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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/548n/15a/143/23a/210/1255a/18b/ /1180/99b 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

Highlights

1. Differential expression analysis reveals 24 DEMs in the SSNHL group.
2. A total of 1083 target genes of these DEMs were predicted.
3. Hsa-miR-34a/15a/23a/210/18b/548n/143 may contribute to the pathogenesis of SSNHL.

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] 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

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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-Na_2-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 Ribo-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 concentration was measured with a Qubit 2.0 Fluorometer; RNA integrity number (RIN) was measured by Bioanalyzer 2100 (Agilent, CA). Sequencing was performed on HiSeq4000 platform to generate paired-end reads (150 bp in length). The raw sequencing data have been uploaded to National Center for Biotechnology Information database under the BioProject accession no SRP115241.

2.4. Sequence quality control and alignment

Raw reads were filtered as follows: adaptor sequences were removed using miRDeep*.[7] Using the online tool FastQC (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 $|\log_2 FC| > 0.5$ 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.

Table 1

Quality statistics of reads.

Sample no.	LxsR1	LxsR2	LxsR3	LxsR4	LxsR5	LxsR6
Number of raw reads	27370520	20443064	25496225	21048198	26673440	22937426
Number of filtered reads	27370520	20443064	25496225	21048198	26673440	22937426
Aligned reads	27365241	20441025	25494651	21045624	26671656	22934864

Note: Sudden sensorineural hearing loss samples: LxsR1, LxsR2, and LxsR3; Normal samples: LxsR4, LxsR5, and LxsR6.

Table 2

The number of unknown and unknown miRNAs.

Sample no.	LxsR1	LxsR2	LxsR3	LxsR4	LxsR5	LxsR6
Known miRNA	531	479	537	470	537	506
Unknown miRNA	1255	915	1353	1303	1569	1427

Note: Sudden sensorineural hearing loss samples: LxsR1, LxsR2, and LxsR3; Normal samples: LxsR4, LxsR5, and LxsR6.

After comparison, we found 397 common miRNAs among the 6 samples. Further differential expression analysis reveals 24 of them were differentially expressed between the SSNHL group and control group, among them, miR-296, miR-3667, miR-15a, miR-1180, miR-18b, miR-451a, miR-24-1, miR-210, miR-99b, miR-190a, miR-660, miR-3940, and miR-34a were upregulated, and miR-1-1, miR-1-2, miR-548ay, miR-95 miR-1255a, miR-143, miR-23a, miR-548n, miR-3679, miR-3074, 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), linoleic acid metabolism (hsa00591), 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-143, hsa-miR-23a, hsa-miR-210, hsa-miR-1255a, hsa-miR-18b, hsa-miR-1180, and hsa-miR-99b had the most target genes; genes *YWHAG*, *GSK3B*, *CDC42*, *NR3C1*, *LCK*, *UNC119*, *SIN3A*, *NFKB2*, *NEDD4*, and *KRT15* interacted with most other genes among all the predicted target genes (Table 6).

Table 4

GO functional annotation of the target genes of differentially expressed microRNAs.

GO term	GO term	Gene	*P* value
CC	Vesicle	59	3.00×10^{-4}
CC	Cytoplasmic vesicle	57	3.17×10^{-4}
CC	Cytoplasmic membrane-bounded vesicle	49	8.35×10^{-4}
CC	Membrane-bounded vesicle	50	9.44×10^{-4}
CC	Cell soma	20	2.02×10^{-3}
BP	Salivary gland development	5	3.21×10^{-3}
CC	Dendrite	19	3.40×10^{-3}
BP	Membrane organization	35	4.03×10^{-3}
CC	Synapse	32	6.51×10^{-3}
CC	Neuron projection	31	6.91×10^{-3}

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.[12] 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.[13] Here, *UNC119* was predicted to be a target gene of this miRNA. *UNC119* encodes photoreceptor synaptic protein HRG4, a photoreceptor protein predominantly localized to the photoreceptor synapses.[14] Previously, Francies 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.[15] This, it is possible that miR-34a may be involved in SSNHL, and *UNC119* is one of its target genes.

Previously, Kwon et al have reported that miR-15a-5p was significantly upregulated in the liver and pancreas of CMP-Neu5Ac hydroxylase (Cmah)-null mice compared with the normal mice expressing Cmah,[16] suggesting a link between the gene *Cmah* and miR-15a-5p. Furthermore, Hedlund et al reported reduced hearing sensitivity in the Cmah−/− mice.[17] 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.[18,19] However, miR-15a was found to be upregulated here,

Table 3

The 24 differential expression miRNAs between sudden sensorineural hearing loss samples and normal samples.

miRNA	$\log_2$ fold change	*P* value	miRNA	$\log_2$ fold change	*P* value	miRNA	$\log_2$ fold change	*P* value
miR-296	1.446	.010	miR-143	−0.912	.026	miR-23a	−0.579	.033
miR-1-1	−1.831	.015	miR-1180	1.342	.026	miR-18b	1.154	.037
miR-3667	1.159	.015	miR-548n	−0.882	.030	miR-451a	0.549	.037
miR-1-2	−1.824	.0191	miR-3679	−1.075	.030	miR-24-1	0.830	.038
miR-15a	1.05	.0208	miR-210	1.067	.031	miR-3074	−0.963	.041
miR-548ay	−1.022	.021	miR-99b	0.705	.031	miR-190a	0.773	.043
miR-95	−1.146	.021	miR-660	0.858	.031	miR-4742	−0.888	.044
miR-1255a	−1.63	.024	miR-3940	1.197	.033	miR-34a	0.850	.048

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	6.57×10^{-3}	*GGT6, PTGES2, CYP2C19, CYP2C18, CYP2C9, CYP2C8, PTGS1, PLA2G1B, ALOX5, PLA2G2F*
hsa04610:Complement and coagulation cascades	10	.0244	*CR1, THBD, MASP1, F13A1, F8, 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, TGFB3, HSPA1B, NFKB2, TGFB2, CDC42, TNFRSF1A, RAC2, ELK4, SOS2, PLA2G1B, NFATC4, CHP, FGF1, CACNA2D1, TAOK2, PTPN5, FGF22, FLNC, CACNA2D2, DUSP2, MAPK7, CD14, PLA2G2F*

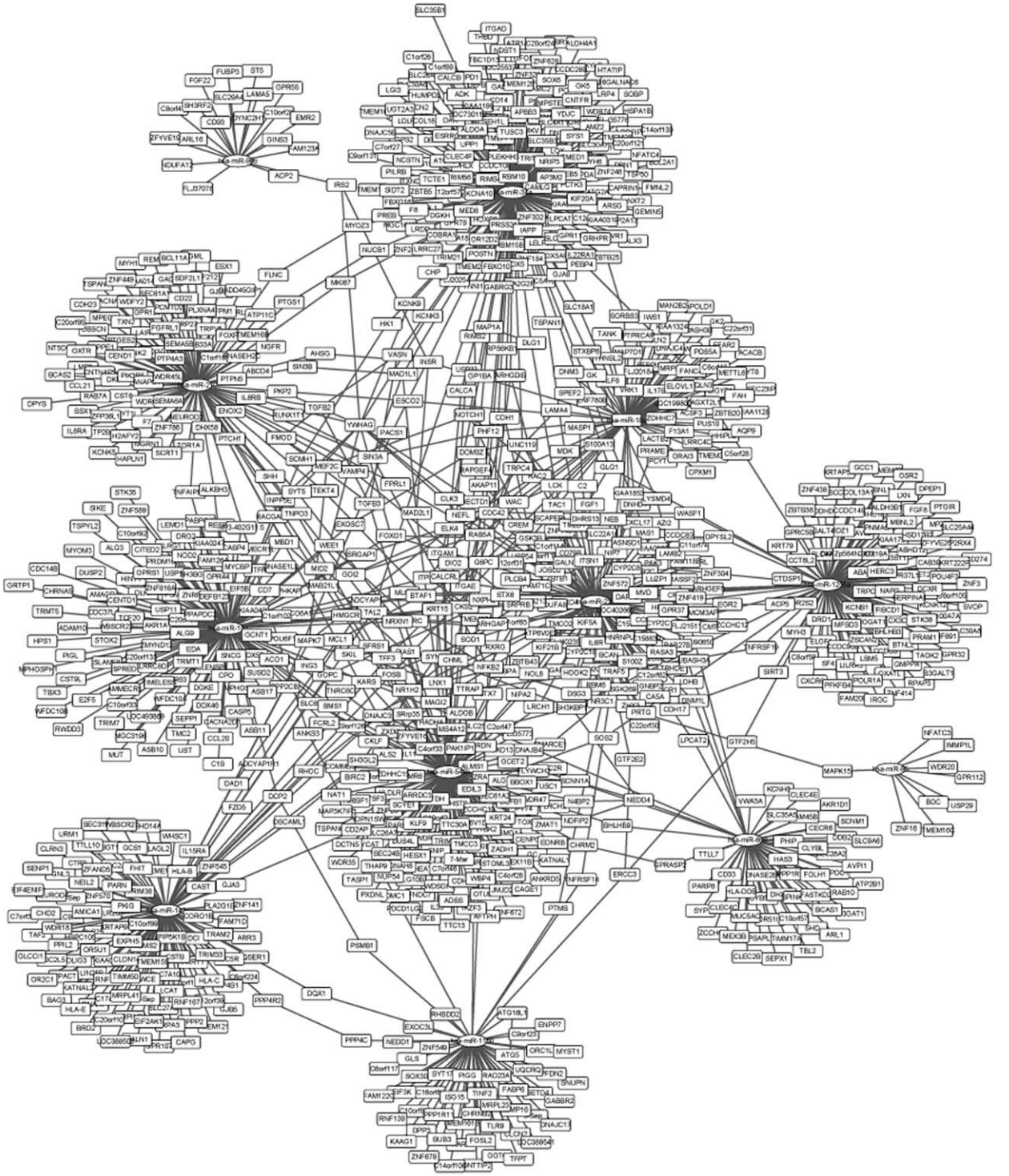

Figure 1. MicroRNA-target-protein-protein-interaction network consisting of differentially expressed miRNAs and targets genes with interaction. An ellipse indicates a microRNA; a rectangle represents a target gene.

Table 6

Small-molecule drugs were found negatively correlated with differentially expressed genes with coefficient <−0.8.

miRNA	Node	Gene	Node
hsa-miR-34a	200	*YWHAG*	12
hsa-miR-548n	144	*GSK3B*	10
hsa-miR-15a	133	*CDC42*	10
hsa-miR-143	114	*NR3C1*	8
hsa-miR-23a	109	*LCK*	7
hsa-miR-210	106	*UNC119*	6
hsa-miR-1255a	106	*SIN3A*	5
hsa-miR-18b	75	*NFKB2*	5
hsa-miR-1180	58	*NEDD4*	5
hsa-miR-99b	200	*KRT15*	5

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.[20] However, hsa-miR-210 expression was found to increase in the patients with SSNHL here. Furthermore, this miRNA was speculated to regulate 2 genes *YWHAG* and *SIN3A* in SSNHL. *YWHAG* 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.[21]*SIN3A* is involved in histone modification and chromatin remodeling that were implicated in the regeneration and loss of inner-ear hair cells,[22] 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 Aymé-Gripp symptoms.[23] 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,[24] 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.[25] 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 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.

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