Schizophrenia-associated microRNA-148b-3p regulates *COMT* and *PRSS16* expression by targeting the *ZNF804A* gene in human neuroblastoma cells

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Abstract. Zinc finger protein 804A (*ZNF804A*) has been identified by genome-wide association studies as a robust risk gene in schizophrenia, but how *ZNF804A* contributes to schizophrenia and its upstream regulation remains unknown. Previous studies have indicated that microRNAs (miRs) are key factors that regulate the expression levels of their target genes. The present study revealed significantly increased expression of miR-148b-3p in the peripheral blood of patients with first-onset schizophrenia compared with healthy controls, and bioinformatics analysis predicted that the *ZNF804A* gene is a target of miR-148b-3p. Therefore, the present study investigated the possible upstream regulation of *ZNF804A* by miR-148b-3p in the human neuroblastoma SH-SY5Y cell line, and assessed the implications for schizophrenia. The results revealed significantly reversed expression levels of miR-148b-3p (P=0.0051) and *ZNF804A* (P=0.0218) in the peripheral blood of patients with first-onset schizophrenia compared with healthy individuals. Furthermore, it was demonstrated that miR-148b-3p directly targeted *ZNF804A* via binding to conserved target sites in the 3'-untranslated region of *ZNF804A* mRNA, where it inhibited the endogenous expression of *ZNF804A* at both the mRNA (P=0.048) and protein levels (P=0.013) in SH-SY5Y cells. Furthermore, miR-148b-3p was revealed to regulate the expression levels of catechol-*O*-methyltransferase (*COMT*) and serine protease 16 (*PRSS16*) by targeting *ZNF804A* in SH-SY5Y cells. Collectively, the present results indicated that there was a direct upstream regulation of the schizophrenia risk gene *ZNF804A* by miR-148b-3p, which contributed to the regulation of the downstream genes *COMT* and *PRSS16*. Thus, the miR-148b-3p/*ZNF804A*/*COMT*/*PRSS16* pathway may play an important role in the pathophysiology of schizophrenia, and may serve as a potential target in drug discovery and gene therapy for this disorder.

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Introduction

Schizophrenia is a serious mental disorder characterized by affective and cognitive symptoms, and affects ~1% of the population with an estimated heritability of 64-80% (1). Genome-wide association studies (GWASs) have revealed a large number of risk genes in schizophrenia (2). The potential interaction of these genes constitutes pivotal signaling pathways and complex networks of regulation implicated with schizophrenia (3).

Zinc finger protein 804A (*ZNF804A*), located on chromosome 2q32.1, was the first schizophrenia susceptibility gene identified by GWASs (4). Subsequent larger GWASs confirmed the association of *ZNF804A* with schizophrenia, and extended its association to other psychiatric diagnoses, including bipolar disorder (5,6). Although *ZNF804A* regulates a number of genes and pathways associated with schizophrenia (7), the upstream regulation of *ZNF804A*, as well as its implication in schizophrenia, have not been fully investigated.

MicroRNAs (miRNAs/miRs), a class of endogenous non-coding single-strand RNAs that are 9-23 nucleotides in length, are of interest as key transcriptional regulators of ~1/2 of human genes (8). Moreover, genetic variants in the human *MIR137* gene locus increase schizophrenia risk and have

genome-wide significance (9). It has been revealed that miR-137 downregulates the presynaptic target gene synaptotagmin-1 and impairs synaptic plasticity in the hippocampus (10). Furthermore, ephrin B2 is a validated target gene of miR-137, and the aberrant expression of miR-137 in the peripheral blood of patients with schizophrenia provides further evidence for its involvement in schizophrenia etiology and diagnosis (11).

The present study revealed significantly different expression profiles for miR-148b-3p in peripheral blood of patients with first-onset schizophrenia compared with healthy controls. Thus, the present study investigated the possible upstream regulation of *ZNF804A* by miR-148b-3p, as an underlying molecular mechanism in schizophrenia. Therefore, SH-SY5Y neuroblastoma human cells were used in the present study. SH-SY5Y is a thrice cloned subline of the SK-N-SH cell line that was originally established from a bone marrow biopsy from a patient with neuroblastoma in the 1970s, and has been widely used for examining the cellular mechanisms underlying the etiology of neuropsychiatric disorders, including schizophrenia and bipolar disorder (12-14).

Materials and methods

Human peripheral blood collection and reverse transcription-quantitative PCR (RT-qPCR). The study was approved by the Medical Ethics Committee of Xi'an Jiaotong University Health Science Center and was in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects in the Affiliated Hospitals of Xi'an Jiaotong University (Xi'an, China) from May 2014 to April 2015. A total of 88 unrelated subjects were enrolled in the present study, including 44 patients with schizophrenia and 44 healthy controls, the details of whom were described previously (11). Peripheral blood samples from the participants (5 ml) were collected in EDTA-coated tubes and were then placed on ice. Total RNA was isolated within 2 h of collection using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The first strand of cDNA for miRNA was synthesized using Mir-X miRNA First-Strand Synthesis Kit (Clontech, USA), and the first strand of cDNA for mRNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). RT-qPCR was performed using FastStart Universal SYBR Green Master (Rox; Roche Diagnostics) and an Mx3005P qPCR system (Agilent Technologies, Inc.) according to the following procedure: 95˚C for 10 min; followed by 40 cycles of 95˚C for 10 sec, 60˚C for 30 sec, 72˚C for 30 sec; and a final dissociation curve analysis stage. Gene expression and miRNA expression were normalized to GAPDH and U6 small nuclear RNA expression, respectively. Each sample was tested in triplicate, and all data were analyzed using the $2^{-\Delta\Delta Cq}$ method (15). Primer information for genes and miRNAs is summarized in Table I.

Bioinformatics prediction. Human miR-148b-3p sequences were obtained from miRBase 22.1 (mirbase.org/). *ZNF804A* mRNA 3'-untranslated region (3'-UTR) sequences (Chr2:184,939,027-184,939,487, NM_194250) were obtained from the UCSC genome browser Human GRCh38/hg38 (http://genome.ucsc.edu/cgi-bin/hgTracks). TargetScan 6.2 (http://www.targetscan.org/), DIANA TOOLS 5.0 (http://diana.imis.athena-innovation.gr/DianaTools/index.php) and miRanda (August 2010 Release) (http://www.microrna.org) were used to predict miR-148b-3p and *ZNF804A* interaction, which was based on the presence of binding sites in the seed region, the efficacy of targeting and the probability of conserved targeting (16). A Venn diagram of individual prediction among the three software programs was calculated and drawn using Bioinformatics & Evolutionary Genomics online software 1.0 (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Recombinant construction and site-directed mutagenesis. A 461-bp-long 3'-UTR fragment of the *ZNF804A* gene, which contained the putative miR-148b-3p binding site, was synthesized by Chang Jing Bio-Tech, Ltd. The sequence was inserted into a pmirGLO Dual-Luciferase miRNA Target Expression vector (Promega Corporation) downstream of the firefly luciferase gene, between the *Sac*I and *Xba*I sites. The constructed wild-type vectors were validated by restriction enzyme digestion and Sanger sequencing. For restriction enzyme digestion, the following reaction components were mixed and maintained at 37˚C for 4 h: 10 μl 10X M buffer, 1 μl SacI, 1 μl XbaI (cat. Nos. 1078A and 1093A, respectively; both Takara Biotechnology Co., Ltd.) and 1 μg constructed wild-type vector. The total volume was adjusted to 20 μl by adding ddH_2O. The digestion products were separated on a 1% agarose gel using DL2000 (cat. no. 3427; Takara Biotechnology Co., Ltd.) as a DNA marker. For Sanger sequencing, the forward primer (5'-GACGAGGTGCCTAAAGGACT-3'), constructed vectors, and BigDye™ terminator v3.1 (cat. no. 4336913; Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for sequencing reaction to generate fluorescently labeled DNA fragments with different sizes. Subsequently, the DNA fragments were separated by capillary electrophoresis, during which the fluorescent labels were excited by laser and transformed to chromatogram. The Sanger sequencing results were identified to be consistent with the GRCh38/hg38 reference sequence from the UCSC genome browser (genome.ucsc.edu/cgi-bin/hgTracks) and the NCBI database (ncbi.nlm.nih.gov/gene/91752). The mutated *ZNF804A* 3'-UTR sequence, containing all six nucleotides in the putative seed-pair region, was synthesized according to A-T, C-G substitution criteria (17). The mutated product was constructed by inserting the mutant sequence into the pmirGLO Dual-Luciferase miRNA Target Expression vector, and it was validated by restriction enzyme digestion and Sanger sequencing.

Cell culture. The SH-SY5Y cell line was purchased from American Type Culture Collection (cat. no. CRL-2266), and has been authenticated by short tandem repeat profiling. Another neuroblastoma cell line, SK-N-BE(2), and 293T cells were obtained from the Cell Bank of the Chinese Academy of Sciences, and were authenticated. All cells were maintained in a humidified incubator at 37˚C with 5% CO_2. 293T cells were cultured in DMEM with high glucose (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Penicillin-Streptomycin solution; HyClone; GE Healthcare Life Sciences). SH-SY5Y and SK-N-BE(2) cells were cultured

Table I. Primer information of miRNA and genes for RT-qPCR.

Gene name	Accession no.	Primer for RT-qPCR	Location for the first nucleotide of the primer[a]	Sequence (5'→3')	Annealing temperature (˚C)
Hsa-miR-148b-3p	NR_029894	Forward	Chr12:54337278	TCAGTGCATCACAGAACTTTGT	60
ZNF804A	NM_194250	Forward	Chr2:184933670	GAAAACAGGAAAAGGCACTCC	60
		Reverse	Chr2:184935847	CACAACAACTCGTTGGGAAAT	
COMT	NM_000754	Forward	Chr22:19962760	CATTGACACCTACTGCGAGC	59
		Reverse	Chr22:19963678	TCTCGATGGTGATGAGCCTC	
PRSS16	NM_005865	Forward	Chr6:27255074	CTTGGACATGGCACCTGAG	58
		Reverse	Chr6:27255274	CTCTCCTTTGCCAGCTTGAG	
GAPDH	NM_002046	Forward	Chr12:6,537,347	CCAAGGTCATCCATGACAACT	60
		Reverse	Chr12:6,537,683	CAGGGATGATGTTCTGGAGAG	
U6 snRNA	NR_004394	Forward	Chr15:67,840,042	CTCGCTTCGGCAGCACA	60
		Reverse	Chr15:67,839,949	AACGCTTCACGAATTTGCGT	

[a]UCSC Browser, Dec 2013 (http://genome.ucsc.edu/cgi-bin/hgGateway). RT-qPCR, reverse transcription-quantitative PCR; miR/miRNA, microRNA; Chr, chromosome; *ZNF804A*, zinc finger protein 804A; NC, negative control; *COMT*, catechol-*O*-methyltransferase; *PRSS16*, serine protease 16.

in a 1:1 mixture of DMEM:nutrient mixture and F-12 medium (DMEM/F-12) containing 1.5 mM L-glutamine (HyClone; GE Healthcare Life Sciences); the mix was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Co-transfection and dual luciferase reporter assays. 293T cells were seeded on 48-well plates at a density of $5x10^4$/well and cultured at 37˚C for 24 h before transfection. A miR-148b-3p mimic (5'-UCAGUGCAUCACAGAACUUUGU-3'), a mimic negative control (NC; 5'-UUCUCCGAACGUGUCACGUTT-3'), a miR-148b-3p inhibitor (5'-ACAAAGUUCUGUGAUGCACUGA-3') and an inhibitor NC (5'-CAGUACUUUUGUGUAGUACAA-3') were obtained from Chang Jing Bio-Tech, Ltd. To evaluate the interaction between miR-148b-3p and the *ZNF804A* 3'-UTR, combinations of 50 nM mimic or mimic NC with 1 μg wild-type and mutant UTR vectors were co-transfected by using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 and 48 h after transfection, cells were harvested. The activity of the luciferase reporter gene was detected using the Dual-Luciferase® Reporter Assay system (Promega Corporation). Each sample was tested in triplicate, and the relative luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity, which was normalized against a blank control.

Cell transfection and RT-qPCR. SH-SY5Y cells were seeded on 12-well plates at a density of $2.5x10^5$/well and cultured at 37˚C for 24 h, followed by transfection with 50 nM miR-148b-3p mimic, 50 nM mimic NC, 50 nM miR-148b-3p inhibitor or 50 nM inhibitor NC, by Lipofectamine™ 2000. For comparison, blank and mock controls using the sample volume of media (1 ml) and transfection reagent (2 μl) were prepared. Each experimental group was transfected in triplicate. After a 24-h transfection period, total RNA was isolated using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified with NanoDrop 2000 (Thermo Fisher Scientific, Inc.). The same experimental procedure was performed in SK-N-BE(2) to verify the results in SH-SY5Y. RT-qPCR for mRNA expression was performed as aforementioned.

Western blotting. Following 48 h of transfection, proteins were extracted from SH-SY5Y cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing the protease inhibitor phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 20 μg protein samples was loaded onto each lane of 8% SDS-PAGE gel, followed by electrophoresis and transfer to PVDF membranes (EMD Millipore). Membranes were probed with the following primary antibodies overnight at 4˚C: Rabbit polyclonal anti-ZNF804A (1:1,000; cat. no. ab229346; Abcam), mouse monoclonal anti-GAPDH (1:1,000, cat. no. TA-08; OriGene Technologies, Inc.) or rabbit polyclonal anti-β-actin (1:1,000; cat. no. ab0035; Abways Technology). Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; cat. no. 31430; Thermo Fisher Scientific, Inc.) or goat anti-rabbit IgG (1:5,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) secondary antibody at room temperature for 2 h. The target proteins were detected by an enhanced chemiluminescence system (EMD Millipore) and scanned using the GeneGnome XRQ system (Syngene) with GeneTools analysis software 4.0; Syngene, and the densitometry of specific western blotting bands was analyzed with GeneTools analysis software. Protein expression was determined by comparing the band intensity of ZNF804A to that of the internal control, GAPDH. The same experimental procedure was performed in SK-N-BE(2) to verify the results in SH-SY5Y. Each experiment was performed in triplicate.

Ad-ZNF804A construction and cell infection. The human *ZNF804A* gene fragment was amplified by PCR and cloned into the adenoviral vector pHBAd-EF1-MCS-GFP (Hanbio

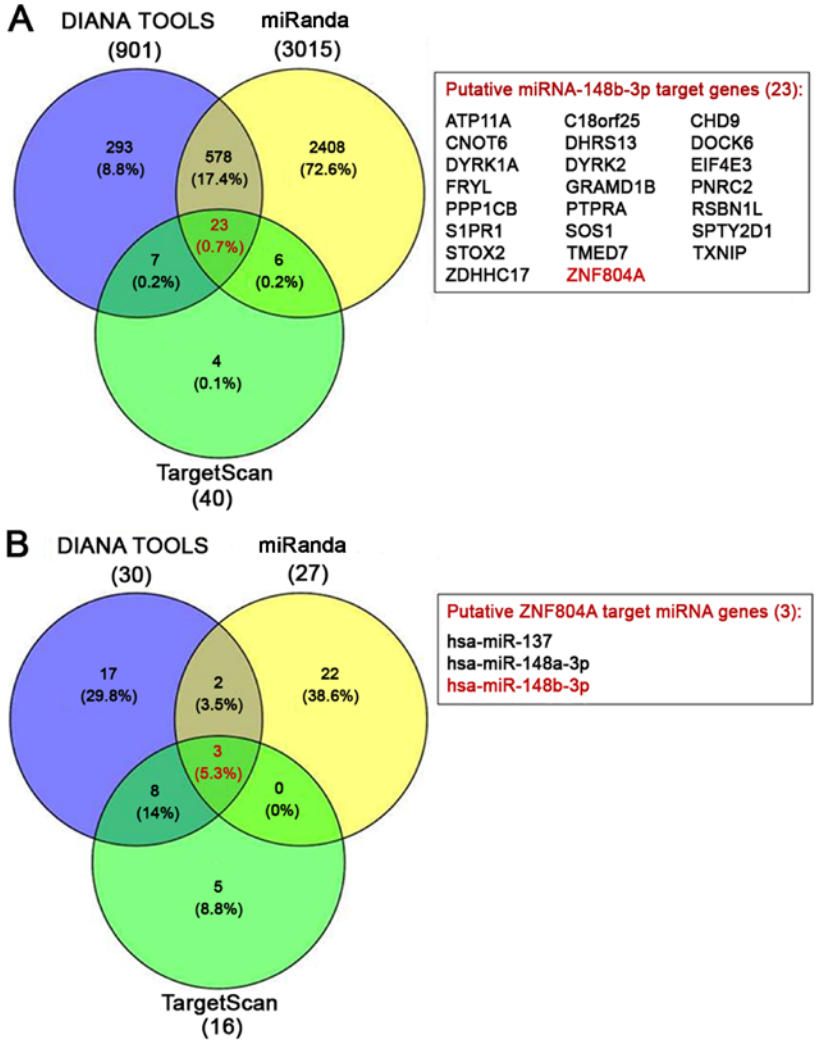

Figure 1. Venn diagrams of bioinformatics results for miR-148b-3p and *ZNF804A*. (A) *ZNF804A* is one of 23 shared target genes among the three databases. (B) miR-148b-3p is one of three shared miRNAs among the three databases. miR/miRNA, microRNA; *ZNF804A*, zinc finger protein 804A.

Biotechnology Co., Ltd.). The forward primer, 5'-GTGACC GGCGCCTACGAATTCGCCACCATGGAGTGTTACTAC ATTGT-3' and reverse primer, 5'-TCGGGATCCCGCCCGGGG GAAGAGAGGTTGCAAAGGGA-3' as well as human genome DNA template were used to amplify the *ZNF804A* gene fragment using KOD Plus (Toyobo Life Science) according to the following procedure: Pre-denatured at 95˚C for 5 min; then 27 cycles of 94˚C for 20 sec, 55˚C for 20 sec and 72˚C for 90 sec; and final extension at 72˚C for 10 min. Subsequently, the recombinant plasmid and pHBAd-BHG plasmid (Hanbio Biotechnology Co., Ltd.) were co-transfected into 293T cells using LipoFiter™ (Hanbio Biotechnology Co., Ltd.) to generate recombinant *ZNF804A* adenovirus, which was named 'Ad-*ZNF804A*'. Ad-green fluorescent protein was used as a NC. SH-SY5Y cells were pre-transfected with miR-148b-3p mimics (50 nM), and then infected with Ad-*ZNF804A* and Ad-NC for 24 h. The multiplicity of infection (MOI) was 50, and the blank group was used as a control. Total RNA was harvested using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) after 48 h, and RT-qPCR for gene expression was performed as aforementioned.

Statistical analysis. Data are presented as the mean ± SEM. Statistically significant differences among groups were detected with one-way ANOVA. Fisher's Least Significant Difference test or Tukey's test were carried out for post hoc analyses. Student's t-tests were used for comparisons between two groups that fitted normal distribution and had a homogeneity of variance. For data that did not fit normal distribution and had a homogeneity of variance, a non-parametric test (Mann Whitney test) was performed. Correlation analysis was performed using Pearson's correlation. Data analyses were performed using GraphPad Prism 5 software (GraphPad Prism Software, Inc.) or SPSS software version 18.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bioinformatic prediction of ZNF804A as a target of miR-148b-3p. TargetScan, DIANA TOOLS and miRanda predicted 40,901 and 3,015 putative target genes of miR-148b-3p, respectively. *ZNF804A* was one of 23 shared target genes identified by all prediction tools (Fig. 1A). Furthermore, 16, 30 and 27 miRNAs that target the *ZNF804A* gene were predicted by TargetScan, DIANA TOOLS and miRanda, respectively. Moreover, miR-148b-3p was one of three shared miRNAs identified by all prediction tools (Fig. 1B). It was demonstrated

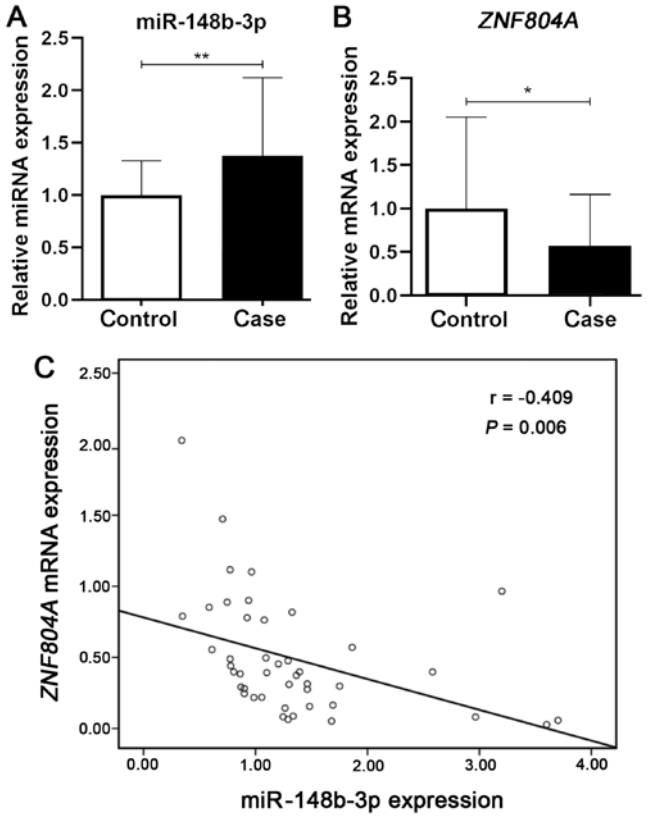

Figure 2. Altered expression levels of miR-148b-3p and *ZNF804A* in the peripheral blood of patients with schizophrenia and healthy controls. (A) Increased expression of miR-148b-3p. (B) Decreased expression of *ZNF804A*. (C) Correlation analysis between miR-148b-3p and *ZNF804A*. *P<0.05, **P<0.01. miR/miRNA, microRNA; *ZNF804A*, zinc finger protein 804A.

that all three databases predicted that miR-148b-3p interacted with the *ZNF804A* 3'-UTR from nucleotides 198-204. The binding site sequence of the *ZNF804A* 3'-UTR is broadly conserved among vertebrates, such as humans, chimpanzees, rhesus monkeys, rodents, rabbits, pigs, cats, elephants and chickens, suggesting that it has important roles in the regulation of gene function (genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr2%3A184939027%2D184939487&hgsid=820743199_5AAECt1MXtmem7EgvQiIixlwuapI).

miR-148b-3p and ZNF804A have aberrantly reversed expression in the peripheral blood of patients with schizophrenia. It was determined that the expression of miR-148b-3p was 1.35-fold higher in the peripheral blood of patients with schizophrenia compared with healthy controls (P=0.0051; Fig. 2A). Furthermore, the expression of *ZNF804A* was significantly decreased in patients with schizophrenia compared with healthy controls (P=0.0218; Fig. 2B). In addition, a correlation analysis between miR-148b-3p and ZNF804A was performed and a moderate negative correlation was identified (r=-0.409; P=0.006; Fig. 2C).

Biological verification of miR-148b-3p directly targeting ZNF804A. PmirGLO reconstructed vectors were generated and were then assessed by restriction enzyme digestion and Sanger sequencing. For this, one product of restriction enzyme digestion was the pmirGLO vector, which was 7,350 bp, and the other product was 461 bp, which was the same size as the PCR product before cloning into the vector (Fig. 3A). The sequencing results of the amplified fragments in the wild-type construct were consistent with the GRCh38/hg38 reference sequences from the UCSC genome browser and the NCBI database (Fig. 3B). Furthermore, the sequence in the binding sites was mutated according to the A-T, C-G substitution criterion (Fig. 3C).

The present results indicated that the luciferase activity in 293T cells co-transfected with the miR-148b-3p mimic was significantly decreased after 24 h (P<0.001; Fig. 4A) and 48 h (P<0.001; Fig. 4B) compared with the NC group. However, the luciferase activity did not reveal a significant difference for the mutant group with miR-148b-3p mimic compared with the NC group, thus suggesting that miR-148b-3p directly binds to the *ZNF804A* 3'-UTR and inhibits its expression.

miR-148b-3p inhibits the endogenous expression of ZNF804A. The results of RT-qPCR experiments revealed that the miR-148b-3p mimic significantly increased the expression of miR-148b-3p compared with the NC, mock or control groups, while the miR-148b-3p inhibitor significantly decreased miR-148b-3p expression. These results demonstrated the transfection efficiency with the successful gain or loss of miR-148b-3p function in SH-SY5Y cells (Fig. 5A). Moreover, *ZNF804A* expression was significantly decreased in the miR-148b-3p mimic group compared with the NC group (P=0.048). In addition, *ZNF804A* expression was increased significantly in the group transfected with the miR-148b-3p inhibitor compared with the inhibitor NC group (P=0.044; Fig. 5B). Therefore, the present results indicated that endogenous expression of *ZNF804A* may be regulated by miR-148b-3p at the mRNA level.

Western blotting results indicated that the miR-148b-3p mimic significantly decreased *ZNF804A* protein expression compared with the NC (P=0.013), mock or control groups (Fig. 6A and C). However, treatment with the miR-148b-3p inhibitor significantly increased *ZNF804A* protein expression (P=0.001; Fig. 6B and D). Collectively, the present results indicated that the endogenous expression of *ZNF804A* was downregulated by miR-148b-3p at the protein level.

miR-148b-3p regulates the schizophrenia susceptibility genes catechol-O-methyltransferase (COMT) and serine protease 16 (PRSS16) by targeting ZNF804A. SH-SY5Y cells were transfected with the miR-148b-3p mimic, mimic NC, miR-148b-3p inhibitor and inhibitor NC, to identify whether *COMT* and *PRSS16* are regulated by miR-148b-3p. It was demonstrated that transfection with the miR-148b-3p mimic induced a significant decrease in *COMT* compared with the NC (P=0.025), mock or control levels, and *COMT* expression was increased significantly in the miR-148b-3p inhibitor group compared with the inhibitor NC group (P=0.040; Fig. 7A). Furthermore, transfection with the miR-148b-3p mimic significantly increased the mRNA expression of *PRSS16* compared with the NC (P<0.0001), mock or control levels, and *PRSS16* expression was significantly decreased in the miR-148b-3p inhibitor group compared with the inhibitor NC group (P=0.047; Fig. 7B). Moreover, western blotting results

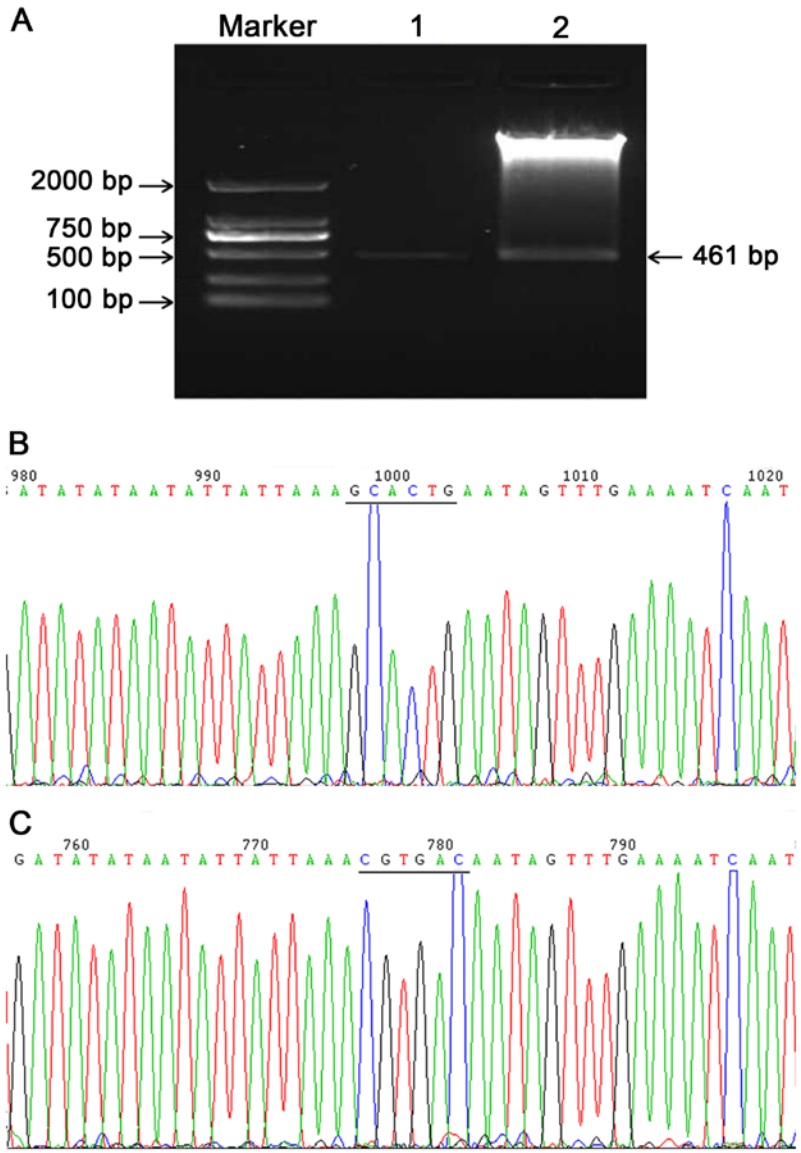

Figure 3. Results of restriction enzyme digestion and sequencing. (A) Results of restriction enzyme digestion. The lane 'Marker' was DL2000. The 461-bp PCR product representing the *ZNF804A* 3'-untranslated is in lane 1, and the restriction enzyme digestion result of the wild-type construct is in lane 2. (B) Sequencing results of the wild-type construct. (C) Sequencing results of the mutant construct. *ZNF804A*, zinc finger protein 804A.

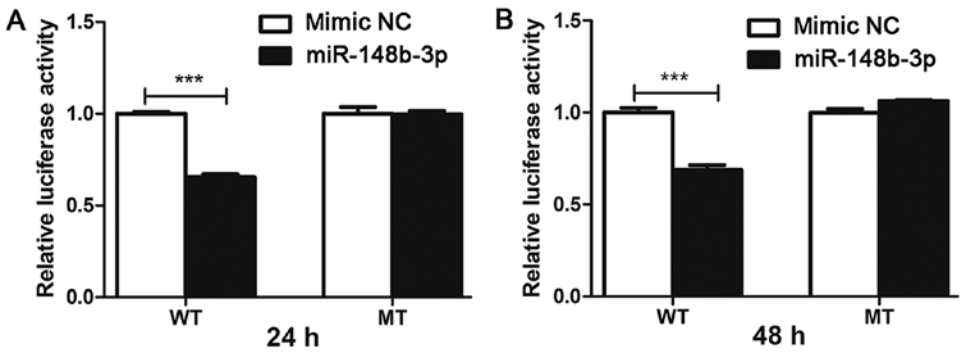

Figure 4. miR-148b-3p directly targets the *ZNF804A* gene. (A) Results of the dual luciferase reporter assay after 24 h, following transfection with the miR-148b-3p mimic or the mimic NC, and co-transfected with the WT or the MT constructs in 293T cells. (B) Results of the dual luciferase reporter assay after 48 h. ***P<0.001. miR, microRNA; *ZNF804A*, zinc finger protein 804A; NC, negative control; WT, wild-type; MT, mutant.

of the quantification of the protein expression of COMT were consistent with the RT-qPCR data (Fig. S1).

Similar experiments were performed in another neuroblastoma cell line, SK-N-BE(2). It was revealed that the results

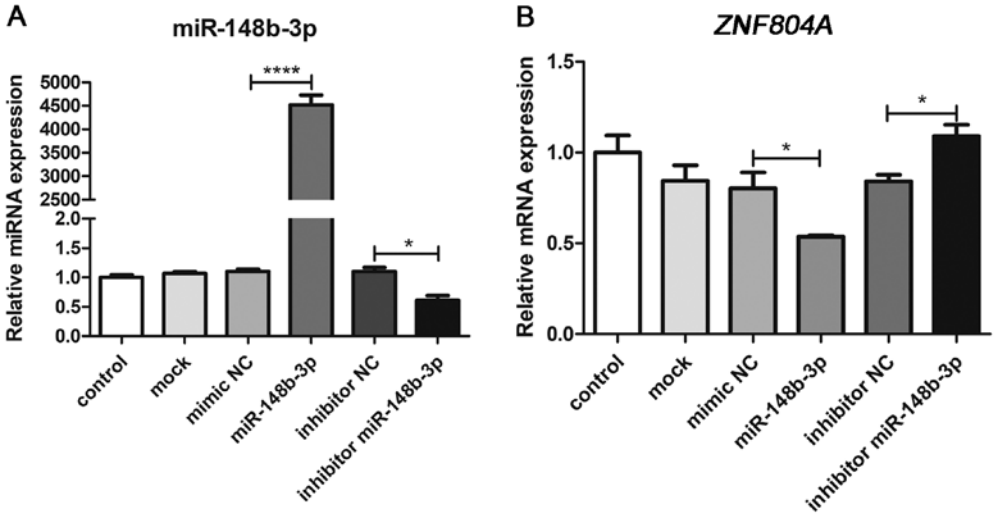

Figure 5. Endogenous mRNA expression of *ZNF804A* following gain or loss of miR-148b-3p function in human neuroblastoma cells. (A) Reverse transcription-quantitative PCR results following miR-148b-3p expression in SH-SY5Y cells. (B) mRNA expression of *ZNF804A* in SH-SY5Y cells. *P<0.05, ****P<0.0001. miR/miRNA, microRNA; *ZNF804A*, zinc finger protein 804A; NC, negative control.

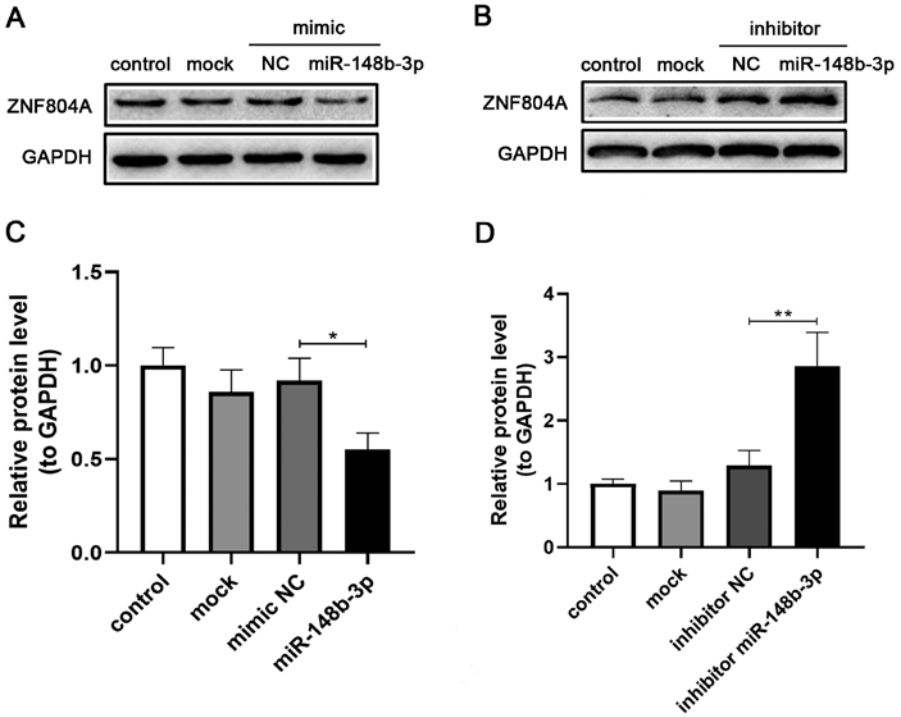

Figure 6. Effect of miR-148b-3p on the endogenous protein expression of *ZNF804A* in SH-SY5Y cells. (A) Western blotting results demonstrated the effects of the miR-148b-3p mimic treatment on *ZNF804A* expression. (B) Western blotting revealed the effects of the miR-148b-3p inhibitor treatment on *ZNF804A* expression. (C) Quantification of western blotting results presented in A. (D) Quantification of western blotting results presented in B. GAPDH was used as an internal control in both experiments. Statistical analyses were performed on three independent results. *P<0.05, **P<0.01. miR, microRNA; *ZNF804A*, zinc finger protein 804A; NC, negative control.

obtained in the SK-N-BE(2) cell line (Fig. S2) were consistent with those generated in the SH-SY5Y cell line.

It has been previously reported that *COMT* and *PRSS16* are regulated by ZNF804A (7). To assess whether miR-148b-3p regulates *COMT* and *PRSS16* by targeting the *ZNF804A* gene, *ZNF804A* expression was rescued in miR-148b-3p-expressing SH-SY5Y cells using an Ad-*ZNF804A* vector. It was revealed that the infection efficiency was as high as 90% when the MOI of adenovirus was 50 (Fig. 8A). Moreover, the mRNA expression level of *ZNF804A* revealed that the transfections were successful (P<0.001; Fig. 8B). The results of the gene expression experiments demonstrated that the transfection of the miR-148b-3p mimic, in addition to the infection of the *ZNF804A* expression vector, rescued the expression levels of *COMT* and *PRSS16*, which indicated that gene regulation was mediated by miR-148b-3p (Fig. 8C and D). Therefore, the present results suggested that miR-148b-3p regulated *COMT* and *PRSS16* by inhibiting *ZNF804A*.

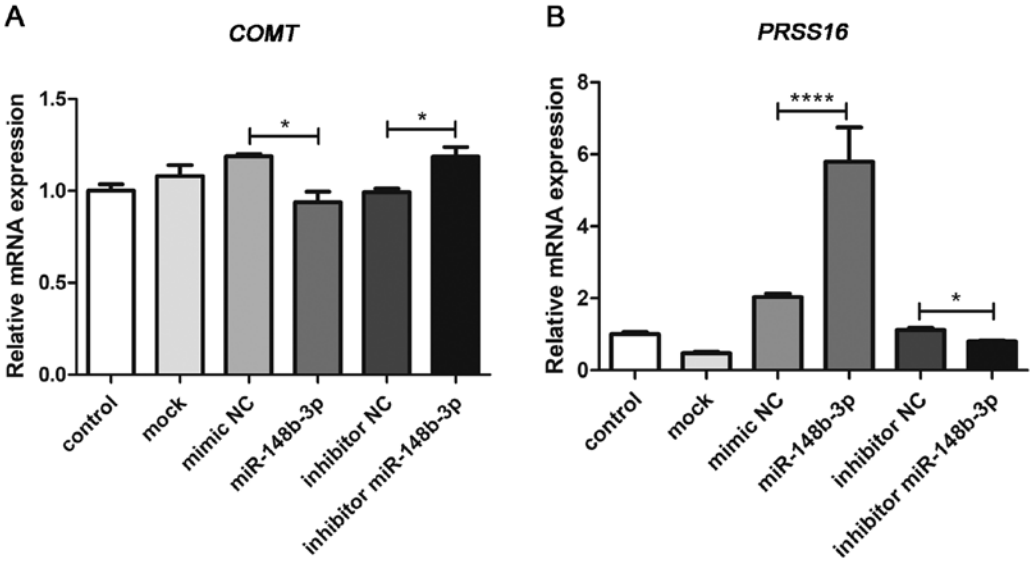

Figure 7. Effect of miR-148b-3p on the expression levels of *COMT* and *PRSS16* in SH-SY5Y cells. (A) mRNA expression of *COMT* in SH-SY5Y cells. (B) mRNA expression of *PRSS16* in SH-SY5Y cells. *P<0.05, ****P<0.0001. miR, microRNA; *ZNF804A*, zinc finger protein 804A; NC, negative control; *COMT*, catechol-*O*-methyltransferase; *PRSS16*, serine protease 16.

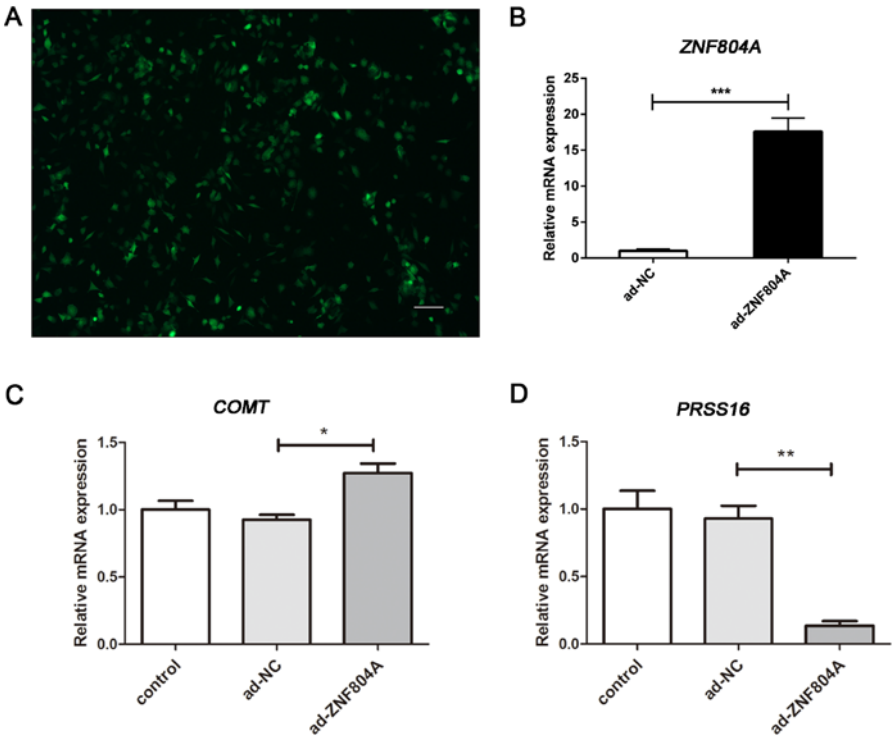

Figure 8. miR-148b-3p regulation of *COMT* and *PRSS16* is rescued by *ZNF804A* in SH-SY5Y cells. (A) Infection results of recombinant *ZNF804A* adenovirus. Multiplicity of infection=50. (B) mRNA expression of *ZNF804A* in SH-SY5Y cells. (C) mRNA expression of *COMT* in SH-SY5Y cells. (D) mRNA expression of *PRSS16* in SH-SY5Y cells. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA; *ZNF804A*, zinc finger protein 804A; NC, negative control; *COMT*, catechol-*O*-methyltransferase; *PRSS16*, serine protease 16.

Discussion

The present study revealed that the expression of miR-148b-3p was significantly increased in the peripheral blood of patients with first-onset schizophrenia compared with healthy controls. Bioinformatics analyses and dual luciferase reporter assays indicated that *ZNF804A* may be directly targeted by miR-148b-3p. In human neuroblastoma cells transfected with a miR-148b-3p mimic or a mimic NC, expression of *ZNF804A* at both the mRNA and protein levels was influenced by miR-148b-3p expression. Furthermore, two susceptibility genes, *COMT* and *PRSS16*, were determined to be indirectly regulated by miR-148b-3p via *ZNF804A* in SH-SY5Y cells. It has been previously shown that dopamine (DA) dysfunction influences both cortical and subcortical circuitry, thus facilitating different

symptomatology of schizophrenia (18). SH-SY5Y cells possess numerous characteristics of DAergic neurons, such as the ability to synthesize DA and express DA transporters (19,20). Furthermore, under certain conditions SH-SY5Y cells can differentiate into a more pronounced DAergic neuronal phenotype, which present several of the characteristics of primary cultures of neurons (20,21). Therefore, this cell line has been widely used for schizophrenia research (19-21).

miR-148b-3p belongs to the miR-148/152 family, which regulates the growth and development of healthy tissues (22). Differential expression between members of the miR-148/152 family in patients with tumor or type 1 diabetes compared with healthy controls indicates their important roles in the initiation and process of different diseases (22). In our previous study, it was revealed that miR-148b-3p expression was upregulated in the post-mortem dorsolateral prefrontal cortex of the schizophrenia group compared with healthy controls (23). Moreover, miR-148b has been revealed to affect neural stem cell proliferation, and their differentiation into new neurons and astrocytes (24).

While the precise function of *ZNF804A* is unknown in psychiatric disorders, the biological function and molecular mechanisms of *ZNF804A* have been revealed by previous studies (25,26). Furthermore, the neuronal function and molecular mechanisms of *ZNF804A* have been revealed by two landmark studies (25,26). Using neurons derived from human neural progenitor cells (NPCs), human induced pluripotent stem cells or primary rat cortical neurons, Deans *et al* (25) revealed that ZNF804A localizes to synapses, and regulates neurite formation and dendritic spine structure. In addition, Zhou *et al* (26) revealed that the rodent homologue of *ZNF804A* (*Zfp804a*) is required for NPC proliferation and neuronal migration, and also identified a novel role of *ZNF804A* in modulating protein translational efficiency during neurodevelopment.

COMT is a downstream gene of *ZNF804A* that codes for catechol-*O*-methyltransferase, which degrades extracellular catecholamines (27). DA is a catecholamine and is also a principal neurotransmitter in the central nervous system (28). In pharmacology, *COMT* is a biological target for the treatment of various central and peripheral nervous system disorders, including Parkinson's disease, depression and other DA-related diseases (29). The DAergic system has been implicated in neuropsychological processes that are impaired in patients with schizophrenia, and these impairments are correlated with structural abnormalities observed in the brains of these patients (30,31). In addition, DA is a powerful activator of resting effector T cells by acting on its receptors on immune effector cells (28). Both altered immune functions and abnormal DAergic systems have been observed in patients with schizophrenia (32). *COMT*, as a susceptibility gene for schizophrenia, also represents an important target for the treatment of the disease (33). *PRSS16* is located at 6p22-p21.3 on the chromosome near the major histocompatibility complex class I region, and encodes a serine protease that is abundantly expressed in the thymus; this serine protease plays an important role in immune function (34). Moreover, a GWAS indicated that the single-nucleotide polymorphisms rs6932590 and rs13219354 in the *PRSS16* loci are associated with schizophrenia risk (35).

Thus, it is speculated that miR-148b-3p may affect neurotransmitter synthesis and immune function by regulating the expression levels of *COMT* and *PRSS16*, which are downstream of *ZNF804A*, in schizophrenia. In the present study, it was revealed that expression of miR-148b-3p was aberrantly higher in the peripheral blood of patients with schizophrenia compared with healthy controls. Moreover, this expression pattern may reflect pathological processes in the brain, as significant gene expression overlap is found between whole blood and brain tissue (36). Brain-derived exosomes and their miRNA cargo may be key players in the cross-talk between the brain and the immune system in psychiatric disorders (37). Furthermore, the secretion of brain or immune cell exosomes is partially regulated by neurotransmitters (38,39). Exosomes or liposomes packaged with a specifically selected miRNA cargo may readily cross the blood-brain barrier, and therefore represent ideal carriers for nucleic acid-based drugs (40,41). The present results indicated that the miR-148b-3p/*ZNF804A*/*COMT*/*PRSS16* pathway may play an important role in the pathophysiology of schizophrenia, and that miR-148b-3p may be a novel drug target in schizophrenia.

In conclusion, the present results indicated that miR-148b-3p directly regulated the expression of *ZNF804A*, and as a result influenced the expression levels of *COMT* and *PRSS16*, which are downstream genes of *ZNF804A*. Therefore, this regulatory pathway may affect the immune function of patients with schizophrenia. It was also speculated that the miR-148b-3p/*ZNF804A*/*COMT*/*PRSS16* pathway may play an important role in the pathophysiology of schizophrenia. In addition, the present results may facilitate the development of drug discovery and gene therapy for schizophrenia.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to confidentiality of another study in our group, but are available from the corresponding author on reasonable request.

Authors' contributions

SW, PW, RT, PY, XY, YL, QS, FN, JH, RZ, YT and JM were involved in conception and design of the study, as well as

acquisition, analysis and interpretation of data. JM, YT, RZ and SW drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Xi'an Jiaotong University Health Science Center, in accordance with the Declaration of Helsinki. Informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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