MicroRNA-222 carried by plasma-derived exosomes as an earlier biomarker to alert the abnormal phosphorylation of Tau caused by shRNA-PCSK9 and its correlation with Aβ1-42Ab.

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Research Article

Keywords: hypercholesterolemia, human subtilisin converting enzyme 9, cognitive impairment, microRNA-222

Posted Date: January 12th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2437137/v1

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**Abstract**

The clinical adverse reaction monitoring data of Human subtilisin converting enzyme 9 gene (proprotein converting enzyme subtilisin/kexin type 9, PCSK9) inhibitors showed that there were neurocognitive adverse events. In order to early warning of the potential danger of PCSK9 inhibitors to cognitive impairment in the process of regulating lipid homeostasis, this study aimed to explore the microscopic effects of plasma exosomes. MicroRNA-222 (miR-222) as an early warning marker for shRNA-PCSK9-induced cognitive impairment. The hypercholesterolemia mouse model (high-fat diet, HFD-model group) was prepared by feeding with high-fat diet. The HFD-models were then divided into shRNA control group (HFD-shRNA-control group) and shRNA PCSK9 group (HFD-shRNA-PCSK9 group). The shRNA-PCSK9 was constructed, injected intravenously into the body, and the expression of PCSK9 mRNA was detected by real-time PCR (RT-PCR) and immunohistochemistry (IHC). Tau protein and phosphorylation in brain tissue were observed by IHC. Western blot (WB) was used to detect Tau protein and P-Tau protein. Serum amyloid Aβ1-42Ab levels were determined by enzyme-linked immunosorbent assay (ELISA). The kits extract plasma exosomes step by step, identify the exosome morphology by negative staining electron microscopy, and determine the size of exosomes by NTA technology. RT-PCR technique was used to detect the expression level of miR-222 carried in plasma exosomes. The HFD-model model was prepared by feeding a high-fat diet for 13 weeks, and the serum total cholesterol (TC) and low-density lipoprotein (LDL-C) contents were significantly increased. At the same time, the expression of PCSK9 mRNA in the brain tissue of HFD-model mice was significantly increased. After shRNA-PCSK9 lentivirus interference, PCSK9 mRNA expression was inhibited, and IHC observed that shRNA-PCSK9 induced abnormal expression and hyperphosphorylation of Tau protein in brain tissue, indicating that the pathological changes of neurofibrillary tangles have occurred. However, at this time, serum Aβ1-42Ab has not been significantly increased, and it has not yet been of significance for the diagnosis of cognitive impairment. The microRNA in plasma exosomes was extracted, and RT-PCR results showed that the expression of miR-222 carried in the exosomes of the HFD-shRNA-PCSK9 group was significantly lower than that of the HFD-shRNA-control group. At the same time, MiR-222 levels in plasma-derived exosomes were inversely correlated with Aβ1-42Ab in serum. MiR-222 carried by plasma-derived exosomes provides an early warning marker for shRNA-PCSK9-induced cognitive impairment.

**Introduction**

Human subtilisin converting enzyme 9 gene (proprotein converting enzyme subtilisin/kexin type 9, PCSK9) inhibitors reduce LDL-C levels in patients with ischemic stroke and reduce the risk of cardiovascular and cerebrovascular events [1]. Monoclonal preparations such as Alirocumab (Allisy), Evolocumab (elouzumab) and Bococizumab have been used in clinic. However, the monitoring data of clinical adverse reactions showed that the incidence of neurocognitive adverse events was 0.8% in the subjects who received Alirocumab (0.7% in the placebo group). Alirocumab treatment resulted in memory loss and confusion in 0.2% of patients (0.1% in placebo group) [2]. At the same time, Evolocumab subjects also reported neurocognitive adverse reactions such as amnesia and mental disorders [3].
Cognitive dysfunction generally refers to cognitive impairment of different degrees caused by various reasons. Neuroentanglement is one of its characteristic pathological changes, and its main component is a double-stranded spiral filament composed of abnormally phosphorylated Tau protein. Amyloid β-protein (Aβ) is also a characteristic pathological change of cognitive impairment. Plasma Aβ level and abnormal phosphorylation of Tau protein have been recognized as diagnostic markers of cognitive impairment. Besides, plasma neurofilament light chain (Nfl) and glial fibrillary acidic protein (GFAP) are cognitive impairment markers independent of Aβ. It can be seen that there is still much room for research and development of biomarkers of cognitive impairment with different potential predisposing factors and different pathological types. The purpose of this study is to explore cognitive impairment related to PCSK9 inhibitors, that is, to find out the potential danger of PCSK9 inhibitors to cognitive impairment in the process of regulating lipid homeostasis. The so-called anti-paralysis should not forget to cure "paralysis".

The regulatory relationship between miR-222 and PCSK9 has been demonstrated in the literature [4], and the relationship between inhibition of miR-222 and the decrease of cholesterol synthesis rate has been made clear [5]. It has been reported that the expression of miR-222 in cerebrospinal fluid of patients with Alzheimer's disease (AD) is obviously changed [6–8], while that of patients with vascular dementia (VD) is abnormal [9]. Therefore, miR-222 in cerebrospinal fluid as an early warning marker of cognitive impairment has a preliminary foundation. Exosomes carry molecular information such as nucleic acid and protein of corresponding source cells, and abundant tiny RNAs are secreted in cerebrospinal fluid and blood circulation as biological diagnostic markers by exosomes, which seal the blood-brain barrier [10]. Measurement of miR-222 carried by plasma exosomes can provide early warning for cognitive impairment induced by PCSK9 inhibitors.

Materials And Methods

Animals, Model

Adult male C57BL/6J mice (8-weeks-old) were purchased from Beijing Huafukang Biotechnology include Company Limited (LicenseNo.: SCXX (Beijing) 2019-0008). Adult male SD (8-weeks-old) were purchased from Beijing Weitonglihua include Company Limited (LicenseNo.: SCXX (Beijing) 2016-0011). The high-fat diet feed (HFD) was purchased from Beijing Keao Xieli Feed Co., Ltd. (license number: SCXX (Beijing) 2019-0003). The mice were housed in groups of four to five per cage with ad libitum access to food and water, and were maintained under a 12-h light/dark cycle (lights on at 8:30 p.m., of at 8:30 a.m.) at a stable temperature (22 ± 2°C). The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all the procedures were approved by the Animal Ethics Committee of Tianjin Institute of Medical and Pharmaceutical Sciences (Tianjin, China; approval no.IMPS-EAEP-Z-MS20023-01 ). Animals were fed HFD (the basic diet was composed of sucrose 20%, lard 15%, cholesterol 2% and bile salt 0.3%) for 13 weeks to make hypercholesterolemia model.
The animals were divided into normal control group (NFD-control group) and hypercholesterolemia model group (HFD-model group). The normal control group was fed with normal diet (NFD-control), while the hypercholesterolemia model group was fed with high-fat diet (HFD-model). After the model was established, HFD-model rats were divided into shRNA blank control group (HFD-shRNA-control group) and shRNA PCSK9 group (HFD-shRNA-PCSK9 group).

**Method for constructing shRNA-PCSK9**

Packaging HIV plasmid mixture, GeneCopoeia 293Ta lentivirus packaging cell line (GenecopoeisCatNo. CLV-PK-01), EGFP positive control plasmid, EndoFectinTM transfection reagent; TiterBoostTM titer enhancer (500x), DMEM medium containing glucose, glutamine and sodium pyruvate, Fetal bovine serum (Thermoscientific catNo. sh300700.02), Opti-MEM I serum-free medium (invitrogen cat No.31985-062/31985-070), Polyamine (sigma-aldrich catNo. H9268), crystal violet (sigma-aldrich catNo. C3886), Penicillin-streptomycin double antibody (Sigma-Aldrich CatNo. P4333). Our previous experiment [13] has verified and screened out the effective silencing sequence of shRNA-PCSK9, and the sequence is shown in Table 1:

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Symbol</th>
<th>Location</th>
<th>Length</th>
<th>Target Sequence</th>
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<tbody>
<tr>
<td>CSHCTR001-1-LVRH1GP(OSNEG20)</td>
<td>-</td>
<td>-</td>
<td>19</td>
<td>Gcttcgcgcgcctgaagtcctta</td>
</tr>
<tr>
<td>MSH040042-33-LVRH1GP(OS637077)</td>
<td>Pcsk9</td>
<td>1228</td>
<td>21</td>
<td>ggagttatattcgaagagctca</td>
</tr>
</tbody>
</table>

The plasmid was synthesized by oligonucleotide annealing, plasmid PCSK9 linearization extraction, target fragment cloning to PCSK9 plasmid expression vector, vector transformation to competent cells, amplification of transfected competent cells, and plasmid restriction enzyme identification. Optimization steps of lentivirus production using 293Ta tool cells. The lentivirus yield of each 10cm culture plate added with eGFP or mCherry positive control group is 10ml supernatant, with 1–10×10⁷ infected units per ml. The lentivirus titers were measured by H1299 cell line or drug sieve after culturing packaging cells, preparing DNA/EndoRectin lentivirus mixture, transfecting packaging cells, harvesting lentivirus and testing titer.

Silent transfection of shRNA-PCSK9 lentiviral vector: 10µl/ time was absorbed by microinjection, injected from tail vein of C57BL/6J mice and SD rats, and injected again at 48h and 72h. Blank lentivirus was injected with HFD-shRNA-control, and shRNA-PCSK9 lentivirus was injected with HFD-shRNA-PCSK9. Forty-eight hours after the last injection, the kidney tissue was taken and stored at -80°C for later use.

0.1g sample was taken, total RNA was extracted, after quality inspection, 5µlRNA was electrophoresed with 1% agarose gel, the integrity of RAN was detected, the residual genomic DNA in RNA was digested, and after reverse transcription, it was determined by PCR instrument, and the data was analyzed by
$2^{-\Delta\Delta CT}$ method. PCSK9 primer is designed as: PCSK9-F5'-3': GCATCCAAACACCCCT, 125bp, 3'-5':TGCCTCGGACACTAA, 125bp

### Table 2
RT-PCR primers used in the study

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>PCSK9</td>
<td>GCATCCAAACACCCCT</td>
<td>TGCCTCGGACACTAA</td>
</tr>
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</table>

**Extraction Of Plasma Exosomes**

At the end of the experiment, the circulating blood of mice/rats were taken, fully anticoagulated by EDTA anticoagulation tube, centrifuged at 3000 rcf/min×15min, and the plasma was sucked. Sample of plasma was 1ml/tube at 4°C and centrifuged at 3000 rcf/min×15min. Take out the precipitate and discard it, collect the supernatant, move it into another set of 1.5ml centrifuge tubes, centrifuge again at 4°C and 10000 rcf/min×20min, collect the supernatant, and discard the precipitate. Transfer to another clean centrifuge tube, add 1mL of extract A, cover the centrifuge tube tightly, mix it upside down for about 1min, then add 0.5mL of extract B, cover the centrifuge tube tightly, mix it upside down for about 1min, make the liquid fully mix, and put it in a refrigerator at 2–8°C for standing overnight. The next day, centrifuge at 4°C and 10000 rcf/min×60min, remove the supernatant, and collect the precipitate. Add exosome preservation solution C to the precipitate, and resuspend the precipitate to obtain exosome samples for later identification and biological determination.

**Exosome Identification Method**

The extracted exosomes were observed by transmission electron microscope and analyzed by particle size. Take out 10 µL of exosomes, absorb 10 µL of samples, drop them on a copper net for precipitation for 1 min, filter paper to absorb floating liquid, 10 µL of uranyl acetate drop them on a copper net for precipitation for 1 min, filter paper to absorb floating liquid, dry them at room temperature for several minutes, and carry out electron microscope detection and imaging at 100 kv to obtain the imaging results of transmission electron microscope. Take frozen samples, thaw them in water bath at 25°C, and place them on ice. Exosome samples were diluted with 1 × PBS and directly used for NTA detection.

**MicroRNA Extraction Method**

After thawing the exosome sample, add 1ml Trizon, mix well, add 200 µl chloroform, shake for 15s, stand for 5min at 4°C, centrifuge at 12,000 rpm for 10min, suck the upper clear water layer, add 150 µl absolute ethanol, place it in RM column at 12,000 rpm for 30s at 4°C, discard RM column, and add 400 µl absolute ethanol. Add 700 µl RWT solution at 12,000 rpm for 30s, 4°C, and discard the waste liquid; Add 500 µl RW2 solution, centrifuge at 4°C at 12,000 rpm for 30s, and discard the waste liquid; Add 500 µl RW2
solution, centrifuge at 4°C at 12,000 rpm for 30s, and discard the waste liquid. Idle at 12,000 rpm for 60s at 4°C, Drying at room temperature for 2min, adding 30 µl Rnase-Free water, standing for 2 min, at 12000 rpm for 120s at 4°C, and measuring RNA concentration.

**MiRNA-222 Content In Plasma Exosomes Measure Method**

Total RNA was extracted from tissue samples with ultrapure RNA extraction reagent. Add 2ml RNAiso Plus to each 0.1g sample, completely cover the sample, and let stand at room temperature for 5min. Centrifuge at 4°C and 12,000 rpm for 5 minutes, and transfer the supernatant to a new 1.5mL centrifuge tube. Add chloroform at the ratio of 200ul chloroform per 1 ml RNAiso Plus, shake vigorously until the solution is fully emulsified, and stand at room temperature for 5 minutes. Centrifuge at 4°C and 12,000 rpm for 15 minutes. Add equal volume of isopropanol and mix well. After standing at room temperature for 10 minutes, centrifuge at 4°C and 12,000 rpm for 10 minutes. Remove the supernatant, add 1mL of 75% ethanol (RNase-Free Water) precooled at -20°C, and clean the precipitate. Centrifuge at 4°C and 12,000 rpm for 5 minutes, and remove the supernatant with a pipette. Dissolve the precipitate with RNase free H₂O, and store the RNA solution in a refrigerator at -80°C. The ratio of A260/A280 of RNA was measured by ultraspectrophotometer (1.9–2.1). The expression of miRNA and mRNA was detected by RT-PCR with TaKaRa code DRRO47A reverse transcription kit, and the relative quantitative analysis of data was carried out by $2^{-\Delta\triangle CT}$ method.

**Western Blotting And Immunohistochemistry**

Total protein was extracted by RIPA method, and protein concentration was detected by BCA kit. 10% separation gel, 5% concentrated gel, 80V 30min concentration, 120V 90min separation, 200mA film transfer for 2h, 5% skimmed milk sealed at room temperature for 30min, washed with TBST once, first antibody incubated overnight at 4°C, washed with TBST buffer the next day, second antibody incubated at room temperature for 1h, imaged and developed by chemiluminescence gel imaging analysis, and analyzed by Image J software.

Conventional paraffin sections are dewaxed to water; 3% H₂O₂ blocked endogenous peroxidase at room temperature for 10 minutes. Microwave repair antigen,Sealing with 5% BSA for 20 minutes,Adding tau and p-tau antibodies dropwise,Overnight at 4°C. Adding secondary antibody after washing PBS, DAB color development after PBS washing. Lignin re-dyeing, ethanol dehydration, xylene transparency, neutral gum sealing.

**Statistical Analysis**

Date are expressed as mean ± SEM and analyzed using GraphPad 6 statistical software. The measurement data is expressed by mean standard deviation, independent sample T test is used for two
groups of analysis, and one-way ANOVA is used for multiple groups of analysis. \( P < 0.05 \) is statistically significant.

**Results**

**Characteristic description of hypercholesterolemia mouse model**

After 13 weeks of feeding with high-fat diet, a hypercholesterolemia mouse model was established. The results showed that serum TC and LDL-C in HFD-model group were significantly higher than those in NFD-control group (Fig. 1A and B). To explore the expression characteristics of PCSK9 in the brain, PCSK9 mRNA was measured in the brain tissue of this model mouse. Interestingly, through RT-PCR tests, we found that the expression level of PCSK9 mRNA in hippocampus and cortex of HFD-model group was significantly higher than that of NFD-control group (Fig. 1C).

**Transfection effect of shRNA-PCSK9**

To reduce the expression of PCSK9 mRNA, sh-RNA PCSK9 was injected into hypercholesterolemic mice via tail vein, and the expression of PCSK9 mRNA in kidney tissue was measured by RT-PCR, which achieved the purpose of inhibiting PCSK9 mRNA. RT-PCR results showed that the expression of PCSK9 mRNA in HFD-shRNA-PCSK9 group was significantly lower than that in HFD-shRNA-control group \( (P < 0.05) \), indicating that shRNA PCSK9 effectively inhibited the expression of PCSK9 mRNA (Fig. 2).

**Hyperphosphorylation of tau protein in brain of hypercholesterolemic mice induced by shRNA-PCSK9**

The expression of PCSK9 mRNA was inhibited by shRNA-PCSK9, which induced abnormal expression and hyperphosphorylation of tau protein in the brain tissue of hypercholesterolemic mice. In HFD-shRNA-PCSK9 group, brown-yellow positive cells were found in hippocampus and cortex (Fig. 3A and B). Tau positive expression was obvious, and tau phosphorylation was abnormally excessive (Fig. 3C and D). At this time, however, the ELISA results of serum dyslexia marker Aβ 1-42ab showed that there was no significant difference between HFD-shRNA-PCSK9 group and HFD-shRNA-control group (Fig. 3E).

**Extraction And Identification Of Plasma Exosomes In Hypercholesterolemic Mice**

According to the instructions of serum exosome extraction kit, complete the exosome extraction process (Fig. 4A). Under negative transmission electron microscope, “teacup-shaped vesicles” were seen as exosomes, with an average particle size of 122.8nm and a concentration of 4.9E + 12 Particles/mL.
The protein electrophoresis bands showed TSG101, HSP70 and CD63 exosomes biomarkers (Fig. 4C), which confirmed that the plasma exosomes of hypercholesterolemic mice could be obtained by this step.

**The effect of shrpcsk9 on miRNA-222 expression in plasma exosomes of hypercholesterolemic mice**

The measurement of miRNA-222 in exosome showed that there was no significant difference between miRNA-222 in HFD-shRNA-control group and HFD-model. Compared with shRNA-control, the expression of miRNA-222 in shRNA-PCSK9 group was significantly decreased, indicating that the expression level of miRNA-222 was affected by the silencing of PCSK9 mRNA (Fig. 5).

**Characteristic Description Of Hypercholesterolemia SD Rats Model**

Because the amount of mouse plasma exosome samples is insufficient, it is not enough to analyze the correlation between miRNA-222 and cognitive impairment indexes of each animal. Therefore, we established the hypercholesterolemia model in rats, in order to obtain enough exosomal samples and complete the follow-up analysis.

Hypercholesterolemia mouse model was established. Oil red O staining showed that the red lesions were lipid droplets in Liver of hypercholesterolemic rats (Fig. 6A). The results showed that serum TC and LDL-C in HFD-model group were significantly higher than those in NFD-control group (Fig. 6B and C). To explore the expression characteristics of PCSK9 in the brain, ICH and RT-PCR was measured in the brain tissue of this model rats. Interestingly, through ICH tests, we found that the expression level of PCSK9 in hippocampus and cortex of HFD-model group was significantly higher than that of NFD-control group (Fig. 6D). ShRNA PCSK9 effectively inhibited the expression of PCSK9 mRNA (Fig. 6E).

**The expression of miRNA-222 and correlation anlysis between miRNA-222 in plasma-derived exosomes and Aβ1-42Ab**

We performed correlation analysis between exosomal biomarkers and found that the levels of miRNA-222 in HFD-shRNA-PCSK9 was lower significantly than HFD-control group and HFD-shRNA-control (Fig. 7A). In detail, in the discovery data set, the miRNA-222 levels in blood-derived exosomes were inversely correlated with Aβ1-42Ab in serum ($R^2 = 0.78, P<0.01$, Fig. 7B).

The role of PCSK9 inhibitor in blood lipid management and neurocognition is a double-edged sword. There is a contradiction between advantages and disadvantages in regulating lipid homeostasis and inducing cognitive impairment. So-called "correcting paralysis" should not forget to cure "paralysis". Early warning markers of cognitive impairment are especially important for prevention.
The purpose of this study is to explore the biomarkers of cognitive impairment induced by PCSK9 inhibitors in the process of regulating lipid homeostasis, and try to find out and intervene as soon as possible. What is the relationship between PCSK9 and lipid regulation and cognitive impairment? It can be analyzed from two aspects: the nerve function and cholesterol metabolism of PCSK9. Firstly, PCSK9 was first called the neuron apoptosis converting enzyme − 1 protein, which is a protein related to the differentiation and apoptosis of nervous system cells. PCSK9 reduces the production of Aβ through the degradation of nerve cell BACE1, thus reducing the apoptosis of nerve cells and preventing the occurrence of AD. If PCSK9 is inhibited, it will lead to the risk of soluble Aβ deposition in cerebral capillaries and arterial walls, which will lead to the decline of cognitive ability. According to other reports, the polymorphism of rs2470409 of PCSK9 gene is related to cognitive impairment, and allele A is the protective factor of cognitive impairment [11]. Secondly, cholesterol metabolism in brain is the common basis of AS and cognitive impairment. In April, 2020, Nature proposed the mechanism of blood-brain barrier damage caused by apolipoprotein E(APOE4), and revealed a new target of Alzheimer's disease independent of Aβ protein and tau protein [12]. The mechanism of PCSK9 in regulating brain lipid homeostasis is also based on ApoER pathway (which is different from LDLR pathway of PCSK9 in liver and circulating blood). The previous data of the research group confirmed that the endogenous PCSK9 in brain is based on apolipoprotein E(ApoE) [13]. It can be seen that the functions of PCSK9 in regulating nerve cells and lipid metabolism become the basis of cognitive impairment induced by PCSK9 inhibitors.

Blood-based biomarkers of cognitive impairment have received great attention. For example, (1) The clinical value of plasma Aβ1–42 and Aβ1–40 is gradually being verified, which is another progress after cerebrospinal fluid Aβ1–42 and Aβ1–40 as biomarkers. Aβ is produced by the division of amyloid precursor protein in the brain, and the deposition of Aβ plaque will appear before cognitive decline. With the deposition of Tau protein, it marks the occurrence of neurological dysfunction. (2) Hyperphosphorylated tau (P-tau). P-tau can be measured in cerebrospinal fluid and plasma, but there are strong differences in the concentration of phosphorylated tau at different sites in plasma. Normal brain tissue contains 2 ~ 3 mol phosphate per mole of Tau protein, but there are 2 ~ 3 times of P-tau in the brain of patients with cognitive dysfunction. Phosphorylation is the most common post-translational modification of Tau. Up to now, 85 phosphorylation sites of Tau protein have been found. To some extent, the phosphorylation of Tau protein is the normal mechanism for neurons to keep active, but excessive phosphorylation leads to the deposition and aggregation of Tau protein, and finally forms double helix. Tau protein is an important pathogenic mechanism leading to cognitive dysfunction. The hyperphosphorylation of Tau protein destroys its ability to bind microtubules, leading to axonal diseases, impairing the function of neurons and synapses, and increasing the formation of tau oligomerization, aggregation and tangles. PTau has been proved to be able to detect the continuous pathological changes of Alzheimer's disease, so it can be used as a specific diagnostic marker of Alzheimer's disease. (3) Nerve filament light chain (Nfl): NFL is an axon scaffold protein, and NFL in cerebrospinal fluid and blood is the first nerve-specific biomarker with clinical value. (4) Glial fibrillary acidic protein (GFAP): The concentration of GFAP in plasma or serum of patients with cognitive impairment increased. However, the predictive value of GFAP is independent of plasma Aβ42/40, which is due to the activation of astrocytes,
and it is not a unique biomarker of cognitive impairment pathophysiology. Based on the above important value of biomarkers based on blood cognitive impairment, the research and development of biomarkers with different potential factors and different types of cognitive impairment warning and diagnosis values is urgently needed clinically.

Exosomes carry molecular information such as nucleic acid and protein of corresponding source cells, and have powerful biological information in the diagnosis and treatment of various diseases. Abundant miRNAs are secreted in cerebrospinal fluid and blood circulation by exosomes sealed by blood-brain barrier, which is a potential marker of cognitive impairment. On the one hand, it can spread toxic Aβ and excessive P-tau between cells, and may induce cell apoptosis, resulting in the loss of neurons; On the other hand, exosomes seem to be able to reduce brain Aβ load by being taken up by glial cells. Through bioinformatics and in vitro functional analysis, three miRNAs at the 3′-UTR binding site of PCSK9 were selected, and the regulatory relationship between miRNA-222 and PCSK9 has been verified [4]. The relationship between the inhibition of miRNA-222 and the decrease of cholesterol synthesis rate has been made clear [5]. These data suggest that miRNA-222 can be used as an early warning marker of cognitive impairment induced by PCSK9 inhibitors. The data of this study verified this scientific reasoning. PCSK9 shRNA transfection silenced PCSK9 mRNA expression in hypercholesterolemic mice, and induced the significant increase of Tau protein expression in hippocampus and cortex of HFD-model mice and the hyperphosphorylation of Tau. This Tau protein lesion will start in the olfactory cortex of the brain and gradually spread to the whole brain [14], which suggests the pathological changes of cognitive impairment. At the same time, the expression of miRNA-222 in plasma exosomes of HFD-shRNA PCSK9 mice decreased significantly, but the difference of plasma Aβ did not show statistical significance.

Compared with cerebrospinal fluid, blood exosomes have advantages as biomarkers, and blood collection is easier and less invasive. Studies have supported that there is consistency between blood exosomes biomarkers and cerebrospinal fluid: blood exosomes Aβ42, T-tau and P-Tau also have the ability to diagnose cognitive impairment [15]. In view of this, the results of this study show that miRNA-222 in blood exosomes is a biomarker for early warning of cognitive impairment induced by PCSK9 inhibitors, which has a suggestive significance for clinical application. In the future, more mechanism research and clinical prospective research will be carried out in order to provide more data support for clinical application.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MiR-222</td>
<td>microRNA-222</td>
</tr>
<tr>
<td>RT-PCR</td>
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</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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TC    total cholesterol
LDL-C low-density lipoprotein
PCSK9 proprotein convertase subtilisin/kexin type 9
HFD    high-fat diet feed
P-tau Hyperphosphorylated tau

Declarations

All of the authors with their respective contributions to the study

Conceptualization, L.W. and D.H.; investigation, H.W., Z.W., N.L. and X.C.; writing manuscript, L.W.; supervision, H.W., L.L.

Author Contribution

Lei Wand, Linna Liu contributed equally to this work.

Funding

This work was financially supported by Tianjin Health Development Planning Commission of Science and Technology Fund Projects (grant no. MS20023)

Authors' contributions

All authors read and approved the final version of the manuscript.

Data Availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

Acknowledgements Not applicable.

References

Figures
Figure 1

Blood lipid level and PCSK9 mRNA expression in brain tissue of hypercholesterolemic mice model prepared by high-fat diet. 

A Serum total cholesterol content $n=10$. 

B Serum low density lipoprotein content $n=10$. 

C Relative expression of PCSK9 mRNA in hippocampus and cortex. $n=3$. Compared with NFD-control, *$p<0.05$, **$p<0.01$
Figure 2

The effect of shRNA-PCSK9 on silence PCSK9 mRNA in hypercholesterolemic mice. Compared with HFD-shRNA–control: *p<0.05.
Figure 3

Abnormal expression and hyperphosphorylation of Tau protein in brain of hypercholesterolemic mice induced by shRNA PCSK9. **A** Immunohistochemical pattern of Tau protein hyperphosphorylation (P-Tau) in hippocampus and cortex of hypercholesterolemic mice induced by shRNA-PCSK9(*200). **B** Immunohistochemical study of abnormal high expression of Tau protein in hippocampus and cortex of hypercholesterolemic mice induced by shRNA-PCSK9. **C** The relative expression of Tau and P-tau protein in hippocampus of hypercholesterolemic mice induced by shRNA-PCSK9 n=3 . Compared with HFD-shRNA–control: **p<0.01. **D** Electrophoretic bands of Tau and P-tau proteins in hippocampus (n=3). **E** Serum Aβ1-42Ab level in hypercholesterolemic mice (n=7).
Figure 4

Extraction and identification of plasma exosomes in hypercholesterolemic mice. A technical process of exosome extraction (drawn by Figdraw). B Under negative staining transmission electron microscope, "teacup-shaped vesicles" can be seen as exosomes, The NTA measurement shows that the particle size is 122.8nm. C Exosome biomarkers TSG101, HSP70 and CD63 can be seen in the protein bands of exosome markers.
Figure 5

Changes of miRNA-222 in plasma exosomes of hypercholesterolemic mice induced by shRNA-PCSK9

Note: The expression level of miRNA-222 in plasma exosomes of hypercholesterolemic mice was significantly decreased (P<0.01) (n=3) after silencing the expression of PCSK9 mRNA by shRNA-PCSK9 transfection. Compared with HFD-shRNA-control: **p<0.01.
Figure 6

Characteristics of hepatic lipid droplet deposition and intracranial PCSK9 expression in HFD rats. A Oil Red O staining in the liver of HFD rats (*200). B-C Serum TC levels and LDL-C levels n=10. Serum TC and LDL-C in HFD-model group were significantly higher than those in NFD-control group. Compared with NFD-control: **p<0.01. Serum TC and LDL-C levels decreased significantly after shRNA-PCSK9 injection. Compared with HFD-shRNA-control, ##p<0.01. D Immunohistochemical pattern of PCSK9 in...
hippocampus and cortex of hypercholesterolemic mice induced by shRNA-PCSK9 (*200). E The relative expression of PCSK9 mRNA in hippocampus and cortex of hypercholesterolemic mice induced by shRNA-PCSK9. Compared with NFD-control: **p<0.01. Compared with HFD-shRNA-control, ##p<0.01 n=3

**Figure 7**

The levels of exosomal miRNA-222 biomarkers are significantly correlated with the levels of Aβ1-42Ab in serum. A The expression level of miRNA-222 in plasma exosomes of hypercholesterolemic mice was significantly decreased (p<0.01) (n=10) after silencing the expression of PCSK9 mRNA by shRNA-PCSK9 transfection. Compared with HFD-shRNA-control: ##p<0.01. Compared with HFD-control: ++p<0.01. Compared with NFD-control: **p<0.01. B show significantly correlations of miRNA-222 from exosomes and Aβ1-42Ab in serum (n=30).