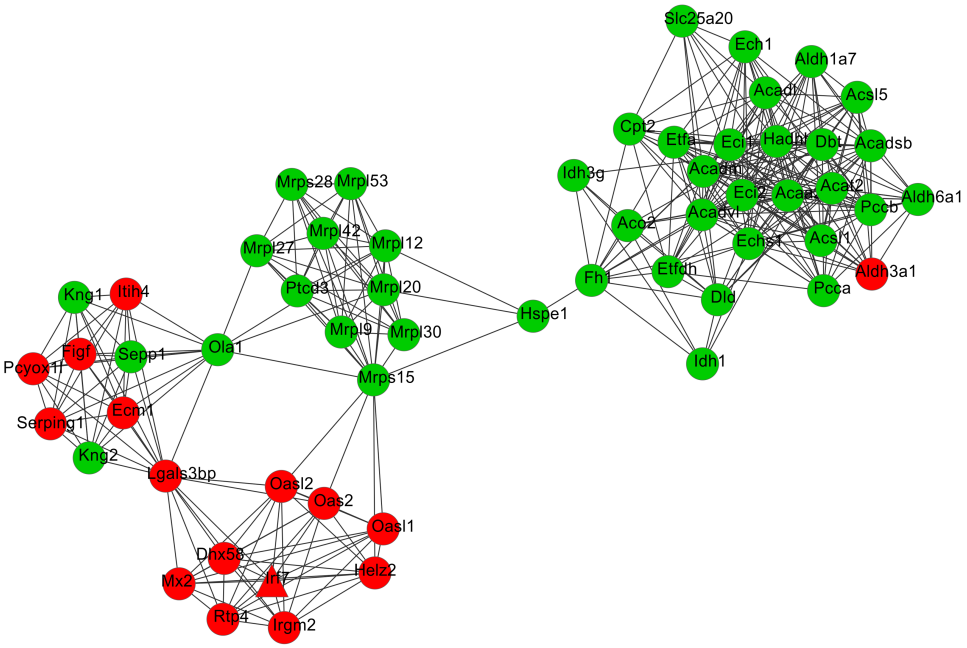

Figure 4 **The most significant module 2 extracted from the protein–protein interaction network.** The red and green nodes represent the upregulated and downregulated genes, respectively.

Full-size DOI: 10.7717/peerj.6856/fig-4

Table 4 KEGG pathways for differentially expressed genes in module analysis.

Module	Term	*P*-value	Genes
1	mmu00190:Oxidative phosphorylation	1.80E−55	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu05012:Parkinson's disease	2.20E−54	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu05010:Alzheimer's disease	9.89E−52	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu05016:Huntington's disease	4.97E−50	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu04932:Non-alcoholic fatty liver disease (NAFLD)	1.49E−45	UQCRC2, NDUFB3, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2, UQCR10…
	mmu01100:Metabolic pathways	1.92E−27	UQCRC2, NDUFB3, ATP5E, NDUFB4, NDUFB5, NDUFB8, NDUFB9, COX7B, CYC1, NDUFB2…
	mmu04260:Cardiac muscle contraction	3.23E−08	UQCRC2, UQCR10, UQCR11, UQCRH, COX7B, CYC1, COX6B1, COX6C
	mmu00020:Citrate cycle (TCA cycle)	0.008597	SDHB, SUCLG1, SDHD
2	mmu00071:Fatty acid degradation	1.63E−18	ECI1, ECI2, ACAA2, ACADSB, CPT2, ACADM, ECHS1, ACADL, ACAT2, HADHB…
	mmu01212:Fatty acid metabolism	1.89E−14	ACADVL, ACAA2, ACADSB, ACSL1, ACADM, CPT2, ECHS1, ACADL, ACAT2, ACSL5…
	mmu00280:Valine, leucine and isoleucine degradation	4.29E−14	ACAA2, ALDH6A1, DBT, ACADSB, ACADM, DLD, ECHS1, ACAT2, PCCB, PCCA…
	mmu01200:Carbon metabolism	9.82E−11	ALDH6A1, ACADM, IDH3G, ACO2, DLD, ECHS1, IDH1, FH1, ACAT2, PCCB, PCCA
	mmu01130:Biosynthesis of antibiotics	1.52E−10	ACAA2, ACADM, ACO2, ECHS1, ACAT2, HADHB, DBT, IDH3G, DLD, IDH1, FH1, PCCB, PCCA
	mmu00640:Propanoate metabolism	1.43E−07	ALDH6A1, ACADM, ECHS1, ACAT2, PCCB, PCCA
	mmu01100:Metabolic pathways	1.91E−07	ACAA2, ALDH6A1, ACADSB, ACADM, ACO2, ECHS1, ACADL, ACAT2, ALDH3A1, HADHB…
	mmu00630:Glyoxylate and dicarboxylate metabolism	9.74E−06	ACO2, DLD, ACAT2, PCCB, PCCA
	mmu00020:Citrate cycle (TCA cycle)	1.46E−05	IDH3G, ACO2, DLD, IDH1, FH1
	mmu00410:beta-Alanine metabolism	5.01E−04	ALDH6A1, ACADM, ECHS1, ALDH3A1
	mmu03320:PPAR signaling pathway	5.45E−04	ACSL1, ACADM, CPT2, ACADL, ACSL5
	mmu03010:Ribosome	5.86E−04	MRPL12, MRPS15, MRPL27, MRPL9, MRPL30, MRPL20
	mmu04146:Peroxisome	6.26E−04	ECI2, ACSL1, ECH1, IDH1, ACSL5
	mmu01210:2-Oxocarboxylic acid metabolism	0.003631	IDH3G, ACO2, IDH1
	mmu00062:Fatty acid elongation	0.006757	ACAA2, ECHS1, HADHB
	mmu00620:Pyruvate metabolism	0.014816	DLD, FH1, ACAT2

Notes.
KEGG, Kyoto Encyclopedia of Genes and Genomes.

in the cochlea. Selp and Itga5 in the basal section were positively, but Sell in the apical section was negatively correlated with greater hearing loss. In line with the above findings, our present study also identified several ECM and adherens genes to be differentially expressed, with the upregulation of LAMA1, ICAM1 and ITGA5, suggesting these genes may be underlying targets for treatment of AHL. Our hypothesis had been preliminarily demonstrated in the study of *Ramunni et al. (2006)* who found inhibition of adhesion

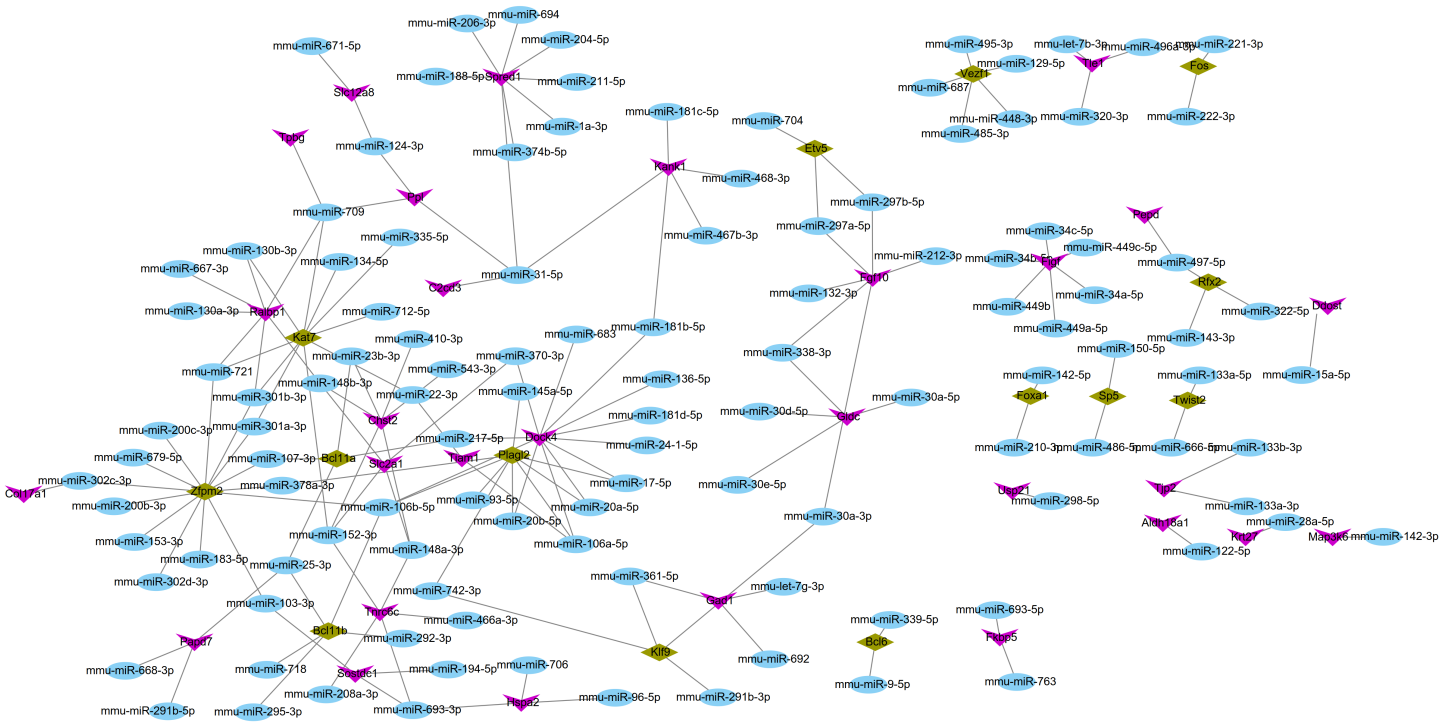

Figure 5 **The miRNA-mRNA regulatory network for the upregulated genes.** Blue: miRNAs; purple: upregulated genes; hazel-green: upregulated transcription factors.

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molecules (sE-selectin, sVCAM-1 and sICAM-1) by a single session of LDL/fibrinogen apheresis led to a complete hearing recovery.

In addition, the upregulation of adherens genes may favor the interaction between leukocytes and inner ear endothelial cells, promoting the inflammation and hearing loss (*Kanzaki et al., 2014*; *Ramunni et al., 2006*), indicating inflammation related pathways may also be a target for AHL. In accordance with our expected, TNF signaling pathway and Cytokine-cytokine receptor interaction pathways were also significantly enriched for upregulated genes (TNFSF13B; FOS; ICAM1) in this study. It had been reported that TNF-α and its receptors (TNFR1, TNFR2) were higher expressed in the cochlea of vibration- or noise-induced hearing loss (*Fuentessantamaría et al., 2017*; *Zou et al., 2005*). Use of TNF-α inhibitor preserved the hearing threshold by improvement of cochlear blood flow (*Arpornchayanon et al., 2013*). The expression of FOS was found to be dynamically changed after deafness, with lower level in the auditory cortex 15 days (compensation mechanism), but increased from 2 weeks and stabilized three months after permanent auditory deprivation in adult rats (*Pernia et al., 2017*). As a TF, FOS may participate in inflammation by regulating its target genes, such as ICAM-1, CSF1 and CCL5 which were all important inflammatory proteins for hearing loss (*Trune et al., 2015*).

PPARs are ligand-activated TFs belonging to the nuclear receptor superfamily. Extensive studies have shown that PPAR participates in various biological functions such as cell proliferation, apoptosis and differentiation by regulating its target genes (*Chung et al.,*

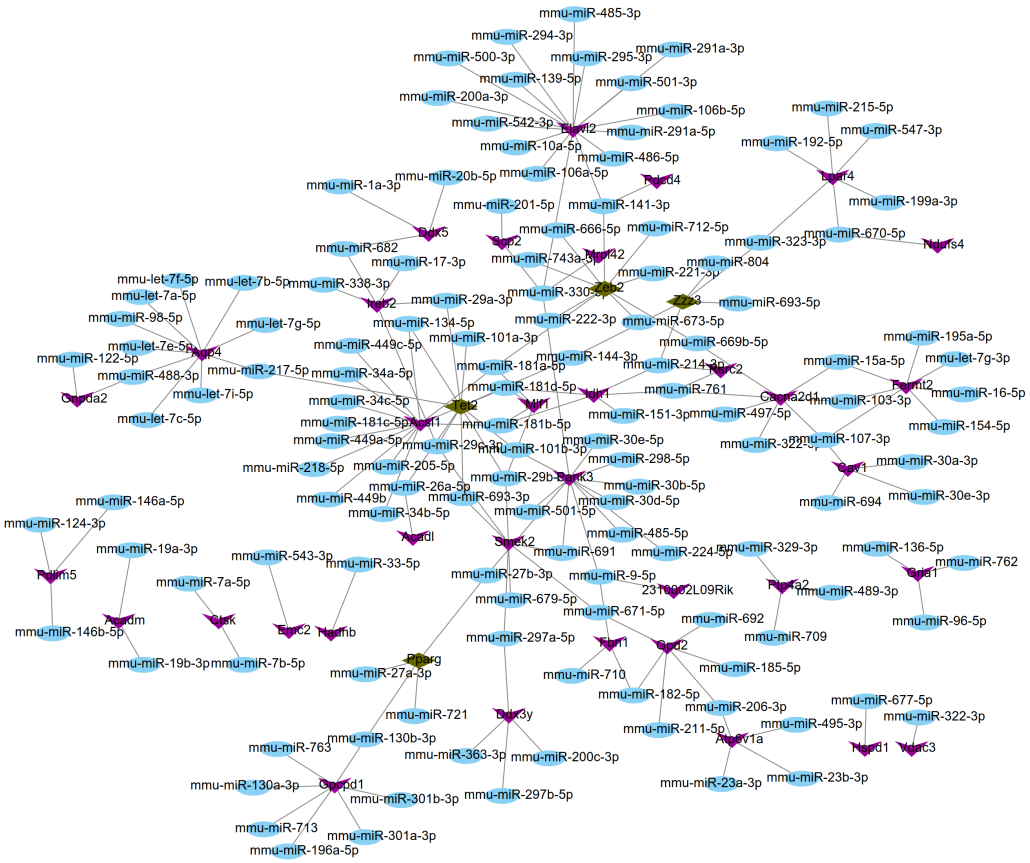

Figure 6 **The miRNA-mRNA regulatory network for the downregulated genes.** Blue: miRNAs; purple: downregulated genes; hazel-green: downregulated transcription factors.

Full-size DOI: 10.7717/peerj.6856/fig-6

2008). For example, there is evidence to reveal that PPAR expression is inversely correlated with inflammatory cytokines IL-1β and TNF-α in aging rats (*Gelinas & Mclaurin, 2005*). The PPARγ agonist ameliorates aging-related renal and cerebral artery injuries by inhibiting the inflammatory genes, reducing ECM production, and attenuating oxidative stress (*Sung et al., 2006*; *Wang et al., 2014*; *Yang et al., 2009*). In this study, we also found PPAR γ was downregulated in the cochlear tissues of Cmah-null mice and our PPI network showed PPARG could interact with SOD2, FOS and ICAM1, implying PPARG mediated pathways may be also a considerably important mechanism for AHL and activation of PPARG may be an underlying therapeutic method for patients with AHL, which has not been reported previously.

MicroRNAs (miRNAs) are a class of small RNAs (18–25-nucleotide) that down-regulate the expression of target genes via binding to the 3′-untranslated region (UTR) and then participate in the cellular processes. There has been evidence to indicate miRNAs participate in the pathogenesis of AHL, including miR-34a (*Huang et al., 2017*; *Pang et al., 2017*) and miR-29b (*Xue et al., 2016*). These two miRNAs were involved in AHL by regulating ROS homeostasis-related gene SIRT1 and then influencing cochlear hair cell apoptosis. However,

Table 5 Small molecule drugs predicted by the Cmap database.

Cmap name	Mean	*N*	Enrichment	*P*
adiphenine	−0.727	5	−0.958	0
DL-PPMP	−0.672	1	−0.95	—
decitabine	−0.617	1	−0.915	—
topiramate	−0.596	1	−0.899	—
5186324	−0.578	1	−0.886	—
5213008	−0.573	1	−0.883	—
sulindac sulfide	−0.56	1	−0.869	—
BW-B70C	−0.555	1	−0.865	—
5186223	−0.553	1	−0.863	—
isoxicam	−0.665	5	−0.852	0.00016
cefamandole	−0.574	4	−0.845	0.00105
isoflupredone	−0.623	3	−0.815	0.01256
5140203	−0.5	1	−0.815	—
tyrphostin AG-1478	−0.497	1	−0.812	—
12,13-EODE	−0.497	1	−0.812	—
STOCK1N-35696	−0.582	2	−0.808	0.07269
lisuride	−0.57	5	−0.808	0.00058
5149715	−0.483	1	−0.801	—
Prestwick-692	−0.629	4	−0.799	0.0032
carbimazole	−0.568	3	−0.792	0.01831
PF-00539745-00	−0.517	3	−0.79	0.01893
Prestwick-691	−0.562	3	−0.78	0.02195
indoprofen	−0.519	4	−0.779	0.00493
5162773	−0.457	1	−0.778	—
iloprost	−0.494	3	−0.759	0.02876
5151277	−0.427	1	−0.755	—
splitomicin	−0.423	1	−0.752	—
Prestwick-1082	−0.625	3	−0.743	0.03481
clorsulon	−0.567	4	−0.739	0.00915
vigabatrin	−0.539	3	−0.738	0.03684
3-acetamidocoumarin	−0.538	4	−0.736	0.00959
thiamphenicol	−0.541	5	−0.734	0.00274
levobunolol	−0.48	4	−0.731	0.01056
oxolamine	−0.551	4	−0.72	0.01241
cinchonine	−0.494	4	−0.716	0.01327
trimethobenzamide	−0.536	5	−0.714	0.00415
atracurium besilate	−0.559	3	−0.712	0.04853
levomepromazine	−0.517	4	−0.71	0.01456
chloropyrazine	−0.492	4	−0.708	0.0151
tranexamic acid	−0.548	5	−0.703	0.00505
isometheptene	−0.517	4	−0.701	0.01657

(*continued on next page*)

Table 5 (*continued*)

Cmap name	Mean	*N*	Enrichment	*P*
benzbromarone	−0.492	3	−0.698	0.05574
heptaminol	−0.544	5	−0.687	0.00685
trihexyphenidyl	−0.406	3	−0.68	0.06572
Prestwick-642	−0.4	4	−0.68	0.02304
viomycin	−0.53	4	−0.678	0.02383
naringenin	−0.463	4	−0.663	0.0291
colistin	−0.415	4	−0.655	0.03288
guanabenz	−0.488	5	−0.649	0.0131
canadine	−0.442	4	−0.647	0.0369
sulmazole	−0.442	3	−0.646	0.08926
Gly-His-Lys	−0.512	3	−0.642	0.09278
terazosin	−0.444	4	−0.637	0.04176
sulfadimethoxine	−0.501	5	−0.628	0.01852
iopamidol	−0.411	4	−0.622	0.05101
PHA-00745360	−0.432	8	−0.62	0.00172
iodixanol	−0.423	3	−0.619	0.11656
ribavirin	−0.469	4	−0.614	0.05616
atractyloside	−0.485	5	−0.593	0.03218
mycophenolic acid	−0.42	3	−0.591	0.15331
Prestwick-1103	−0.472	4	−0.59	0.07607
aciclovir	−0.415	6	−0.587	0.01712
triflupromazine	−0.446	4	−0.578	0.08749
rifampicin	−0.415	4	−0.577	0.08825
acemetacin	−0.477	4	−0.571	0.09443
bumetanide	−0.459	4	−0.57	0.09533
josamycin	−0.407	5	−0.551	0.05727
trapidil	−0.404	3	−0.544	0.23612
proguanil	−0.463	3	−0.524	0.27656

as a crucial gene identified to be associated with ROS in AHL of our study, there was no study to investigate the miRNAs that regulate PPARG in AHL. Thus, we also predicted the potential miRNAs that regulate PPARG by using the miRwalk database. As a result, miR-130b-3p, miR-27a-3p, miR-27b-3p and miR-721 were screened. miR-27b-3p has been shown to target PPARG to inhibit cell proliferation, but increase the inflammatory response to promote cell apoptosis (*Lee et al., 2012*). Nevertheless, there were no studies on the relationship between PPARG and others miRNAs in cell apoptosis, which may be our future research direction.

Furthermore, we also identified the potential drugs for inhibiting PPARG, consisting of the most negatively correlated adiphenine, DL-PPMP, decitabine and topiramate. Several studies have demonstrated topiramate could attenuate oxidative damage, inflammation and neuronal cell death (*Motaghinejad & Shabab, 2016*; *Tian et al., 2015*), indicating topiramate may also be an underlying drug for PPARG-related AHL.

However, there were some limitations in this study. First, the sample size in the microarray data GSE70659 was small. Another microarray or sequencing experiments should be performed to further screen crucial mechanisms for AHL. Second, we only preliminarily identified the AHL-related genes, miRNAs and drugs. Additional *in vivo* and *in vitro* experiments (PCR, Western blotting, knockout and overexpression design) are necessary to confirm their expression and their functions.

CONCLUSION

Our present study preliminarily reveals Cmah deficiency may lead to AHL by downregulating PPARG, which may then induce the higher expressions of ECM and adhesion (ICAM1) and pro-inflammatory (FOS, TNFSF13B), but lower expression of anti-oxidative genes (SOD2). Downregulation of miR-130b-3p, miR-27, miR-721 and the use of topiramate may be potential treatment strategies for ALH by upregulating PPARG.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Juhong Zhang conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Na Wang performed the experiments, prepared figures and/or tables, approved the final draft.
- Anting Xu conceived and designed the experiments, authored or reviewed drafts of the paper, approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data is available in Supplemental Information 1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.6856#supplemental-information.

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