Transcriptome sequencing of PBMC reveals Hsa-miR-124-5p regulates cell cycle and metabolism of lipids via targeting CSTF2/ TXNRD1 in hearing loss patients

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Research Article

Keywords: hearing loss, miRNA-seq, hsa-miR-124-5p, miRNA-mRNA

Posted Date: March 29th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1439086/v1

Abstract

Background

Hearing loss (HL) is a public health event, which seriously affects the happiness of people's life. Hsa-miR-124-5p has not been reported in HL. This study aimed to construct a miRNA-mRNA network associated with HL.

Methods

Subjects were screened through clinical audiology experiments. MiRNA-seq was performed on the peripheral blood mononuclear cells (PBMC), and the differentially expressed miRNAs (DEMs, *P*-Value < 0.05, |log FC | ≥1) were obtained by analysis. We selected the most significantly up-regulated DEMs for research. We predicted the downstream target genes of the most significantly up-regulated DEMs through miRDB, mirDIP, mirtarbase and TargetScan databases, and four database overlapping genes were considered as downstream target genes. Enrichment analysis was performed on overlapping genes. Then, a protein-protein interaction (PPI) network of overlapping genes was performed through STRING database, aimed to pick out hub genes. Finally, we construct the miRNA-mRNA network.

Results

There were 6 clinical participants, 3 hearing loss patients and 3 healthy subjects.There are 29 DEMs, of which hsa-miR-124-5p is the most significantly up-regulated DEMs. 13 downstream target genes corresponding to hsa-miR-124-5p were screened, which were significantly enriched in cell cycle and metabolism of lipids. PPI analysis was performed on 13 target genes, and two hub genes (CSTF2/ TXNRD1) were finally obtained. Most importantly, miRNA-mRNA networks containing hsa-miR-124-5p/CSTF2 and hsa-miR-124-5p/TXNRD1 were successfully constructed.

Conclusions

In summary, hsa-miR-124-5p may be a novel biomarker for hearing loss patients and play a important role in hearing loss patients by targeting CSTF2 and TXNRD1. Hsa-miR-124-5p may participate in cell cycle and metabolism of lipids biological process in hearing loss patients.

Introduction

Hearing loss is a relatively common type of disease in clinical diagnosis and treatment, and its incidence is on the rise [1]. In 2016, The Lancet reported that the second biggest obstacle was mainly hearing loss [2]. Mild hearing loss is reported to be more common in the United States, accounting for approximately 23 percent of people over the age of 12. Moderate hearing loss is more common in people over the age of 80 [3]. In recent years, with the development of medicine and the update of instruments, the therapeutic effect of deafness has been improved, but the prevalence of deafness is still high [4]. We explored some miRNA biomarkers related to hearing loss. We hope these biomarkers will contribute to the diagnosis and treatment of hearing loss.

MicroRNAs (miRNAs) is one of the non-coding RNAs that can affect gene expression by regulating the level of messenger RNA (mRNA) [5]. The mechanism of miRNA regulating biological process includes methylation of miRNA promoter, genetic polymorphism, and interactions with RNA binding protein (RBP), etc [6]. MiRNA plays an important role in balancing physiological processes, affects all genetic pathways of cell cycle checkpoint, cell proliferation and apoptosis, and has a wide range of target genes [7].

Misregulation of miRNA expression can lead to many diseases. Increased miRNA (hsa-miR-124-5p) in aged human blood has been reported and confirmed by polymerase chain reaction (PCR) [8]. Hsa-miR-124-5p may also play an important role in type 2 diabetes [9]. In acute myeloid leukemia, hsa-miR-124-5p inhibited KG-1a cell proliferation and cell cycle progression and promoted apoptosis through BMSCs-derived exosomes [10]. However, hsa-miR-124-5p, as a miRNA, has not been reported in hearing loss. Our research will focus on the biological process of hsa-miR-124-5p in hearing loss.

Currently, miRNA-seq is a widely used sequencing technology, mainly used in clinical practice, which reveals the existence and quantity of DEMs through large-scale sequencing [11]. Nowadays, microarray studies have shown that miRNAs are differentially expressed in hearing loss patients and normal people [12]. However, so far, the involvement of miRNAs in metabolism of lipids in hearing loss patients has been rarely reported. The mechanisms of miRNAs in metabolism of lipids of hearing loss patients remain unclear.

Therefore, it will be of great significance to study hsa-miR-124-5p in hearing loss metabolism of lipids mechanism. For hearing loss patients, potentially important miRNAs and downstream target genes related to hearing loss need to be identified. Understanding the physiological process of hearing loss helps in the diagnosis and treatment of deafness.

Materials And Methods

Population and ethics statement

This study was based on patients with otolaryngology and head and neck surgery in Zhongshan Hospital Affiliated to Xiamen University (February 2021 to February 2022). Institutional ethics committee approval for this project was provided prior to the start of this study and was in accordance with the Declaration of Helsinki. This study was performed with the approval of the Institutional Ethics Committee of Zhongshan Hospital Affiliated to Xiamen University (Approval number: 2021-077), and written and oral informed consents were obtained from patients or their relatives.

Subject hearing test

We performed hearing tests on randomly selected subjects. Three deaf patients and three healthy subjects were selected for inclusion in this study. All patients underwent audiometry (Otometrics, Model 1066) and audiograms were recorded. After the subjects were determined to be eligible for our study, the peripheral blood of the patients was drawn and temporarily stored in EDTA anticoagulant tubes.

PBMC isolation and rNA preparation

Patient blood was stored in EDTA anticoagulant tubes immediately after ex vivo and temporarily stored at 4℃. Take 3ml of blood from the EDTA anticoagulation tube into a 15ml freshly packaged centrifuge tube, add 3 times the volume of red blood cell lysate, place at 4℃ for 15min, centrifuge at 4℃ for 10min, draw the cell pellet after layering, add 1ml trizol to the cell pellet and mix well. Add 270ul chloroform to the centrifuge tube, vortex until the solution is emulsified and milky white, let stand for 5 minutes, centrifuge, suck the supernatant into a new EP tube, add an equal volume of isopropanol, mix well and let stand for 10 minutes, after centrifugation Remove the supernatant, add 50ul of nucleic acid-free plum water to the precipitation, and store the samples after fully dissolving.

RNA-seq

MiRNA-seq sequencing was entrusted to Aimo Gene Xiamen Biotechnology Co., Ltd. MiRNA-seq experiments were performed on peripheral blood mononuclear cells provided by subjects. According to the manufacturer's instructions, the experimental protocol was performed according to standard procedures provided by Illumina, including library preparation and sequencing experiments. Small RNA sequencing libraries were prepared using TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA). After the library preparation was completed, the constructed library was sequenced using Illumina novaseq6000, and the sequencing read length was single-ended 1X50bp. After the library is constructed, use Qubit2.0 for preliminary quantification, dilute the library to 1 ng/ul, and then use Agilent 2100 to detect the insert size of the library. After the insert size is as expected, use the Q-PCR method to determine the effective concentration of the library. Perform accurate quantification (library effective concentration > 2nM) to ensure library quality. The measured raw data (Raw Data) has a certain proportion of interference data (Dirty Data). In order to make the results of information analysis more accurate and reliable, data filtering is first performed on the raw data. Including removal of low-quality bases, linker sequences, and short sequences (less than 18bp). The raw data generated by sequencing needs to be preprocessed. We use TrimGalore to filter out unqualified sequences to obtain valid data (clean data). The reads obtained by sequencing were compared with the database, and the RNA types in the sequencing reads were identified. At the same time, in order not to affect downstream analysis as much as possible, we removed sequences identified as non-miRNAs. The subsequent differential expressed miRNAs (DEMs) were further analyzed.

Bioinformatic analysis

MiRNA-seq data quality inspection was done with fastqc (0.11.7), data filtering was done with trim_galore (0.6.0), repeated sequence removal was done with (RepeatMasker) 4.0.9, and non-miRNA was removed with infernal (1.1.3), miRNA alignment was done using bowtie (1.0.0), predicted secondary domain was done using RNAfold (2.4.14), De novoe precursor scoring was done using miRDeep2 (2.0.0.8) [13], and differential expression was done using R:DEseq2/edgeR (1.24.0) [14] done. Statistical analysis, graphing was done using R (3.6.1) [15]. Sequencing was performed using an Illumina novaseq6000. The original data processing process was completed by Xiamen Aimo Gene Biotechnology Co., Ltd. We further filtered hearing loss differentially expressed genes (HL-DEMs), the threshold were set at P-Value < 0.05 and |log FC|≥1, and the HL-DEMs obtained according to this criterion were included in our study. We selected the most up-regulated miRNAs for corresponding predictions. Aims to find downstream mRNAs.

MiRNA target gene prediction

The miRNA-mRNA was identified through the miRDB (http://www.mirdb.org/), mirDIP (http://ophid.utoronto.ca/mirDIP/index.jsp#r), mirtarbase (http://mirtarbase.cuhk.edu.cn/php/search.php#target) and TargetScan (http://www.targetscan.org/vert_80/) databases. Genes appearing in all four databases were considered target genes for DEMs. The miRDB database was used for miRNA target prediction and functional annotation. Transcriptome-wide target prediction data is available in the miRDB, including 3.5 million predicted targets regulated by 7000 miRNAs in 5 species [16]. mirDIP v4.1 provides nearly 152 million human microRNA target predictions from 30 different sources [17]. miRTarBase provides comprehensive information on experimentally validated miRNA-target interactions (MTIs) [18]. The Targetscan database is used to predict target genes downstream of miRNAs [19].

Go and pathway enrichment analysis of target gene

Metascape [20] (http://metascape.org) is a premium database that introduces a workflow that integrates gene annotation, membership analysis, and meta-analysis of polygenic lists. It's rich set of analysis tools is accessible through a convenient one-click quick analysis interface, and results are communicated through article-like analysis reports.

MCODE analysis, key candidate genes from PPI network

The STRING [21] (https://string-db.org/) database is designed to integrate all known and predicted associations between proteins, including physical interactions and functional associations. Among these associations, protein-protein interactions are particularly important due to their versatility, specificity, and adaptability. Minimum required interaction score: medium confidence (0.400). Network display options: hide disconnected nodes in the network.

MiRNA-mRNA axis

Through the STRING database, we further screened the predicted downstream target genes, and finally obtained hub target genes. We predict that miRNAs may affect hearing loss by regulating hub target genes.

Results

Audiogram

We performed hearing testing on clinical subjects, and all subjects received hearing tests on both ears. The audiogram is shown in Fig. 1.

Bioinformatics

In this study, a total of 29 DEMs were identified ($p < 0.05$, $|\log2FC| \geq 1$), including 17 up-regulated miRNA and 12 down-regulated miRNA (Table 1). The 17 up-regulated DEMs were hsa-miR-124-5p, hsa-novel-chr16_28339, hsa-novel-chr1_3515, hsa-novel-chr11_21630, hsa-miR-598-5p, hsa-novel-chr19_35129, hsa-novel-chr19_34296, hsa-novel-chr19_34928, hsa-miR-7151-5p, hsa-miR-135a-3p, hsa-novel-chr19_34132, hsa-novel-chr1_1231, hsa-miR-4423-3p, hsa-novel- chr11_22905, hsa-novel-chr10_20495, hsa-miR-4464, and hsa-

miR-497-5p. The 12 down-regulated DEMs were hsa-novel-chr11_21171, hsa-novel-chr11_22226, hsa-novel-chr1_2052, hsa-novel-chr21_36490, hsa-novel-chr22_35752, hsa-miR-455-3p, hsa-novel-chr5_10288, hsa-novel-chr1_1685, hsa-miR-216b-5p, hsa-miR-217-5p, hsa-miR-323b-3p, and hsa-miR-1908-5p. We used RStudio software to visualize these DEMs data in volcano plot and heatmap. The volcano plot marked five DEMs with the most significant changes in *P*-value (Fig. 2A). The heatmap shows all DEMs (Fig. 2B).

Table 1
29 differential expressed miRNAs (DEMs) were identified from hearing loss patiernts vs normal subjects, Including 17 upregulated DEMs and 12 downregulated DEMs.

Up-genes	hsa-miR-124-5p	hsa-novel-chr16_28339	hsa-novel-chr1_3515	hsa-novel-chr11_21630	hsa-miR-598-5p	hsa-novel-chr19_35129
	hsa-novel-chr19_34296	hsa-novel-chr19_34928	hsa-miR-7151-5p	hsa-miR-135a-3p	hsa-novel-chr19_34132	hsa-novel-chr1_1231
	hsa-miR-4423-3p	hsa-novel-chr11_22905	hsa-novel-chr10_20495	hsa-miR-4464	hsa-miR-497-5p	
Down-genes	hsa-novel-chr11_21171	hsa-novel-chr11_22226	hsa-novel-chr1_2052	hsa-novel-chr21_36490	hsa-novel-chr22_35752	hsa-miR-455-3p
	hsa-novel-chr5_10288	hsa-novel-chr1_1685	hsa-miR-216b-5p	hsa-miR-217-5p	hsa-miR-323b-3p	hsa-miR-1908-5p

MiRNA target gene prediction

Through four online databases, 13 downstream target genes of hsa-miR-124-5p were obtained, respectively *REV3L, PAGR1, CSTF2, NABP1, CELF2, TXNRD1, FOXG1, SUCO, MPEG1, LCOR, TAOK1, PLEKHA1*, and *ARF1* (Fig. 3 **and** Table 2). Downstream target genes predicted for each database are in **supplementary file**.

Table 2
miRNA-mRNA network of overlapping genes.

miRNA	mRNA
Hsa-miR-124-5p	REV3L
Hsa-miR-124-5p	PAGR1
Hsa-miR-124-5p	CSTF2
Hsa-miR-124-5p	NABP1
Hsa-miR-124-5p	CELF2
Hsa-miR-124-5p	TXNRD1
Hsa-miR-124-5p	FOXG1
Hsa-miR-124-5p	SUCO
Hsa-miR-124-5p	MPEG1
Hsa-miR-124-5p	LCOR
Hsa-miR-124-5p	TAOK1
Hsa-miR-124-5p	PLEKHA1
Hsa-miR-124-5p	ARF1

Gene ontology and pathway analysis

Metascape (https://metascape.org) was used to explore the functional enrichment of target genes downstream of hsa-miR-124-5p. $p < 0.05$ was set as the critical value. Functional annotation of metascape revealed that target genes were mainly enriched in DNA repair ($Log_{10}P$=-4.44), regulation of mitotic cell cycle ($Log_{10}P$=-3.00), and metabolism of lipids ($Log_{10}P$=-2.45) **(**Fig. 4 **and** Table 3**)**.

Table 3
GO and reactome analysis of 13 downstream target genes.

GO	Category	Description	Count	%	-Log10(P)	Log10(q)
GO:0006281	GO Biological Processes	DNA repair	4	30.77	-4.44	-0.09
GO:0007346	GO Biological Processes	regulation of mitotic cell cycle	3	23.08	-3.00	0.00
R-HSA-556833	Reactome Gene Sets	Metabolism of lipids	3	23.08	-2.45	0.00

MCODE analysis, key candidate genes from PPI network

We uploaded all 13 downstream target gene lists to the STRING database and constructed a protein-protein interaction (PPI) network. The results from the STRING database are as follows: Number of nodes: 13, Number of edges: 1, Average node degree: 0.154, Average local clustering coefficient: 0.154, Expected number of edges: 1, PPI enrichment *p*-value: 0.549 (Fig. 5).

MiRNA-mRNA axis

As shown in the Fig. 6, in the PPI network of 13 overlapping genes, we finally selected 2 downstream target genes. Furthermore, miRNA-mRNA network, including 2 mRNAs (CSTF2 and TXNRD1) and 1 miRNA (hsa-miR-124-5p), were constructed successfully.

Discussion

Hearing loss is a global public health event that deserves attention. The specific molecular biological mechanism of miRNA in the pathogenesis of hearing loss remains unclear. By applying bioinformatics methods to analyze the peripheral blood mononuclear cells of deaf patients, we found that hsa-miR-124-5p as a new biomarker is highly expressed in the peripheral blood of Hearing loss patients. In conclusion, in-depth study of the key genes and mechanisms is of great significance for the diagnosis and treatment of deafness.

MiRNA-seq has been widely used in the sequencing of clinical patient samples in recent years. This technology has made a huge contribution to some niche diseases. For example: neuropsychiatric diseases [22], multiple myeloma [23], glucocorticoid-induced osteoporosis [24], etc. However, to date, there have been few reports using this method to study the pathogenesis of hearing loss.

In this study, we took an integrative approach to construct miRNA-mRNA networks and analyzed key downstream target genes and pathways that may be regulated by miRNAs. First, we selected the most up-regulated DEMs and 13 downstream target genes in ARHL patients were identified. We found that 13 downstream target genes predicted by four databases were involved in the DNA repair, regulation of mitotic cell cycle, and metabolism of lipids pathway. Then, we constructed the miRNA-mRNA network.

Thirteen overlapping genes were found to affect hearing loss by participating in lipid metabolism processes. A related study reported that patients with hypercholesterolemia had more severe hearing loss ($p < .05$) [25]. Other studies have reported that sudden sensorineural hearing loss (SSNHL) is associated with increased blood lipid level. Total cholesterol (TC) and triglycerides (TG) are risk factors for hearing loss. The probability of SSNHL in subjects with elevated TC and TG levels increased by 2.20 times (95% CI 1.50–3.24) and 1.50 times (95% CI 1.08–2.08), respectively [26]. Hsa-miR-124-5p is involved in lipid metabolism as a novel biomarker for hearing loss. Hsa-miR-124-5p is highly expressed in patients with hearing loss, and the lipid metabolism in patients with deafness is higher than that in the normal control group. Therefore, we predict that Hsa-miR-124–5p promotes lipid metabolism in patients with hearing loss, causes lipid metabolism disorder, and participates in the biological process of deafness.

The main reason for hearing loss is the damage of auditory cells, and the main pathological change of hearing loss is auditory cells apoptosis [27]. The accumulation of reactive oxygen species in auditory cells is closely related to apoptosis. Our previous studies have found that some clinical drugs can cause the accumulation of ROS in auditory cells, intracellular activated PARP and activated caspase3 increased, which belong to the biological process of apoptosis [28]. We found that the expression of hsa-miR-124-5p was significantly increased in patients with hearing loss, so we speculated that the reason for deafness caused by hsa-miR-124-5p might be through the promotion of ROS accumulation and the change of apoptosis-related marker proteins in cochlear auditory cells.

Conclusion

In summary, we found hsa-miR-124-5p, as a potential regulatory hearing loss miRNA. The molecular role of hsa-miR-124-5p in hearing loss has not been fully elucidated. It is undeniable that our research has some obvious limitations. 1. The number of samples is small. 2. Further functional experiments are needed to support and verify the functions of these hsa-miR-124-5p in hearing loss.

Declarations

Acknowledgements

Thanks to the medical college of Xiamen University for providing experimental platform. We thank Zhongshan Hospital, Xiamen University for providing research funding.

Funding

3502Z20204009/Key Medical and Health Project of Xiamen Science and Technology Bureau

2020J02060/Natural Science Foundation of Fujian Science and Technology Department

2009CXB-29/Study on the Protective Effect and Mechanism of DNMT Inhibitor RG108 on Hair Cell Damage

Authors' contributions

All the authors have designed the thesis. Dongdong Zhang analyzed the sequencing data and completed the preliminary draft. Yixin Sun collated the abstract part of this article, Cong Zou collected clinical samples. Yongjun Hong conducted a listening test. Other authors participated in the design and made recommendations. Chengfu Cai finally corrected the manuscript.

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Ethics declarations

Ethics approval and consent to participate

This study has been approved by the ethics committees of Scientific Research Sub-Committee, Medical Ethics Committee, Zhongshan Hospital, Xiamen University (Approval number: 2021-077). All patients gave their consent to participate in this study.

Consent for publication

All patients gave their consent for the publication.

Competing interests

All authors declare that they have no competing interests.

Availability of data and material

All data for this study are available from the corresponding author.

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Figures

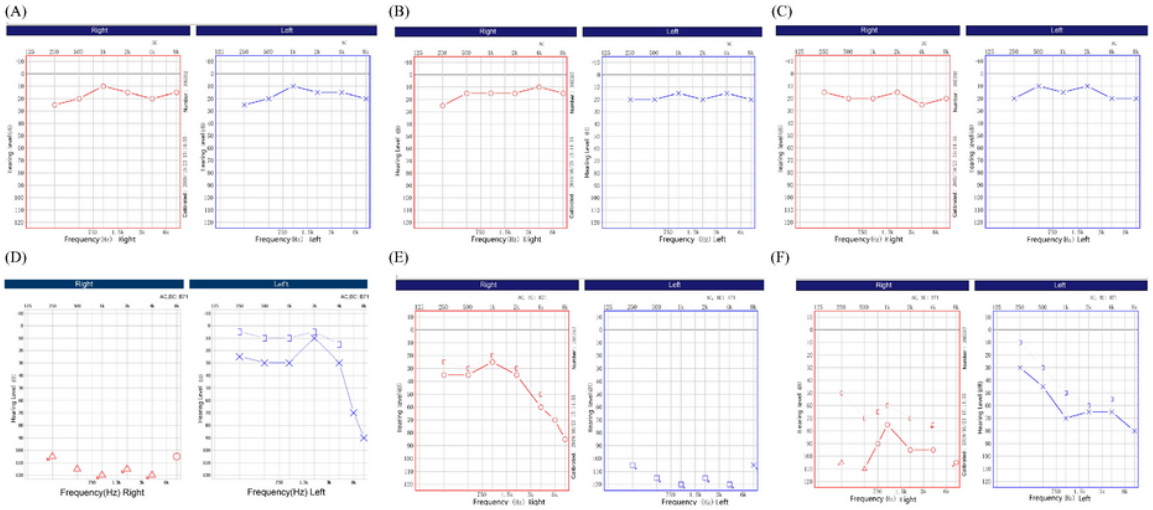

Figure 1

Audiograms of the subjects participating in this study. All patients hide person information. (A-C) Hearing diagrams of healthy subjects. (D-F) Hearing diagrams of patients with hearing impairment. All patients received bilateral hearing test. The red curve represents the right ear, and the blue curve represents the left ear.

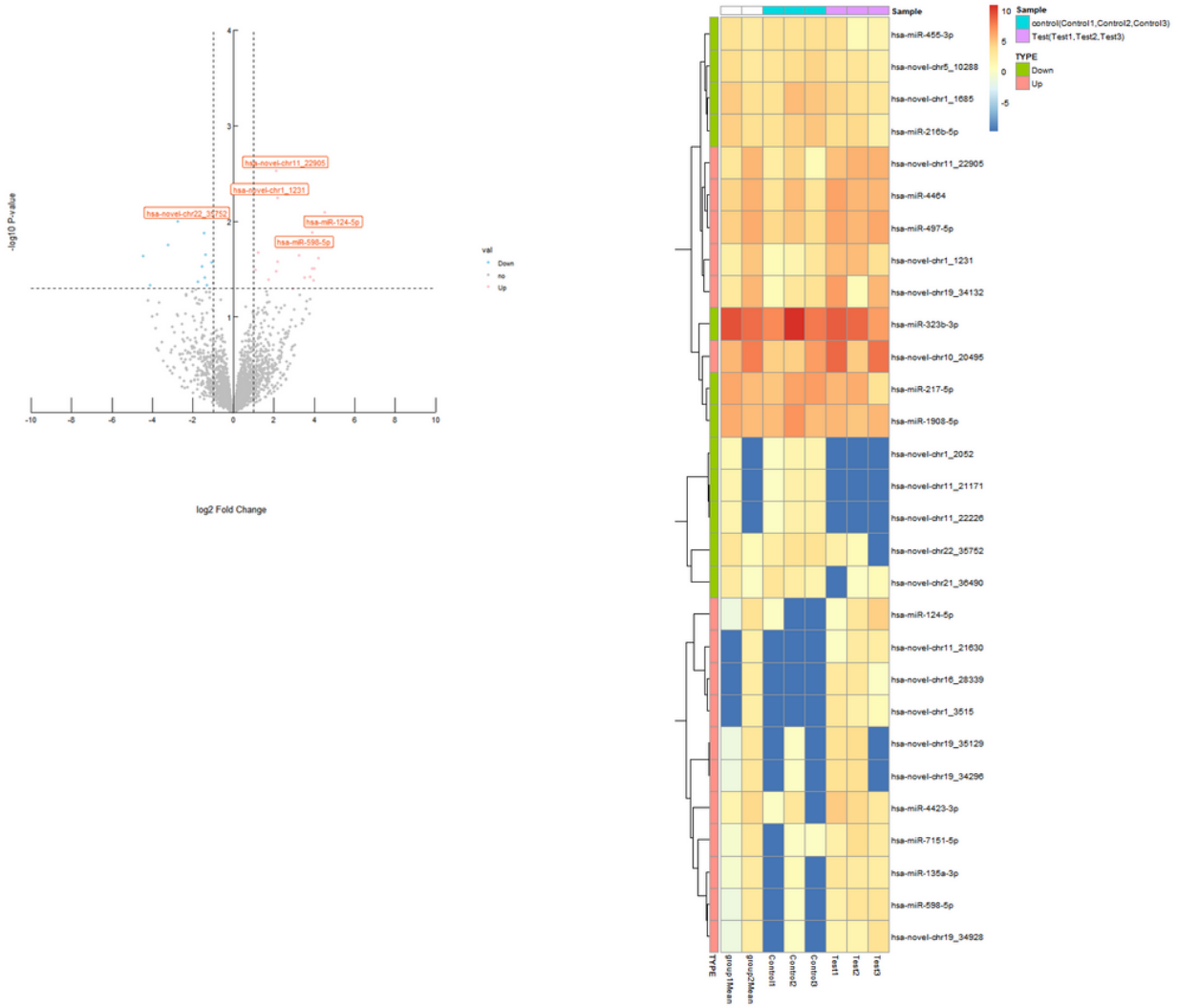

Figure 2

Genomic changes in normal subjects and hearing loss patients. (A) Volcano plot of DEMs. (B) Heatmap of DEMs.

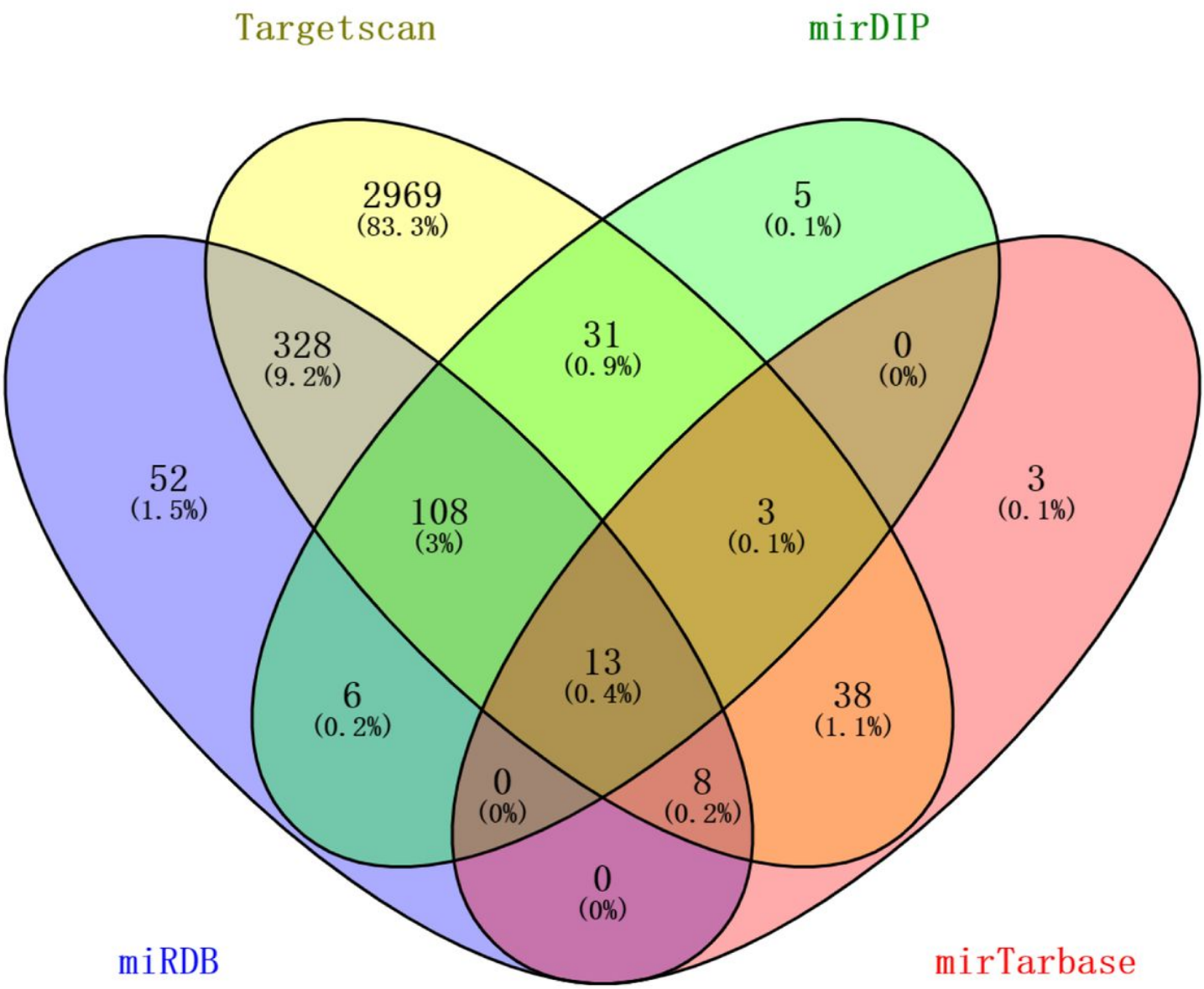

Figure 3

Overlapping genes shared by 4 databases.

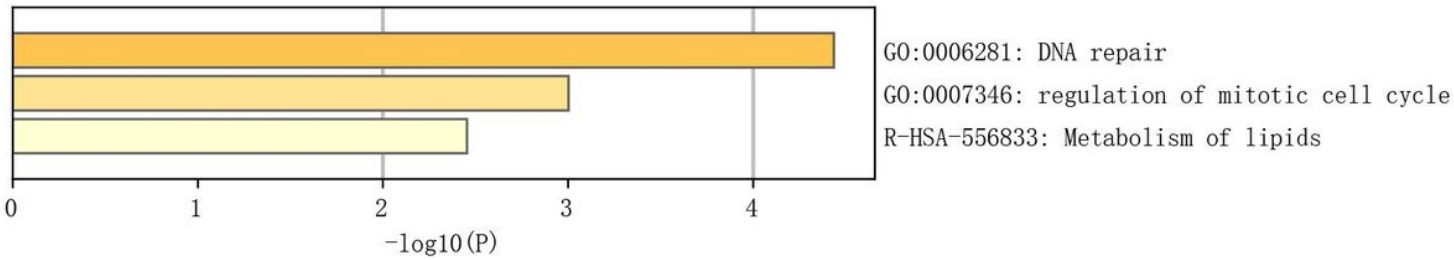

Figure 4

Biological function analysis of DEMs between hearing loss patients and normal subjects.

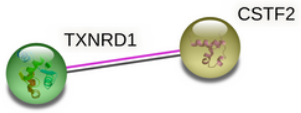

Figure 5

Two downstream target genes by PPI network analysis.

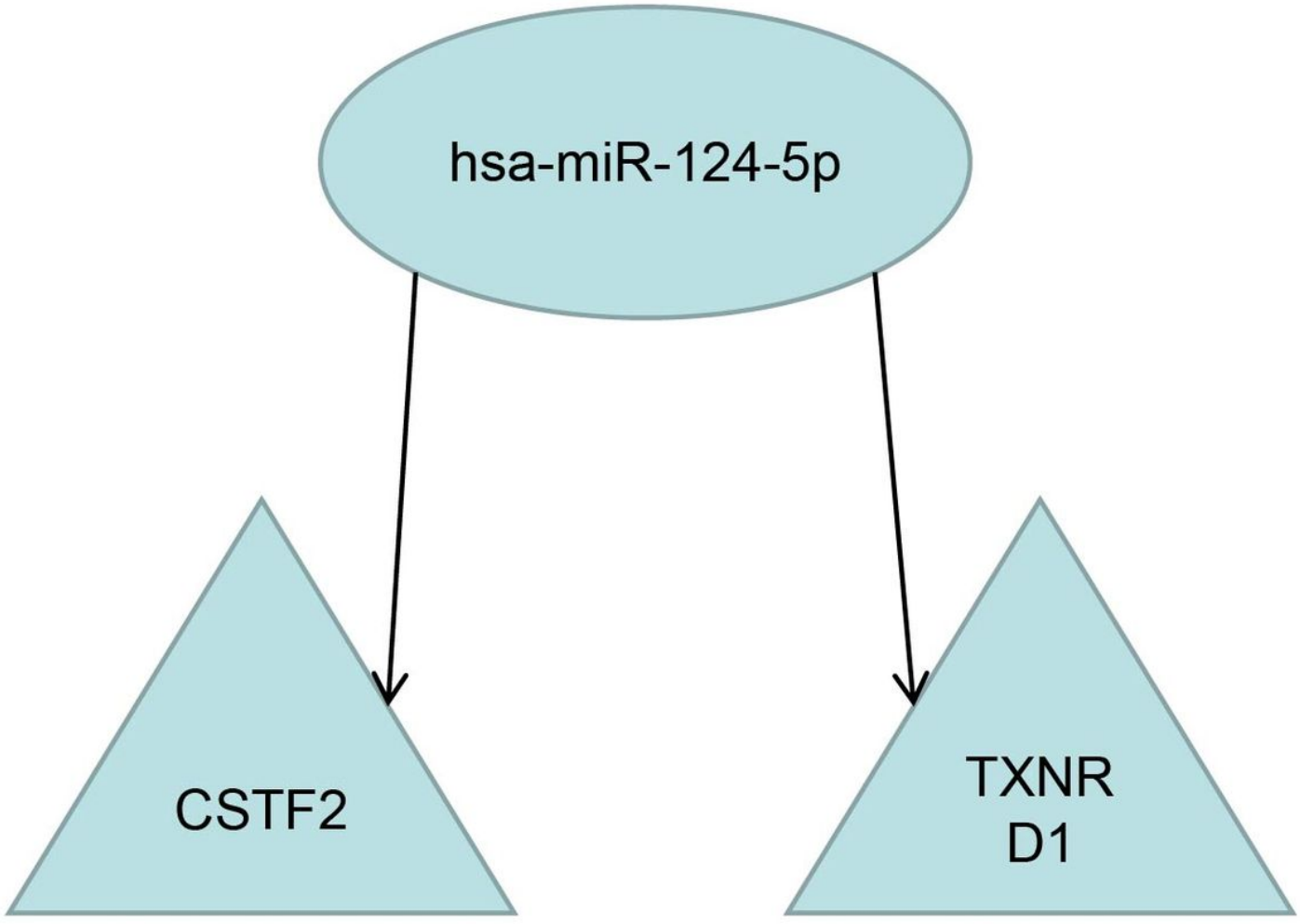

Figure 6

MiRNA-mRNA network. Circle represents miRNA, triangle represents mRNA.