BRIEF COMMUNICATION

Survival of Cochlear Spiral Ganglion Neurons Improved *in vivo* by Anti-miR204 *via* TMPRSS3

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ABSTRACT

Objective: *Sensorineural hearing loss (SNHL) is caused by damage to hair cells followed by degeneration of the spiral ganglion neurons (SGNs), and cochlear implanting is an effective treatment. Unfortunately, the progressive hearing loss is still found due to ongoing degeneration of cochlear SGNs. The aim of this study was to investigate the neuroprotective effect of anti-miR204 on SGNs* in vivo*.*

Methods: *Our recent* in vitro *work suggested that anti-miR204 could be a potential therapeutic strategy in SNHL* via *rescue cochlear SGNs. In order to further our knowledge of miR204 on SGNs* in vivo*, we made a kanamycin ototoxicity model and then virus containing the anti-miR204 gene (AAV1-anti-miR204) was microinjected into the cochlear of the model to monitor the effect.*

Results: *The SGNs were rescued by anti-miR204 in the kanamycin ototoxicity mouse group compared to the sham group. Moreover, expression of TMPRSS3 in SGNs was saved by anti-miR204 treatment.*

Conclusion: *Anti-miR204 might be an alternate way to alleviate the degeneration of cochlear SGNs of kanamycin ototoxicity mice.*

Keywords: MiR204, Kanamycin ototoxicity, sensorineural hearing loss, spiral ganglion neurons TMPRSS3

Supervivencia de las neuronas en el ganglio espiral de la cóclea y su mejoramiento in vivo mediante anti-miR204 via TMPRSS3

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RESUMEN

Objetivos: *La pérdidaneurosensorial de la audición o hipoacusia neurosensorial (HNS) es causada por un daño a las células pilosas, seguido por la degeneración de las neuronas del ganglio espiral (NGE), y el implante coclear es un tratamiento e icaz. Por desgracia, todavía encontramos pérdida progresiva de la audición debido a la continua degeneración de las NGE cocleares. El objetivo de este estudio fue investigar el efecto neuroprotector del anti-miR204 en NGE* in vivo*.*

Métodos: *Nuestro reciente trabajo in vitro sugiere que anti-miR204 podría ser una estrategia terapéutica potencial en la HNS via rescate de las NGE cocleares. Con el objeto de hacer avanzar nuestro conocimiento de miR204 in vivo, creamos un modelo de ototoxicidad de kanamicina, y entonces un virus contentivo del gen anti-miR204 SGNs (AAV1-anti-miR204) fue microinyectado en la cóclea del modelo para monitorear el efecto.*

Resultados: *Las NGE fueron rescatadas por anti-miR204 en el grupo de ratones con ototoxicidad de kanamicina en comparación con el grupode simulación. Por otra parte, la expresión de TMPRSS3 en las NGE fue salvada por el tratamiento anti-miR204.*

Conclusión: *El anti-miR204 puede ser una manera alternativa de paliar la degeneración de las NGE concleares de ratones con ototoxicidad de kanamicina.*

Palabras claves: Ototoxicidad de kanamicina, miR204, pérdida neurosensorial de la audición, neuronas del ganglio espiral, TMPRSS3

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INTRODUCTION

Sensorineural hearing loss (SNHL) is caused by damage of hair cells followed by degeneration of the spiral ganglion neurons (SGNs) and can be improved by cochlear implants (1). Cochlear implantation is the only US Food and Drug Administration-approved treatment for children with marked bilateral sensorineural hearing loss (2). However, the benefit of the cochlear implant depends on the excitability of the SGNs (2).

Type II transmembrane serine proteases (TTSPs) degrade components of the extracellular matrix (ECM) which can be divided by their structures into subfamilies (3, 4). TMPRSS3 belongs to the Hepsin/TMPRSS subfamily (3) which is expressed in SGNs of the mouse cochlear and is a potential diagnostic marker and therapy target on SNHL (5, 6). A previous study from our group suggested that TMPRSS3 is highly expressed in the SGNs of the mouse cochlear (5). In addition, expression of TMPRSS3 in the cochlear was reduced after the kanamycin injection, which indicated that TMPRSS3 may play an important role in normal cochlear function and be in volved in the process of aminoglycoside antibiotic-induced deafness (7). In our recently published work, downregulation of TMPRSS3 results in degeneration of SGNs by transfection of TMPRSS3 siRNA. Further *in vitro* study indicated that miR204 inhibits viability of SGNs by targeting TMPRSS3, which could be employed as a potential therapeutic strategy in SNHL (6). More investigations are necessary to know the effect of miR204 *in vivo*.

In order to further our knowledge of miR204 *in vivo* on SGNs, we made a kanamycin ototoxicity model and then administered anti-miR204 to monitor the effect on SGNs.

SUBJECTS AND METHODS

Animals and establishment of a kanamycin-induced ototoxicity model

The protocol of animal experiment was approved by the Animal Care and Use Committee at Xiangya School of Medicine, Central South University. Fifteen male C57BL/6 mice, age four weeks, were randomly assigned to one of the following three experimental groups: (i) sham group (0.9% saline), (ii) kanamycin group (800 mg/kg kanamycin) and (iii) anti-miR204 group (800 mg/kg kanamycin + AAV1-anti-miR204 injection). Each group had five mice. All surgical procedures were done in a clean, dedicated space. Mice were anaesthetized by carbon dioxide (CO_2). Kanamycin sulphate was injected subcutaneously twice daily for two weeks.

Round window membrane injection

After anaesthesia, the round window membrane (RWM) was gently punctured with a borosilicate capillary pipette and remained in place until efflux stabilized. A DNA fragment containing the miR204 precursor flanked by 500 bp genomic sequence at either end was inserted into lentiviral vector LPP-MmiR3405 (GeneCopoeia, MD, USA). A fixed volume of AAV1-anti-miR204 (0.6 µl or 1.0 µl of a 2.5×10^{13} vg/mL) previously drawn into the Hamilton syringe was gently injected through RWM over 1−2 minutes. The bulla was sealed with dental cement and the wound was sutured.

Histology of cochlear sections

Mice were anaesthetized and their cochleas were isolated, dissected, perfused through oval and round windows by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at pH 7.4, and incubated in the same fixative for two hours. After fixation, the cochleas were rinsed with PBS and immersed in 5% EDTA in 0.1 M phosphate buffer for decalcification. After decalcification, the cochlears were then embedded in paraffin and processed for paraffin section (5 um). Cresyl violet Nissl staining was used to examine the histology of the cochlear with light microscopy.

Western blot analysis

Spiral ganglion neurons after treatment were washed twice with PBS and protein samples were separated by 10% SDS-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were incubated with primary antibodies against TMPRSS3 (1:500, abcam) or β-actin (1:2000, Cell Signalling Technology) overnight at 4 °C. Blots were washed and incubated for one hour with horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody. Immunoreactive protein bands were detected and each band was normalized with respect to its corresponding β-actin band.

Statistical analysis

Data are presented as the mean ± standard deviation from at least three independent experiments. Two-tail Student's *t*-test and analysis of variance (ANOVA) were performed to analyse the data using SPSS 12.0. $p < 0.05$ was considered statistically significant.

RESULTS

Rescue SGNs by anti-miR204 *in vivo*

Our recent paper suggested that miR204 could regulate expression of TMPRSS3 *in vitro* (6). However, we still have no idea whether miR204 could regulate expression of TMPRSS3 *in vivo*. On day seven, mice were anaesthetized and their cochlear were isolated, dissected, and perfused through oval and round windows by 2% paraformaldehyde. As shown in Fig. 1, in kanamycin ototoxicity mouse cochlea, the cells are reduced a lot ($p < 0.05$). However, treatment with AAV1-anti-miR204 led to increased survival of cells in SGNs compared with the sham group ($p < 0.05$).

Anti-miR204 regulates expression of TMPRSS3 in SGNs

The effect of AAV1-anti-miR204 transfection on endogenous TMPRSS3 protein expression was subsequently evaluated in SGNs by Western blot. As shown in Fig. 2, the expression of TMPRSS3 protein was decreased in SGNs without transfection of AAV1-anti-miR204 compared with the sham group. However, there is not much difference after transfection with

AAV1-anti-miR204 compared with the sham group.

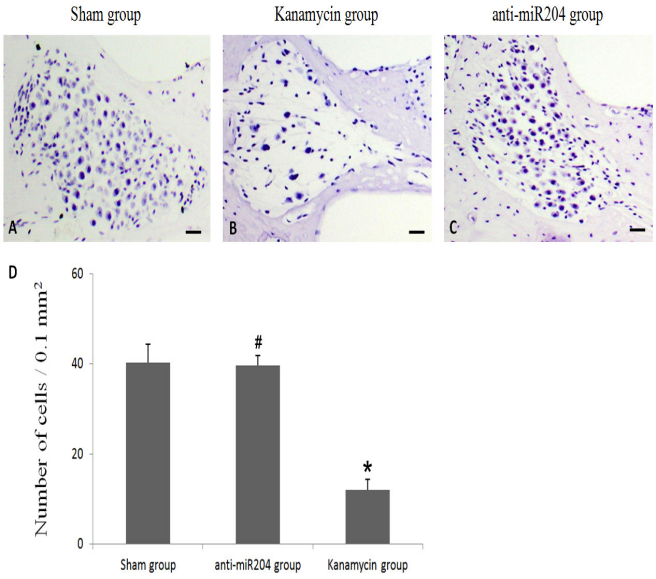

Fig. 1: Cochlear stained with cresyl violet. The cells are missing in the cochlear of kanamycin ototoxicity mice (B) when compared to the sham group mice (A). After treatment with anti-miR204 (C), the cells are saved when compared to the sham group mice (A). The difference is significant (*$p < 0.05$) in the cells in the cochlear between sham group and kanamycin ototoxicity mice group (D). However, there is no significant change (#$p > 0.05$) between sham group and anti-miR204 treatment (D). (Scale bars: 50 μm)

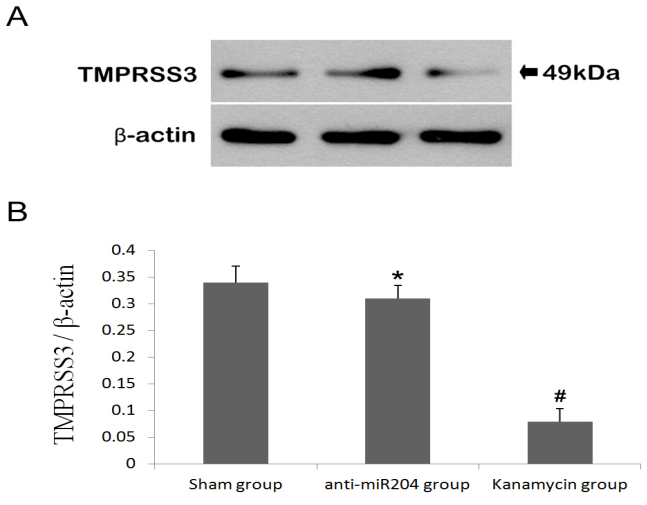

Fig. 2: Expression of TMPRSS3 in spiral ganglion neurons (SGNs). (A) The TMPRSS3 protein expression was downregulated in SGNs without transfection of AAV1-anti-miR-204 when compared to the sham group. (B) TMPRSS3 protein bands were quantitated by densitometry. The expression of TMPRSS3 protein was no different between sham group and anti-miR204 group (*$p > 0.05$). However, reduced expression in the karamycin group was significant when compared to the sham group (#$p < 0.05$).

DISCUSSION

Sensorineural hearing loss is among the most frequent sensory deficits, but they lack effective drug therapies. Cochlear implantation is an effective treatment for children with SNHLs. However, the effect wanes due to the continuous loss of the spiral ganglion neurons.

TMPRSS3, the member of Hepsin/TMPRSS subfamily, is expressed in SGNs of the mouse cochlear and is a potential diagnostic marker and therapy target on SNHL (3−6). In our recently published work, the *in vitro* study was conducted on the role of TMPRSS3 on cell viability of SGNs by transfection of TMPRSS3 siRNA. Our data suggest that downregulation of TMPRSS3 led to degeneration of SGNs (6).

MicroRNAs (miRNAs) are a class of endogenous non-coding RNA in length of 22 nucleotides (nt) on average. The miRNAs could post-transcriptionally regulate gene expression of growth, development, differentiation and many other biological processes (8). More and more evidence reveal that anti-microRNA or silencing microRNA might be an effective means of therapeutic intervention (9−11). Our recent work suggested that miR-204 inhibits viability of SGNs by targeting TMPRSS3 which indicated anti-miR204 could be employed as a potential therapeutic strategy in SNHL *via* rescue cochlear SGNs. In this study, we investigated the neuroprotective effect of anti-miR204 as a promising agent to improve the viability of the auditory neurons *in vivo*. We found that the SGNs were rescued by anti-miR204 in the kanamycin ototoxicity mouse group compared to the sham group. Moreover, expression of TMPRSS3 in SGNs was saved by anti-miR204 treatment.

In conclusion, the results of this study suggest that anti-miR204 might be an effective way to alleviate the degeneration of spiral ganglion neurons of the cochlear of the kanamycin ototoxicity mice.

Conflict of interest

The authors declare there is no conflict of interest.

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