Up-Regulated miR-224-5p Can Target the Expression of Neuritin in Hearing Loss

Yin Song
Jiaxing Center for Disease Control and Prevention
Xue Zhang
Tongxiang Center for Disease Control and Prevention
Jiawei Sun
Shihezi University School of Medicine
Lina Li
Jiaxing Center for Disease Control and Prevention
Xiaofei Zhang
Jiaxing Center for Disease Control and Prevention
Ping Li
Jiaxing Center for Disease Control and Prevention
Jin Huang (bio-huangjin@shzu.edu.cn)
Shihezi University School of Medicine
Yan Yong
Jiaxing Center for Disease Control and Prevention

Research Article

Keywords: Neuritin, hearing loss, miR-224-5p, Bioinformatics, High-throughput sequencing

Posted Date: December 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1038261/v1

Abstract

Objective: This study screened the differentially expressed miRNAs in the mouse cochlea during hearing loss and explored the relationship between miR-224-5p and Neuritin.

Methods: The combination of kanamycin sulfate and furosemide was used to establish a mouse hearing loss model. High-throughput sequencing was used to screen the differentially expressed miRNAs during hearing loss. qRT-PCR was used to identify the expression of differential miRNAs in hearing loss. Western Blot was used to detect the expression of Neuritin protein. Luciferase was used to identify the binding site of miRNA and Neuritin.

Results: The expression of miR-224-5p in the mouse cochlea increased during hearing loss ($p<0.05$). MiR-224-5p mimics can reduce Neuritin protein expression in 293T cells ($p<0.05$). MiR-224-5p can specifically bind to Neuritin ($p<0.05$).

Conclusion: The expression of miR-224-5p increases in hearing loss and targets the expression of Neuritin

Introduction

Hearing loss is the most widely distributed sensory disability in the world, and its negative impact is at the forefront of the global burden of disease[1]. According to WHO's data, the global disability deafness reached 466 million in 2019, and it is expected to exceed 900 million by 2050. Among people over 65 years old, one-third suffer from hearing loss. Hearing loss not only affects the ability to communicate with others and causes people's loneliness, but is also related to Alzheimer's[2] and mental illness[3]. In addition, hearing loss can also have a negative impact on the economy. The number of people at risk of hearing loss is increasing year by year, and we need to find a way to save them.

Sensorineural hearing loss (SNHL) occurs after the sensory hair cells or nerve structure of the cochlea is damaged, which accounts for the majority of hearing loss[4]. The main causes of hearing loss include aging, genetic mutations, noise exposure, exposure to therapeutic drugs with ototoxic side effects, and chronic diseases[5]. The current treatment for sensory hearing loss is mainly to use cochlear implants, but the use of cochlear implants requires patients to still have a certain number of functional hair cells and spiral neurons[6, 7]. Research on the treatment of hearing loss mainly focuses on the local delivery of drugs to the cochlea[8], gene therapy[9] and cell therapy[10]. For hearing loss, no drug-based therapy has been approved by the Food and Drug Administration, and treatment mostly relies on devices such as hearing aids and cochlear implants.

This approach that delivers neurotrophic factors in the cochlea to treat hearing loss has a promising future. Exogenous neurotrophins BNDF and NT-3 have been shown to promote the synaptic regeneration of SGN peripheral fibers in the hair cell, and save the hearing function of adult animals after acoustic trauma [11–13]. Neuritin is a neurotrophic factor closely related to neuroplasticity, which can significantly

promote the growth of neurites and their branch formation, as well as the development and maturity of synapses[14]. Neuritin can also regulate the formation of synaptic circuits[15], inhibit apoptosis, and maintain the survival of neurons[16]. Neuritin participates in the development of the central nervous system, and its expression is closely related to nerve regeneration and repair after injury, learning and memory[17]. Our previous study found that the expression of Neuritin in the mouse cochlea decreased after hearing loss. When exogenous Neuritin protein was administered to the cochlea of mice, the damage of mouse hair cells was reduced, and the hearing function can be restored to a certain extent. What's more, we also observe the same phenomenon in the hidden hearing loss caused by noise.

miRNA is defined as a set of short non-coding RNAs that help regulate gene expression[18]. Since 1993, many miRNAs that play a role in the normal development of the inner and outer ears have been discovered[19, 20]. Most protein-coding genes in vertebrates are regulated by miRNA, so the ability of miRNA to regulate mRNA in the auditory system is worthy of further study[21, 22].

Neuritin, as a neurotrophic factor, shows an improvement effect on hearing loss, and the miRNA that regulates its effect in the cochlea is unclear. For this reason, this article mainly explores miRNAs that may regulate Neuritin expression in hearing loss.

Materials And Methods

All procedures were approved by the Animal Ethical and Welfare Ethics Committee of Hangzhou Normal University and were performed in accordance with relevant guidelines and regulations. All our animal experiments methods were in accordance with ARRIVE guidelines.

1. Construction of hearing loss mice

In this study, a combination of kanamycin sulfate and furosemide was used to establish a model of acute and severe sensorineural hearing loss. Two-month-old CBA mice (without gender distinction) were selected, and each mouse was injected subcutaneously with kanamycin sulfate (Biosharp, BS152) 1,000 $mg{\cdot}kg^{-1}$, and after waiting for half an hour, the mice was injected with furosemide (Sigma, PHR1057) 500 $mg{\cdot}kg^{-1}$ to establish a hearing loss model. In this experiment, the mice were divided into 3 groups, the first group was the control group, the second group was the drug-induced injury group for 12 hours, and the third group was the drug-induced injury group for 24 hours. The animals used in this experiment were CBA mice (SPF grade), and all the experimental animals were purchased from the Experimental Animal Center of Hangzhou Normal University.

2. Auditory Brainstem Response (ABR)

Each CBA mouse was intraperitoneally injected with sodium pentobarbital (50 $mg{\cdot}kg^{-1}$) and placed in a standard shielded soundproof room after anesthesia. The detection electrodes were placed behind the left and right auricles and under the scalp of the mouse to perform the auditory brainstem response of the mouse. The stimulus sound is a short stimulus, the scanning duration is 10 ms, the stimulus

repetition rate is 11 times·s^{-1}, the filter bandpass is 150-1 500 Hz, and the superimposition is 500-1 000 times. Each mouse was tested for binaural hearing, and the second wave threshold was used as the mouse hearing threshold.

3. Preparation of Corti RNA

The cochlea was removed immediately after the mouse was sacrificed by cervical dislocation, and was quickly transferred to a dissecting microscope to strip the outer cochlear bone structure and remove the Corti organ. Combine 6 Corti apparatuses into a set, grind with liquid nitrogen and transfer the powder to a 1.5 mL EP tube. Add 1ML TRNzol Universal ageant to the EP tube, mix well and let stand for 10 minutes. Add 200ul chloroform to the EP tube, shake vigorously for 30s and then let it stand for 10 minutes. Centrifuge at 12000 rpm for 15 minutes. Take the upper liquid to a new 1.5ML EP tube, add 500 mL of isopropanol, mix well and let it stand at room temperature for 10 minutes. Centrifuge at 12000 rpm for 10 minutes. Discard the supernatant, add 1ML of 75% ethanol, and let stand at room temperature for 5 minutes. Centrifuge at 8000rpm for 5 minutes, discard the supernatant, add 20ul DEPC-treated water, and store at -80°C.

4. miRNA sequencing and bioinformatics analysis

The RNA-seq method was used to perform miRNA sequencing on sample RNA, and the sequencing depth of each sample was 20 M. The DEG-seq method was used to analyze the miRNA sequencing results and screen the miRNAs with different expressions. The screening criteria are log2FC>1 and Qvalue<0.001. The miRNA that was up-regulated in the 12-hour group and the 24-hour group was selected as a candidate miRNA. "NRN1" is used as a keyword to predict the miRNA targeting Neuritin gene from 7 different websites (TargetScan, miRDB, DIANA, miRNAMap, miRWalK, miRmap, trabase), and then take the intersection with the candidate miRNA to obtain the possible targeted regulation of Neuritin and Candidate miRNAs that are up-regulated during hearing loss.

5. q-PCR

The stem-loop method was used to synthesize candidate miRNA primers, and U6 was used as an internal reference. According to Takara Reverse Transcription Kit (Takara RR 037A) 10 μL system (5×PrimeScript Buffer 2 μL, PrimeScript RT Enzyme Mix I 0.5 μL, PCR Reverse Primer 0.5μL, Total RNA 2 μL, ddH_2O 5μL) the extracted RNA was reverse transcribed into cDNA. The cycle conditions were: 95℃ 30s (1 cycle), 95℃ 5s, 60℃ 30s (40 cycles)). According to the 20 μL system of Takara qPCR kit(TB Green Premix Ex Taq II 10 μL, PCR Forward Primer 0. 8 μL, PCR Reverse Primer 0. 8 μL, cDNA 2μL, ddH_2O 6.4 μL), the obtained cDNA was subjected to real-time fluorescence quantitative PCR, and the cycling conditions were 95℃ 30s (1 cycle), 95℃ 5s, and 60℃ 30s (40 cycles). Use the fluorescence quantitative PCR instrument (Germany analytik-jena 870wer 3G) to measure the fluorescence of the sample, and calculate the CT value and TM value. The △△t method was used to calculate the differential expression folds of candidate miRNAs between the 12 h group, 24 h group and the control group, and the target miRNAs were screened according to the differential fold.

The primers were designed by the Primer Premier 5.0 Software and as follows:

miR-224-5p-F: 5’- CGCGCGTAAGTCACTAGTGGT -3’

miR-224-5p-RT: 5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACGGA-3’

U6-F: 5’-CTCGCTTCGGCAGCACA-3’

U6-R: 5’-AACGCTTCACGAATTTGCGT-3’

6. Western blot

The synthetic mimics of candidate miRNAs were transfected into 293T cells with Lipofectamine 3000 (ThermoFisher, L3000015), and after 48 hours of culture, the cells were lysed with RIPA (Beyotime, P0013B) to extract total protein. Configure a 15% concentration SDS-PAGE gel, separate about 60-100ug of protein on the SDS-PAGE gel under the conditions of 80 V for 30 min and 110 V for 90 min, and use the semi-dry transfer method to transfer the protein under the conditions of 23 V for 43 min. Onto PVDF membrane (Immobilon, ISEQ00010). Use 5% skimmed milk powder (Biosharp, BS 102) to seal the PVDF membrane for 2 h, then use 5% skimmed milk powder to dilute Neuritin antibody (Abcam, 64186) at a ratio of 1:1000, and incubate overnight at 4°C. On the next day, the goat anti-rabbit secondary antibody (Nakayama Jinqiao) was diluted at a ratio of 1:10000, incubated at room temperature for 2 hours, and an enhanced chemiluminescence system (BIO-RAD, 1705060) was used to detect the immune response zone.

7. Luciferase

Neuritn and miR-224-5P binding sites were predicted through miRmap (https://mirmap.ezlab.org/). Neuritn sequences with a length of 60 bp were cloned into the GP-miRGLO vector. GP-miRGLO vector and miR-224-5P mimics rely on Lipofectamine 3000 (Thermofisher, L3000015) to be transfected into 293T cells. After 48 hours of transfection, the lysate was collected, and the phochillase report system (Dual-Luciferase®Reporter (DLR™) Assay System,E1980) was used to measure the photinus pyralis luciferase and Renilla ReniFormis luciferase activity. Computational fluorescence activity ratio of firefly and sea kidney fluorescence activity. Three biological repeats were performed.

The Neuritn sequences were cloned into the GP-miRGLO vector as follows:

NRN1 -miR-224-5p WT:
GAGAGGGAAAAGGAGAAGGCCAGGGGAATGACTTCAAGAGTGGTGTCCACGTGGGAATCA

NRN1 -miR-224-5p MUT:
GAGAGGGAAAAGGAGAAGGCCAGGGGAAACTGAACAAGAGTGGTGTCCACGTGGGAATCA

Results

1. Hearing loss model construction and identification

CBA mice are commonly used listening loss model mice. We chose the normal 2-month-old CBA mice to build a hearing loss model, which was injected with sulfate pamycin and fuff in combination[23, 24]. The results show that the hearing wave disappears in the 20-70 dB stage of the hearing loss mouse (Fig. 1A), the hearing threshold rises from 20-30 dB to 70-80 dB ($p<0.05$) (Fig. 1B). The hearing waveform and hearing threshold of the control group were still normal. After the hearing loss model is constructed, the mouse ear Neuritin protein was detected at different times by WB. The WB test results show that the expression of Neuritin in mice has decreased significantly after drug intervention ($p<0.05$) (Fig. 1C-D).

2. Analysis and screening of differentially expressed miRNAs

After we obtained the mouse Corti RNA, miRNA was sequenified by using RNA-seq. The sequencing results were analyzed by DEG-seq, and miRNA was screened in accordance with $|log2FC| >1$, Qvalue<0. 01. In the 12h group, 80 significantly up-regulated miRNAs and 169 significantly down-regulated miRNAs were found (Fig. 2A-B). In the 24h group, 40 significantly up-regulated miRNAs and 94 significantly down-regulated miRNAs were found (Fig. 2C-D). After overlapping up-regulated miRNAs of 12h group and 24h group, we found 24 overlapping up-regulated miRNAs (Fig. 2E). For miRNAs that are up-regulated in both groups(Table 1), we believe that its credibility is higher.

Table 1
Co-upregulated miRNAs of 12h group and 24h group.

Name	Log2FC(12h)	Log2FC(24h)
mmu-miR-6395	9.902375114	8.294620749
novel-mmu-miR208-3p	8.347842794	5.787902559
mmu-miR-196a-5p	4.883149314	4.180081616
novel-mmu-miR122-3p	4.804710201	3.253756592
mmu-miR-365-3p	3.827926532	2.736448481
novel-mmu-miR202-3p	3.744036623	2.552801226
novel-mmu-miR365-5p	3.379729601	2.401263919
mmu-miR-1247-3p	2.17951105	2.387730153
mmu-miR-211-5p	2.02469942	2.349374161
mmu-miR-3073a-5p	1.790449884	2.307428525
novel-mmu-miR13-5p	1.751320887	1.916243595
mmu-miR-181a-2-3p	1.620006171	1.56877381
mmu-miR-133b-3p	1.618699686	1.460072816
mmu-miR-21a-3p	1.576811863	1.450963418
mmu-miR-93-3p	1.52420847	1.386963105
mmu-miR-145a-5p	1.463477479	1.304519703
mmu-miR-133a-3p	1.412191078	1.248273853
mmu-miR-224-5p	1.308867767	1.243959048
mmu-miR-1198-5p	1.295626098	1.202791709
mmu-let-7c-2-3p	1.295626098	1.132215524
mmu-let-7a-1-3p	1.209508516	1.132215524
novel-mmu-miR130-5p	1.142836059	1.116049148
mmu-miR-1247-5p	1.115747867	1.083187075
mmu-miR-339-5p	1.051379351	1.065787458

3. Correlation analysis of Neuritin and hearing loss

Seven bioinformatics (TargetScan, mRDB, DIANA, miRNAMap, miRWalK, miRmap, trabase) websites were used to predict miRNAs targeted Neuritin and 1592 miRNAs were found. From 1592 miRNAs and 24 up-regulated miRNAs we found 15 identical miRNAs (miR-6395,miR-211-5p,miR-196a-5p,miR-3073a-5p,miR-133b-3p,miR-21a-3p,miR-1247-3p,miR-181a-2-3p,miR-224-5p,miR-133a-3p,miR-1247-5p,miR-145a-5p,miR-93-3p,miR-339-5p and miR-1198-5p) (Fig. 3A). These 15 miRNAs are related to hearing loss and Neuritin. Rudnicki[25] found that miR-224-5P can induce the occurrence of endonal inflammatory stimulation, which affects hearing. This makes us more believe that miRNA-224-5P may play an important role in hearing loss, so the miR-224-5p is listed as target miRNA.

4. MiRNA-224-5p is upregulated in hearing loss and negative related to Neuritin

We synthesized primers for miR-224-5p, and U6 was used as an internal reference. qRT-PCR was used to detect the expression of miR-224-5p in the cochlea after hearing loss. The results of qRT-PCR showed that the expression of miR-224-5p increased after 12h and 24h of hearing loss in mice ($P<0.05$) (Fig. 4A). After confirming that the expression of miR-224-5p increased, we synthesized mimics of miRNA-224-5p. WB was used to detect Neuritin expression after miRNA-224-5p mimics were transfected into 293T cells. The WB results showed that after miR-224-5p intervention, the expression of Neuritin was inhibited, and there was a significant decrease (Fig. 4B-C). miR-224-5p is not only related to hearing, but also inhibits the expression of Neuritin.

5. miR-224-5p and Neuritn are specific binding

MiR-224-5p showed an inhibitory effect on Neuritin. In order to identify whether this effect is targeted, we conducted a dual luciferase reporter gene experiment. Through the mir-map website, we predicted the binding site of miR-224-5p and Neuritin, and the results are shown in Fig. 5A. We cloned the 60bp Neuritin sequence into the GP-miRGLO vector for dual luciferase experiments(Fig. 5B-C). The results showed that after transfection of the GP-miRGLO vector and miR-224-5p mimics, the expression of Neuritin in the WT group decreased ($p<0.05$) (Fig. 5D). And the expression of Neuritin in the Control group and the MUT group was normal.

Discussion

In this study, we used kanamycin sulfate combined with furosemide to induce sensorineural hearing loss in CBA mice. For a long time, it has been a classic method for building sensorineural hearing loss models that combines aminoglycosides and diuretics[26, 27]. When aminoglycosides and diuretics are used in combination, diuretics can promote the passage of aminoglycosides through the blood-cochlear barrier[28, 29], change the lymphatic potential of the inner ear to make aminoglycosides more effectively diffuse to the cochlea[30, 31], and Reduce the renal clearance of kanamycin[32]. This is a rapid and violent deafness reaction and the cochlear hair cells will fall off after 36 hours. In order to detect changes in the miRNA of hair cells during hearing loss, we must extract RNA before the hair cells fall off. In order to accurately observe the changes of miRNA, we finally selected two time points of 12h and 24h for detection. Research data on miRNAs related to hearing loss is scarce, so we built a hearing loss model to

extract RNA from the Corti for miRNA sequencing. Through RNA-seq sequencing, we obtained miRNAs that were differentially expressed in the 12-hour group and the 24-hour group. In order to increase the accuracy of the results, we took the intersection of the results of the two groups and obtained 24 miRNAs that were up-regulated during hearing loss. In hearing loss, Neuritin expression decreases, and the corresponding miRNA should be up-regulated, so we mainly focus on up-regulation of miRNA. The bioinformatics website was used to predict miRNAs targeting Neuritin. Each website has a different prediction method. In order to get as comprehensive results as possible, we selected 7 websites for prediction (TargetScan, miRDB, DIANA, miRNAMap, miRWalK, miRmap, trabase). In the end, we got 1592 miRNAs that may target Neuritin. Fortunately, among these miRNAs, we found 15 miRNAs that overlap with the up-regulated miRNAs in hearing loss. Investigating 15 miRNAs, we found that miR-224-5p is involved in cochlear inflammation[25], so we are more convinced that miR-224-5p is related to hearing loss. After we identified miR-224-5p as the target miRNA, we first used qRT-PCR to identify the relationship between hearing loss and miR-224-5p, then used WB to identify the relationship between Neuritin and miR-224-5p, and finally used Luciferase to identify the binding site of Neuritin and miR-224-5p. Our results indicate that miR-224-5p is not only up-regulated in hearing loss, but also targeted to inhibit Neuritin expression.

The relationship between Neuritin and hearing continues to be studied. Linhares[33] found that in patients with Subtelomeric 6p25 deletion/duplication, Neuritin duplicated, and its clinical manifestations also showed hearing loss. Leijon[34] found that Neuritin expression changes in SPON of hearing loss mice, and may affect hearing by regulating neuronal survival and plasticity. Liu[35] also found that Neuritin is one of the risk factors for noise-induced hearing loss when analyzing the risk factors for hearing loss. These findings all point to a correlation between hearing loss and Neuritin, which coincides with our experimental results.

Research on miR-224-5p is not limited to hearing loss. Zhu[36] found that LINC00094 inhibited endotoxin-1 expression by up-regulating miR-224-4p/miR-497-5p, promoted the expression of ZO-1, Okrudin and Claudine-5, and finally alleviated BBB Permeability in the AD microenvironment. Du[37] found that MiR-224-5p can reduce the activation of microglial inflammation by regulating the expression of NLRP3. Liu[38] found that miR-224-5p plays a vital role in hypoxic neuronal injury through NR4A1, which may be an important regulatory mechanism of neuronal OGD injury. These clues that miR-224-5p is related to inflammation and nerves suggest that miR-224-5p may play a role in hearing loss. Hearing loss is also an inflammatory stimulus or neurodegenerative disease. Not only that, miR-224-5p is also involved in the development of a variety of tumors, such as breast cancer[39], glioma[40], gastric cancer[41], prostate cancer[42], melanoma[43], etc.

There is no effective treatment for hearing loss. Our discovery suggests a new possibility. We can reduce the expression of miR-224-5p in the cochlea to increase the expression of Neuritin, which can alleviate the occurrence of hearing loss. This will provide new ideas for hearing loss treatment. At the same time miR-224-5p can be used as a new marker of hearing loss.

In summary, the results of our research show that miR-224-5p is up-regulated during hearing loss and can target to inhibit the expression of NRN1. miR-224-5p is promising as a potential marker of hearing loss and a potential therapeutic drug.

Conclusion

miR-224-5p is elevated in hearing loss and targets the expression of Neuritin.

Declarations

Acknowledgments

The authors would like to thank the Animal Center of Hangzhou Normal University for breeding the mice. Dr Lei Yang (Department of Prevention Medicine, School of Medicine, Hangzhou Normal University, Hangzhou, Zhejiang 310036, P.R. China) kindly provided the experimental equipment.

Funding

The present study was supported by the Zhejiang Provincial Natural Science Foundation of China (grant no. LY18H260002 to YH).

Author contributions

JH contributed to the conception or design of the study. YS, XZ and JS contributed to the acquisition, analysis, or interpretation of all cell-level data. YS contributed to the acquisition, analysis and interpretation of the bioinformatics data. YS, YY, LL, PL, XZ and JH contributed to the drafting of the the article and revising it critically for all content. All authors contributed to revising the work critically for important intellectual content.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of interest

All experimental procedures were approved by the Animal Ethics Committee of Hangzhou Normal University.

Statement

All our animal experiments meet the requirements of animal ethics and are supervised by the Animal Ethical and Welfare Ethics Committee of Hangzhou Normal University. Our research accord to the ARRIVE guidelines (PLoS Bio 8(6), e1000412,2010).

References

1. Karikari TK, Charway-Felli A, Hoglund K, Blennow K, Zetterberg H: **Commentary: Global, regional, and national burden of neurological disorders during 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015**. *Front Neurol* 2018, **9**:201.
2. Ray M, Dening T, Crosbie B: **Dementia and hearing loss: A narrative review**. *Maturitas* 2019, **128**:64-69.
3. Blazer DG, Tucci DL: **Hearing loss and psychiatric disorders: a review**. *Psychol Med* 2019, **49**(6):891-897.
4. Walling AD, Dickson GM: **Hearing loss in older adults**. *Am Fam Physician* 2012, **85**(12):1150-1156.
5. Cunningham LL, Tucci DL: **Hearing Loss in Adults**. *N Engl J Med* 2017, **377**(25):2465-2473.
6. Pfingst BE, Bowling SA, Colesa DJ, Garadat SN, Raphael Y, Shibata SB, Strahl SB, Su GL, Zhou N: **Cochlear infrastructure for electrical hearing**. *Hear Res* 2011, **281**(1-2):65-73.
7. Cook JA, Hawkins DB: **Hearing loss and hearing aid treatment options**. *Mayo Clin Proc* 2006, **81**(2):234-237.
8. Ma Y, Wise AK, Shepherd RK, Richardson RT: **New molecular therapies for the treatment of hearing loss**. *Pharmacol Ther* 2019, **200**:190-209.
9. Omichi R, Shibata SB, Morton CC, Smith RJH: **Gene therapy for hearing loss**. *Hum Mol Genet* 2019, **28**(R1):R65-R79.
10. Pauley S, Kopecky B, Beisel K, Soukup G, Fritzsch B: **Stem cells and molecular strategies to restore hearing**. *Panminerva Med* 2008, **50**(1):41-53.
11. Sly DJ, Campbell L, Uschakov A, Saief ST, Lam M, O'Leary SJ: **Applying Neurotrophins to the Round Window Rescues Auditory Function and Reduces Inner Hair Cell Synaptopathy After Noise-induced Hearing Loss**. *Otol Neurotol* 2016, **37**(9):1223-1230.
12. Suzuki J, Corfas G, Liberman MC: **Round-window delivery of neurotrophin 3 regenerates cochlear synapses after acoustic overexposure**. *Sci Rep* 2016, **6**:24907.
13. Wan G, Gomez-Casati ME, Gigliello AR, Liberman MC, Corfas G: **Neurotrophin-3 regulates ribbon synapse density in the cochlea and induces synapse regeneration after acoustic trauma**. *Elife* 2014, **3**.
14. Naeve GS, Ramakrishnan M, Kramer R, Hevroni D, Citri Y, Theill LE: **Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis**. *Proc Natl Acad Sci U S A* 1997, **94**(6):2648-2653.
15. Cantallops I, Cline HT: **Rapid activity-dependent delivery of the neurotrophic protein CPG15 to the axon surface of neurons in intact Xenopus tadpoles**. *Dev Neurobiol* 2008, **68**(6):744-759.

16. Gao R, Li X, Xi S, Wang H, Zhang H, Zhu J, Shan L, Song X, Luo X, Yang L *et al*: **Exogenous Neuritin Promotes Nerve Regeneration After Acute Spinal Cord Injury in Rats**. *Hum Gene Ther* 2016, **27**(7):544-554.
17. Di Giovanni S, Faden AI, Yakovlev A, Duke-Cohan JS, Finn T, Thouin M, Knoblach S, De Biase A, Bregman BS, Hoffman EP: **Neuronal plasticity after spinal cord injury: identification of a gene cluster driving neurite outgrowth**. *FASEB J* 2005, **19**(1):153-154.
18. Patel M, Hu BH: **MicroRNAs in inner ear biology and pathogenesis**. *Hear Res* 2012, **287**(1-2):6-14.
19. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.
20. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: **Identification of novel genes coding for small expressed RNAs**. *Science* 2001, **294**(5543):853-858.
21. Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, Rigoutsos I: **A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes**. *Cell* 2006, **126**(6):1203-1217.
22. Friedman RC, Farh KK, Burge CB, Bartel DP: **Most mammalian mRNAs are conserved targets of microRNAs**. *Genome Res* 2009, **19**(1):92-105.
23. Ma L, Yi HJ, Yuan FQ, Guo WW, Yang SM: **An efficient strategy for establishing a model of sensorineural deafness in rats**. *Neural Regen Res* 2015, **10**(10):1683-1689.
24. Hirose K, Sato E: **Comparative analysis of combination kanamycin-furosemide versus kanamycin alone in the mouse cochlea**. *Hear Res* 2011, **272**(1-2):108-116.
25. Rudnicki A, Shivatzki S, Beyer LA, Takada Y, Raphael Y, Avraham KB: **microRNA-224 regulates Pentraxin 3, a component of the humoral arm of innate immunity, in inner ear inflammation**. *Hum Mol Genet* 2014, **23**(12):3138-3146.
26. West BA, Brummett RE, Himes DL: **Interaction of kanamycin and ethacrynic acid. Severe cochlear damage in guinea pigs**. *Arch Otolaryngol* 1973, **98**(1):32-37.
27. Webster M, Webster DB: **Spiral ganglion neuron loss following organ of Corti loss: a quantitative study**. *Brain Res* 1981, **212**(1):17-30.
28. Ding D, McFadden SL, Browne RW, Salvi RJ: **Late dosing with ethacrynic acid can reduce gentamicin concentration in perilymph and protect cochlear hair cells**. *Hear Res* 2003, **185**(1-2):90-96.
29. Ding D, McFadden SL, Woo JM, Salvi RJ: **Ethacrynic acid rapidly and selectively abolishes blood flow in vessels supplying the lateral wall of the cochlea**. *Hear Res* 2002, **173**(1-2):1-9.
30. van Emst MG, Klis SF, Smoorenburg GF: **Identification of the nonlinearity governing even-order distortion products in cochlear potentials**. *Hear Res* 1997, **114**(1-2):93-101.
31. Asakuma S, Snow JB, Jr.: **Effects of furosemide and ethacrynic acid on the endocochlear direct current potential in normal and kanamycin sulfate-treated guinea pigs**. *Otolaryngol Head Neck Surg (1979)* 1980, **88**(2):188-193.

32. Ohtani I, Ohtsuki K, Omata T, Ouchi J, Saito T: **Potentiation and its mechanism of cochlear damage resulting from furosemide and aminoglycoside antibiotics**. *ORL J Otorhinolaryngol Relat Spec* 1978, **40**(1):53-63.
33. Linhares ND, Svartman M, Rodrigues TC, Rosenberg C, Valadares ER: **Subtelomeric 6p25 deletion/duplication: Report of a patient with new clinical findings and genotype-phenotype correlations**. *Eur J Med Genet* 2015, **58**(5):310-318.
34. Leijon SC, Peyda S, Magnusson AK: **Temporal processing capacity in auditory-deprived superior paraolivary neurons is rescued by sequential plasticity during early development**. *Neuroscience* 2016, **337**:315-330.
35. Liu SY, Song WQ, Xin JR, Li Z, Lei S, Chen YQ, Zhao TY, Wang HY, Xu LW, Zhang MB *et al*: **NRN1 and CAT Gene Polymorphisms, Complex Noise, and Lifestyles interactively Affect the Risk of Noise-induced Hearing Loss**. *Biomed Environ Sci* 2021, **34**(9):705-718.
36. Zhu L, Lin M, Ma J, Liu W, Gao L, Wei S, Xue Y, Shang X: **The role of LINC00094/miR-224-5p (miR-497-5p)/Endophilin-1 axis in Memantine mediated protective effects on blood-brain barrier in AD microenvironment**. *J Cell Mol Med* 2019, **23**(5):3280-3292.
37. Du P, Wang J, Han Y, Feng J: **Blocking the LncRNA MALAT1/miR-224-5p/NLRP3 Axis Inhibits the Hippocampal Inflammatory Response in T2DM With OSA**. *Front Cell Neurosci* 2020, **14**:97.
38. Liu LL, Qiao S, Wang ML, Wu HK, Su YX, Wang KM, Liu XW: **MiR224-5p Inhibitor Restrains Neuronal Apoptosis by Targeting NR4A1 in the Oxygen-Glucose Deprivation (OGD) Model**. *Front Neurosci* 2020, **14**:613.
39. Zang CS, Huang HT, Qiu J, Sun J, Ge RF, Jiang LW: **MiR-224-5p targets EGR2 to promote the development of papillary thyroid carcinoma**. *Eur Rev Med Pharmacol Sci* 2020, **24**(9):4890-4900.
40. Zheng SQ, Qi Y, Wu J, Zhou FL, Yu H, Li L, Yu B, Chen XF, Zhang W: **CircPCMTD1 Acts as the Sponge of miR-224-5p to Promote Glioma Progression**. *Front Oncol* 2019, **9**:398.
41. Fang X, Dong Y, Yang R, Wei L: **LINC00619 restricts gastric cancer progression by preventing microRNA-224-5p-mediated inhibition of OPCML**. *Arch Biochem Biophys* 2020, **689**:108390.
42. Gan BL, Zhang LJ, Gao L, Ma FC, He RQ, Chen G, Ma J, Zhong JC, Hu XH: **Downregulation of miR2245p in prostate cancer and its relevant molecular mechanism via TCGA, GEO database and in silico analyses**. *Oncol Rep* 2018, **40**(6):3171-3188.
43. Wang X, Wang Y, Lin F, Xu M, Zhao X: **Long non-coding RNA LINC00665 promotes melanoma cell growth and migration via regulating the miR-224-5p/VMA21 axis**. *Exp Dermatol* 2020.

Figures

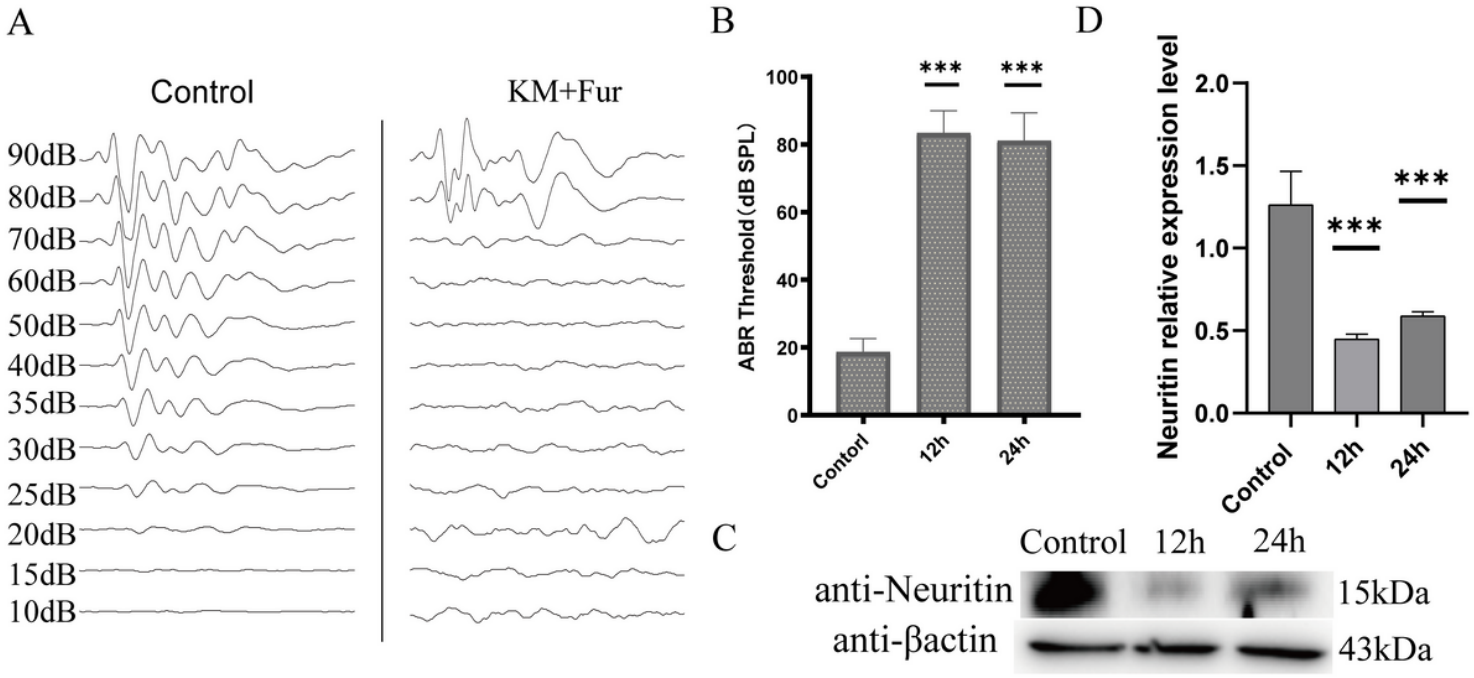

Figure 1

Mouse hearing function and Neuritin expression test results of hearing loss model mice A: ABR test results of mice. B: Statistical results of ABR test results of mice. C: Neuritin expression in mouse cochlea after hearing loss. D: Statistical results of Neuritin expression in mouse cochlea.

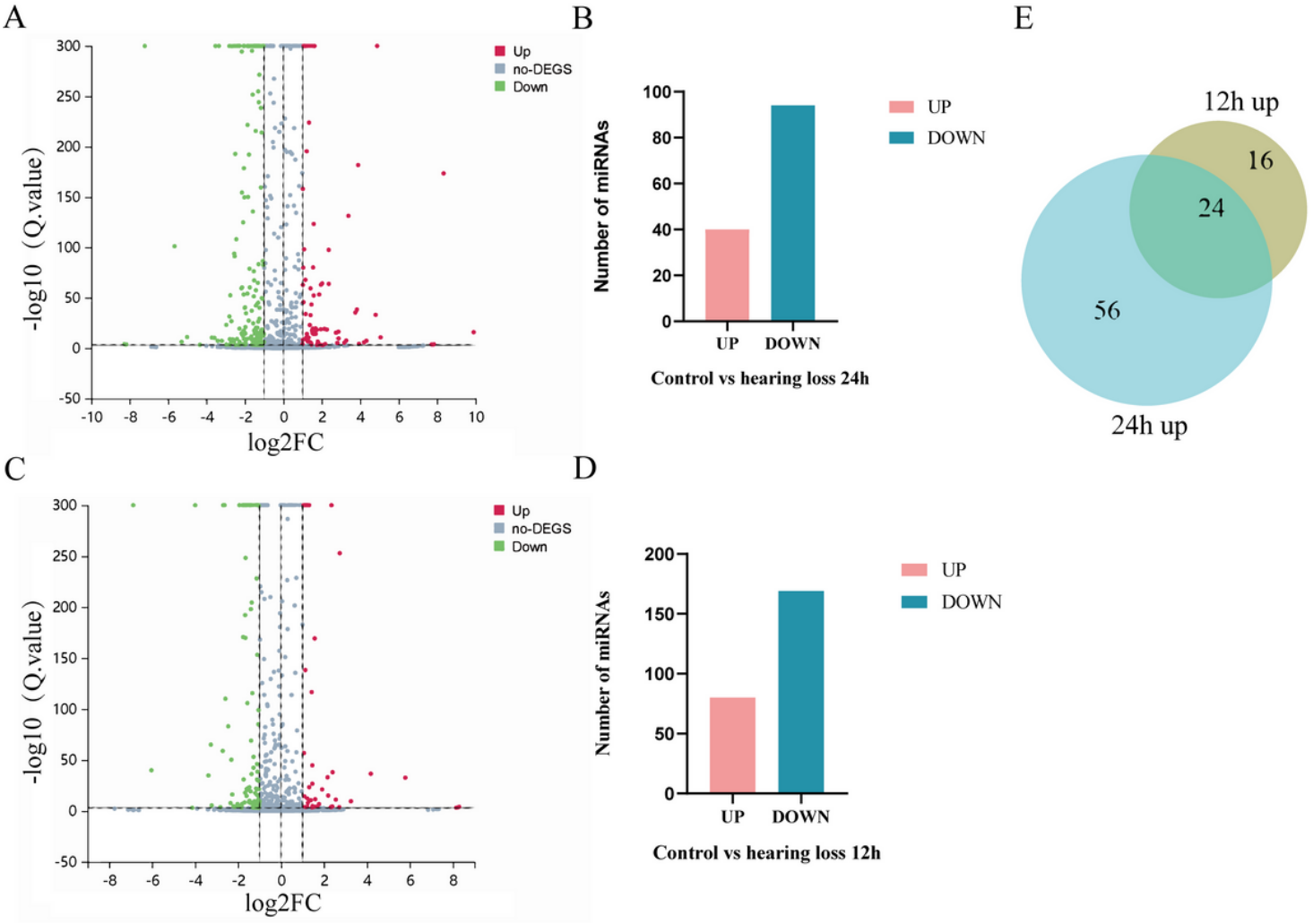

Figure 2

Screening difference expression miRNA A: Difference miRNAs volcano illustration of 12h group. B: Number of differential miRNAs in 12h group. C: Difference miRNAs volcano illustration of 24h group. D: Number of differential miRNAs in 24h group. E: Co-upregulated miRNAs of 12 hours group and 24 hours group. Note: The blue dot representative is lowered, and the red dot is indicated, and the gray point represents a screening.

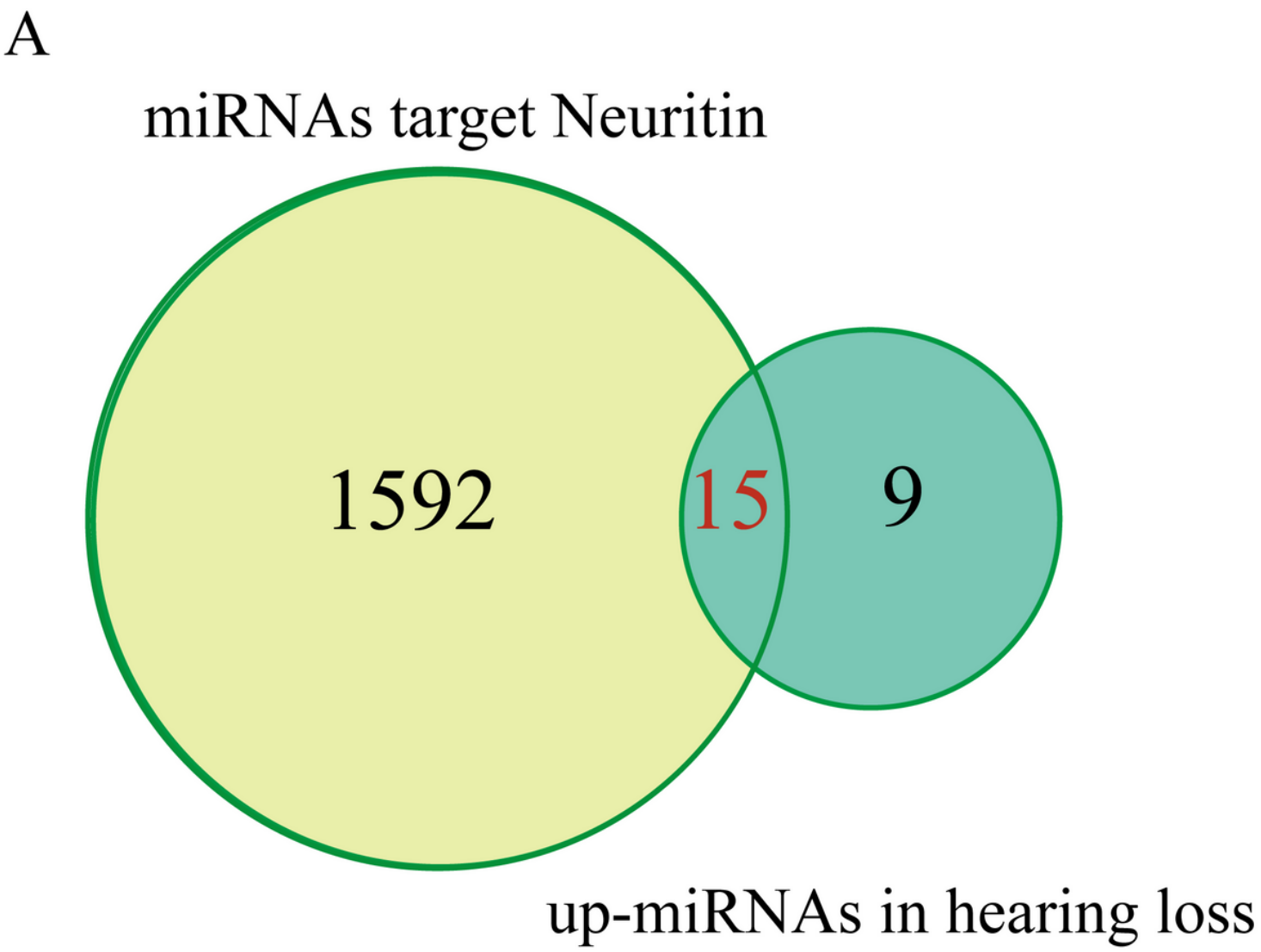

Figure 3

Screening miRNAs related to Neuritin and hearing loss A: The result of miRNAs associated with Neuritin and hearing loss.

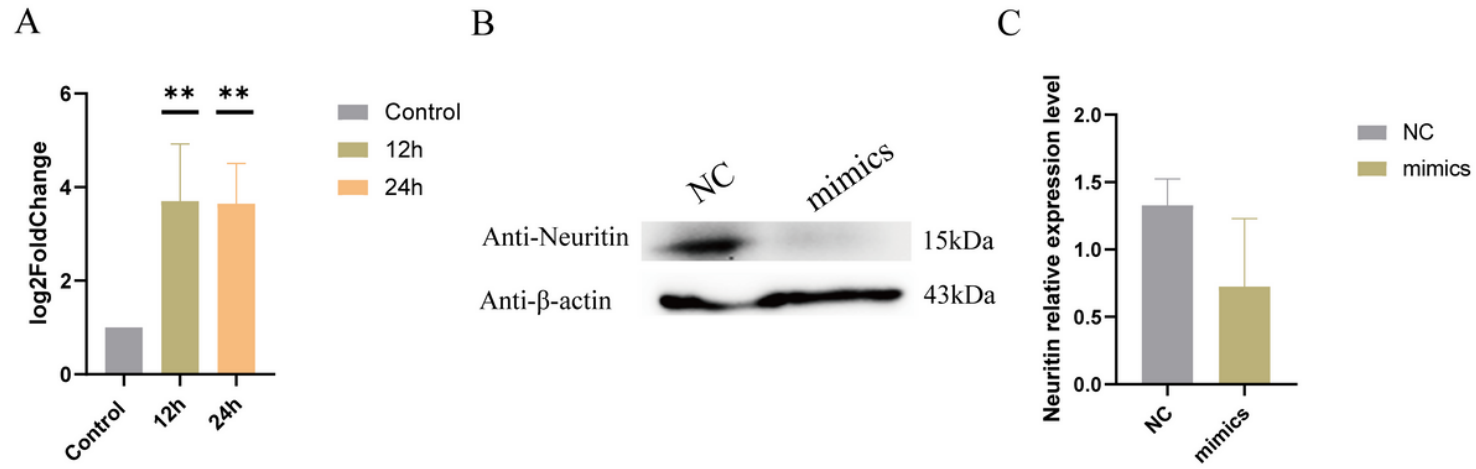

Figure 4

Correlation analysis of miRNA-224-5 with hearing loss and Neuritin A: q-PCR results of miRNA-224-5p after hearing loss. B: WB result of Neuritin after transfected with miR-224-5p. C: Statistical graph of neuritn expression results.

Figure 5

Analysis result of binding site between miR-224-5p and Neuritin A: The prediction result of the binding site of miR-224-5p and Neuritin. B: Construction of GP-miRGLO vector containing normal Neuritin

sequence. C: Construction of GP-miRGLO vector containing MUT Neuritin sequence. D: Results of dual luciferase reporter gene for miR-224-5p and Neuritin.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 12hgroupmiRNA.csv
- 24hgroupmiRNA.csv
- coupgradulatemirna.xlsx
- miRNAstargetNeuritin.xlsx