RNA sequencing uncovers the key microRNAs potentially contributing to sudden sensorineural hearing loss

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Abstract
This study aimed to identify miRNAs that may contribute to the pathogenesis of sudden sensorineural hearing loss (SSNHL) by RNA-seq (RNA-sequencing).

RNA was extracted from SSNHL patients and healthy volunteers, respectively. Sequencing was performed on HiSeq4000 platform. After filtering, clean reads were mapped to the human reference genome hg19. Differential expression analysis of miRNAs between the SSNHL samples and the normal samples was performed using DEseq to identify differentially expressed microRNAs (DEMs). The target genes of the DEMs were predicted using the online tool miRWalk, which were then mapped to DAVID (https://david.ncifcrf.gov/) for functional annotation based on GO database and for pathway enrichment analysis based on KEGG. Finally, a miRNA-target-protein-protein interaction (PPIs) network was constructed using the DEMs and their target genes with interaction.

Differential expression analysis reveals 24 DEMs between the SSNHL group and control group. A total of 1083 target genes were predicted. GO functional annotation analysis reveals that the target genes in the top 10 terms are mainly related to the development of salivary glands, neurotransmission, dendritic development, and other processes. KEGG pathway enrichment analysis reveals that the target genes were functionally enriched in pathways arachidonic acid metabolism, complement and coagulation cascades, linoleic acid metabolism, and MAPK signaling pathway. In the miRNA-target-PPI network, hsa-miR-34a/15a/23a/210/18b/548n/143 had the most target genes; genes YWHAG, GSK3B, CDC42, NR3C1, LCK, UNC119, SIN3A, and NFKB2, interact with most other genes among all the predicted target genes.

Hsa-miR-34a/15a/23a/210/18b/548n/143 is likely to have a role in the pathogenesis of SSNHL.

Abbreviations: DEMs = differentially expressed microRNAs, GO = gene ontology, KEGG = Kyoto Encyclopedia of Genes and Genomes, LPS = lipopolysaccharide, miRNA = microRNA, PPIs = protein-protein interactions, RNA-seq = RNA-sequencing, SHL = sudden hearing loss, SSNHL = sudden sensorineural hearing loss.

Keywords: differentially expressed microRNAs, RNA sequencing, sudden sensorineural hearing loss, target genes

1. Introduction
Sudden hearing loss (SHL) is defined as a rapid onset, occurring over a 72-hour period, of a subjective sensation of hearing impairment in one or both ears.1,2 Sudden sensorineural hearing loss (SSNHL) a predominant subtype of SHL, is sensorineural in nature and mostly idiopathic at presentation, which is presumptively attributed to vascular, viral, or multiple etiologies although definitive etiology is unknown.2 MicroRNA (miRNA) is a short, noncoding RNA that is thought to regulate gene expression through sequence-specific
base pairing with the 3′-untranslated region (3′-UTR) of target mRNA. The miR183 family (miR-96, miR-182, and miR-183) is implicated in the differentiation and function of the mechanosensory hair cells in the vertebrate inner ear.[3,4] And several studies have reported that mutations in the gene miRNA96 is associated with progressive hearing loss, proving the regulatory role of this miRNA in maintaining the normal function of hair cells.[5,6] Aside from this miRNA family, other miRNAs have not been reported in SSNHL. Thus, this study was designed to seek more miRNAs that might contribute to the pathogenesis of this disease by RNA-seq (RNA-sequencing).

2. Materials and methods

2.1. Ethical review
This study has been approved by the Medical Ethics Committee of the Southern Medical University. Written informed consent was provided by each patient and volunteer before sampling. Nine patients with SSNHL were included in this study, and 3 healthy volunteers were recruited as normal controls.

2.2. Patient sampling
Venous peripheral blood samples were collected from 9 patients and 3 healthy volunteers. EDTA-Na2-anticoagulated venous peripheral blood samples were mixed at 1:1:1 from 3 patients as a final sequencing sample. Thus, there were 3 samples in the patient group (SSNHL, named as LxsR1, LxsR2, and LxsR3) and 3 normal controls (named as LxsR4, LxsR5, and LxsR6).

2.3. Total RNA extraction, library construction, and sequencing
Total RNA was extracted from the plasma of the aforementioned 6 samples using miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany). Then, rRNA was removed using Epicentre Ribopure ZeroTM kit (Illumina Inc, San Diego, CA) and the remaining RNA (polyA+, polyA−) was recovered and purified. Afterward, the purified RNA was broken into short segments using random fragmentation reagent (Fragmentation Buffer). Next, reverse transcription was performed to construct cDNA library. RNA integrity number (RIN) was measured by Bioanalyzer 2100 (Agilent, CA). Sequencing was performed on HiSeq4000 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); low quality reads containing ≥2 bases with quality value <20. Next, clean reads were mapped to the human reference genome hg19 using TopHat 2 (http://ccb.jhu.edu/software/tophat/index.shtml).

2.5. Identification of miRNAs differentially expressed in patients with SSNHL
Differential expression analysis of miRNAs between the SSNHL group and the normal control was performed using DEseq.[8] Only miRNAs with |log₂FC| > 0.5 and P < .05 were used as differentially expressed miRNA (DEM).

2.6. Prediction of target genes and functional enrichment analysis
With reference to databases miRWalk, miRanda, RNA22, and TargetScan, the target genes of the DEMs were predicted using the online tool miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/).[9] Adjusted P < .01 was set as cutoff.

2.7. Functional annotation of target genes of DEMs
The target genes of the DEMs were mapped to DAVID (https://david.ncifcrf.gov/) for functional annotation based on GO (gene ontology) database and for pathway enrichment analysis based on KEGG (Kyoto Encyclopedia of Genes and Genomes) database (P value < .05 as cutoff).[10]

2.8. Construction of miRNA-target-protein-protein-interaction (PPIs) network
Based on the Human Protein Reference Database (http://www.hprd.org/), target genes of DEMs with interaction between their encoding proteins were considered as a pair. Then, these target gene-gene pairs were further integrated with the DEMs to construct a miRNA-target-PPIs network, which was visualized with Cytoscape.[11]

3. Result

3.1. Quality control of reads and statistics
The number of raw reads, cleans reads, and reads mapped to human reference genome are listed in Table 1. All the clean reads contained bases with score >Q30. The results of sequencing were reliable.

3.2. Reads alignment and identification of DEMs
With reference to the human reference genome hg19, the clean reads that were aligned to known miRNAs, as well as those novel ones, in each sample were identified, and their numbers were listed in Table 2.
miR-1255a, miR-95, and miR-548ay were the predicted target genes (Table 6).

and hsa-miR-99b had the most target genes; genes miR-210, hsa-miR-1255a, hsa-miR-18b, hsa-miR-1180, and hsa-miR-548n, hsa-miR-15a, hsa-miR-143, hsa-miR-23a, hsa-miR-3679, and miR-4742 were downregulated between SSNHL samples and healthy volunteers (Table 3).

3.3. Prediction of target genes of DEMs and functional analysis

With reference to databases miRWalk, miRanda, RNA22, and Targetscan, a total of 1083 target genes were predicted, forming 1127 miRNA-gene regulation pairs based on the 24 differential expression miRNA.

GO functional annotation analysis reveals that the target genes in the top 10 terms are mainly related to the development of salivary glands (BP, \( P = 3.21 \times 10^{-3} \)), neuro projection (CC, \( P = 6.91 \times 10^{-3} \)), dendritic development (CC, \( P = 3.40 \times 10^{-3} \)), and other processes (Table 4).

KEGG pathway enrichment analysis reveals that the target genes were functionally enriched in pathways arachidonic acid metabolism (hsa00590), complement and coagulation cascades (hsa04610), and MAPK signaling pathway (hsa04010) (Table 5).

3.4. Construction of miRNA-PPIs network

The target genes of the DEMs identified previously formed 141 PPIs. In the miRNA-target-PPIs network (Fig. 1), hsa-miR-34a, hsa-miR-548n, hsa-miR-15a, hsa-miR-23a, hsa-miR-210, hsa-miR-18b, and hsa-miR-99b had the most target genes; genes YWHAG, GSK3B, Cdc42, NR3C1, Lck, UNC119, SIN3A, NFkBa, NEDD4, and KRT15 interacted with most other genes among all the predicted target genes (Table 6).

4. Discussions

Using RNA-seq technique, we first identified DEMs that were speculated to contribute to SSNHL and then predicted their target genes. Among them, DEMs hsa-miR-34a, hsa-miR-548n, hsa-miR-15a, hsa-miR-23a, hsa-miR-210, hsa-miR-18b, and hsa-miR-1180 that were predicted to regulate more target genes may have more critical roles in SSNHL.

MiR-34a has been suggested as a tumor suppressor gene as its inactivation was reported in several types of cancer. A recent study reported that miR-34a level was increasing in the cochlea, auditory cortex, of C57BL/6 mice (a mouse model of age-related hearing loss) during aging, especially in the plasma compared with that in normal mice. Here, UNCI19 was predicted to be a target gene of this miRNA. UNCI19 encodes photoreceptor synaptic protein HRG4, a photoreceptor protein predominantly localized to the photoreceptor synapses. Previously, Frances et al have reported that progressive sensorineural deafness was present in all individuals affected with North Carolina macular dystrophy over the age of 20 years in a family, but hearing was normal in unaffected members, suggesting a relationship between sensorineural hearing and photoreceptors. This, it is possible that miR-34a may be involved in SSNHL, and UNCI19 is one of its target genes.

Previously, Kwon et al have reported that miR-15a-5p was significantly upregulated in the liver and pancreas ofCMP-Neu5Ac hydroxylase (Cmah)-null mice compared with the normal mice expressing Cmah. suggesting a link between the gene Cmah and miR-15a-5p. Furthermore, Hedlund et al reported reduced hearing sensitivity in the Cmah−/− mice. Thus, miR-15a may be involved in the physiological mechanisms of hearing. Activation of Ras-Rac-Cdc42-JNK signaling may be responsible for aminoglycoside-induced death of auditory hair cells. However, miR-15a was found to be upregulated here,
### Table 5

KEGG pathway enrichment analysis of the target genes of differentially expressed microRNAs.

| Pathway name                                      | Number of gene | P value       | Gene                                                                 |
|---------------------------------------------------|----------------|---------------|----------------------------------------------------------------------|
| hsa00590:Arachidonic acid metabolism              | 10             | $6.57 \times 10^{-3}$ | GGT6, PTGES2, CYP2C19, CYP2C18, CYP2C9, CYP2C8, PTGES1, PLA2G1B, ALDOK, PLA2G2F |
| hsa04610:Complement and coagulation cascades      | 10             | .0346         | CR1, THBD, MASP1, F13A1, F1, SERPINA1, SERPIND1, F7, C1S, C2         |
| hsa00591:Linoleic acid metabolism                 | 6              | .0261         | CYP2C19, CYP2C18, CYP2C9, CYP2C8, PLA2G1B, PLA2G2F                   |
| hsa04010:MAPK signaling pathway                   | 25             | .0375         | MEF2C, FGF8, FGF1, HSPA1B, NFATC1, NFATC4, FGF22, HSPA1B, PTGS1, PLA2G1B, PLA2G2F |

Figure 1. MicroRNA-target-protein-protein-interaction network consisting of differentially expressed miRNAs and targets genes with interaction. An ellipse indicates a microRNA; a rectangle represents a target gene.
thus how this miRNA function in SSNHL via Cdc42 needs to be further addressed. Since Cdc42 was functionally enriched in the MAPK signaling pathway, this pathway may also have a role in SSNHL.

Song et al reported a decreased expression of hsa-miR-210 in lipopolysaccharide (LPS)-treated human middle ear epithelial cells, and claimed this miRNA has an important role in LPS-induced inflammatory response of otitis media. However, hsa-miR-210 expression was found to increase in the patients with SSNHL. Furthermore, this miRNA was speculated to regulate 2 genes YWHAAG and SIN3A in SSNHL. YWHAAG encodes 14-3-3 protein gamma, belonging to a highly conserved 14-3-3 protein family. Tra et al have reported the upregulation of this gene in age-related hearing loss. SIN3A is involved in histone modification and chromatin remodeling that were implicated in the regeneration and loss of inner-ear hair cells, thus this gene is related to sensorineural hearing loss. As both the 2 target genes of hsa-miR-210 are involved in SSNHL, this miRNA is also likely to contribute to this disease.

Hsa-miR-18b has also been speculated to have a role in SSNHL, and one of its predicted target gene GSK3B was associated with SSNHL. GSK3 encodes a serine/threonine kinase glycogen synthase kinase 3 beta, and it has been reported that the mutation of GSK3 may impair the MAF (musculoaponeurotic fibrosarcoma oncogene homolog) phosphorylation, which will cause hearing loss among other eponym Aymè-Gripp symptoms. Another gene LCK encoding lymphocyte-specific protein tyrosine kinase was also regulated by this miRNA; however, it has never been reported in SSNHL. Additionally, although hsa-miR-23a has never been reported in SSNHL, one of its target genes NR3C1 has been suggested to be implicated in SSNHL. NR3C1 encoding a glucocorticoid receptor was found to be located in the same interval on chromosome 18, in which a major effect QTL for noise injury to the mouse cochlear lateral wall was reported by Ohlemiller et al suggesting a relationship between NR3C1 and vulnerability of the mammalian cochlea. Furthermore, Lee et al reported DA9801 can ameliorate the hearing impairment caused by diabetes mellitus in STZ-induced diabetic model, and they found NR3C1 and AKT might be responsible for this neuroprotective effect. Thus, we proposed that this miRNAs may also contribute to the pathogenesis of SSNHL. Finally, hsa-miR-548n and hsa-miR-143 were also found to regulate many target genes, however, they have never been reported in SSNHL before. Due to the lack of SSNHL samples, the relationship between hsa-miR-548n and hsa-miR-143 and target genes in process of SSNHL was not confirmed by experiment. But in our future study, we will validate the target genes in the mRNA level by RNA-seq.

Here, we identified several miRNAs (hsa-miR-34a, hsa-miR-15a, hsa-miR-23a, hsa-miR-210, hsa-miR-18b, hsa-miR-548n, and hsa-miR-143), which are likely to have a role in the pathogenesis of SSNHL based on the role of their target genes in this disease. Since our findings are partially drawn by prediction, they need to be further validated.

References

[1] Stachler RJ, Chandrasekhar SS, Archer SM, et al. Clinical practice guideline: sudden hearing loss. Otolaryngology Head Neck Surg 2012;146(3 suppl):S1–35.

[2] Rauch SD. Clinical Practice. Idiopathic sudden sensorineural hearing loss. N Engl J Med 2008;359:833–40.

[3] Soukop GA, Fritsch B, Pierce ML, et al. Residual microRNA expression dictates the extent of inner ear development in conditional Dicer knockout mice. Dev Biol 2009;328:328–41.

[4] Li H, Kloosterman W, Fekete DM. MacroRNA-183 family members regulate sensorineural fates in the inner ear. J Neurosci 2010;30:3254–63.

[5] Mencia A, Modamo-Hobybor S, Redshaw N, et al. Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. Nature genetics 2009;41:609–13.

[6] Lewis MA, Quina E, Glazier AM, et al. An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. Nat Genet 2009;41:614–8.

[7] An J, Lai J, Lehman ML, et al. miDeep*: an integrated application tool for miRNA identification from RNA sequencing data. Nucleic Acids Res 2013;41:727–37.

[8] Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010;11:R106.

[9] Dweep H, Sticht C, Pandey P, et al. miRWalk: database: prediction of possible miRNA binding sites by “walking” the genes of three genomes. J Biomed Inform 2011;44:839–47.

[10] Reissbach T, Speed TP. GOstat: find statistically overrepresented Gene Ontologies within a group of genes. Bioinformatics 2004;20:1464–5.

[11] Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13:2498–504.

[12] Lodigyn D, Tarasov V, Epanchinsm E, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. Cell Cycle 2008;7:5291–600.

[13] Pang J, Xiong H, Yang H, et al. Circulating miR-34a levels correlate with age-related hearing loss in mice and humans. Exp Gerontol 2016;76:56–67.

[14] Ishiba Y, Higashide TN, Mori N, et al. Targeted inactivation of synaptic HRG4 (UNC119) causes dysfunction in the distal photoreceptor and slow retinal degeneration, revealing a new function. Exp Eye Res 2007;84:473–85.

[15] Francis PJ, Johnson S, Edmunds B, et al. Genetic linkage analysis of a novel syndrome comprising North Carolina-like macular dystrophy and progressive sensorineural hearing loss. Br J Ophthalmol 2003;87:893–8.

[16] Kwon DN, Chang BS, Kim JH. MicroRNA dysregulation in liver and pancreas of CMP-Neu5Ac hydroxylase null mice disrupts insulin/PI3K-AKT signaling. Biochem Res Int 2014;2014:236385.

[17] Hedlund M, Tangvorarundtakul P, Takematsu H, et al. N-glycolyneuraminic acid deficiency in mice: implications for human biology and evolution. Mol Cell Biol 2007;27:4340–6.

[18] Bodmer D, Brors D, Pak K, et al. Rescue of auditory hair cells from amingolinosidic toxicity by Clostridium difficile toxin B, an inhibitor of the small GTPases Rho/Rac/Cdc42. Hear Res 2002;172:81–6.

[19] Battaglia A. Ras activation contributes to outer hair cell apoptosis in the basal turn of the cochlea after cisplatin and gentamicin exposure. PhD thesis, University of California, San Diego, CA; 2002.

[20] Song JJ, Kwon SK, Cho CG, et al. Microarray analysis of microRNA expression in LPS-induced inflammation of human middle ear epithelial cells (HMECs). Int J Pediatr Otorhinolaryngol 2011;75:648–51.

[21] Tra Y, Frisina RD, D’Souza M. A novel high-throughput analysis approach: immune response-related genes are regulated in age-related hearing loss. Open Access Bioinformatics 2011;3:107–22.
[22] Friedman LM, Avraham KB. MicroRNAs and epigenetic regulation in the mammalian inner ear: implications for deafness. Mamm Genome 2009;20:581–603.

[23] Niceta M, Stellacci E, Gripp K, et al. Mutations impairing GSK3-mediated MAF phosphorylation cause cataract, deafness, intellectual disability, seizures, and a Down syndrome-like facies. Am J Hum Genet 2015;96:816–25.

[24] Ohlemiller KK, Rosen AD, Gagnon PM. A major effect QTL on chromosome 18 for noise injury to the mouse cochlear lateral wall. Hear Res 2010;260:47–53.

[25] Lee YR, Hong BN, Her YR, et al. Amelioration of auditory response by DA9801 in diabetic mouse. Evid Based Complement Alternat Med 2015;2015:230747.