

Cerebrospinal fluid exosomal miR-152-3p predicts the occurrence of subarachnoid haemorrhage and regulates vascular smooth muscle cell dysfunction

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Abstract

Introduction: Ruptured intracranial aneurysm (RA) can lead to subarachnoid haemorrhage (SAH). This study was to explore the predictive value of cerebrospinal fluid (CSF) derived exosome miR-152-3p and its regulatory role in the human vascular smooth muscle cells (HVSMCs).

Material and methods: Real-time quantitative polymerase reaction was carried out to detect CSF exosome miR-152-3p in 66 patients with unruptured intracranial aneurysms (UA), 69 patients with RA, and 68 patients with hydrocephalus. Clinical predictive value of SAH occurrence was assessed using receiver operating characteristic curve (ROC) and logistics regression analysis. Cell Counting Kit-8 and Transwell were employed to detect the proliferation and migration of HVSMCs. The binding relationship between miR-152-3p and PTEN was confirmed by the dual-luciferase reporter assay.

Results: Compared with hydrocephalus, exosome miR-152-3p was lower in patients with intracranial aneurysms, and among them, RA was lower than in patients with UA ($p < 0.001$). ROC confirmed that exosome miR-152-3p not only distinguishes patients with UA from patients with hydrocephalus but also predicts SAH in patients with intracranial aneurysms. Logistic regression analysis showed that miR-152-3p (OR = 0.039, 95% CI = 0.015-0.106, $p < 0.001$) and aneurysm size (OR = 2.701, 95% CI = 1.045-6.890, $p = 0.040$) were independent predictors of progression for UA to RA. Increased miR-152-3p inhibited the proliferation and migration of HVSMCs. PTEN was the direct target gene of miR-152-3p, which was elevated in CSF-derived exosomes and negatively correlated with miR-152-3p levels.

Conclusions: Our study confirmed that the CSF-derived exosome miR-152-3p was a feasible predictor of SAH and was involved in the dysfunction of HVSMCs.

Key words: intracranial aneurysm, miR-152-3p, cerebrospinal fluid, exosome, predicts.

Introduction

Subarachnoid haemorrhage (SAH) is defined as a sudden filling of the subarachnoid space with blood. As a serious cerebrovascular disease, it accounts for

5-7% of strokes [6] and is associated with 50% of the annual global mortality rate [17]. About 10% of patients died before reaching the hospital and 45% of patients died within 30 days [17]. The aetiology of SAH is complex, but 85% of SAH are caused by a ruptured

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intracranial aneurysm (RA), resulting in intracranial hypertension and decreased cerebral blood flow [29]. However, unruptured intracranial aneurysms (UA) are asymptomatic and the diagnosis relies on expensive and time-consuming radiological techniques [14]. Moreover, the rupture of the intracranial aneurysm is sudden and the exact mechanism of their rupture is not known. In summary, early recognition and prediction of aneurysmal SAH (aSAH) and understanding of its pathological mechanism can help improve its clinical outcome.

MicroRNAs (miRNAs) are a class of small (22 nucleotides) non-coding RNA molecules that regulate the disease processes by regulating their targets. Moreover, miRNAs are among the main active components carried by exosomes from different sources [25]. Exosomes are bilayer membrane vesicles with a diameter of 40–160 nm that transfer proteins, lipids, DNA, RNA (miRNA, lncRNA) into recipient cells to regulate their biological processes and affect the progression of the disease [15]. Exosome miR-124 is involved in the neuronal transmission of SAH and attenuates early brain injury [2]. Cerebrospinal fluid (CSF) exosome miR-630 regulates endothelial cell function in SAH [20]. In addition, exosomal miRNAs have shown advantages over traditional miRNAs as biomarkers for disease diagnosis, prediction, and prognosis. For example, miRNAs are protected by RNase because they are encapsulated by exosomes. Secondly, primitive miRNAs are encapsulated in exosomes, which can better reflect the specific information of parental cells [10]. For example, serum exosome miR-19a-3p is a diagnostic biomarker for the occurrence of the intracranial aneurysm [9].

miR-152-3p has been reported to be involved in neuronal damage in ischemic stroke [27]. Low expression of serum-derived exosome miR-152-3p in acute ischemic stroke patients correlates with its progression [18]. Remarkably, Stylli *et al.* identified differentially expressed miRNAs in CSF from non-SAH patients and SAH patients (combined vasospastic or non-vasospastic) and confirmed a significant down-regulation pattern of miR-152 in miRNAs [19]. Therefore, we hypothesized that the CSF-derived exosome miR-152-3p may have a potential clinical value in aSAH and may be involved in the progression of the disease. For this reason, we conducted studies to search for biomarkers that could identify RA for predicting the development of SAH and attempted to explore their potential mechanisms by regulating miR-152-3p levels *in vitro*.

Material and methods

Ethics statement

The study was conducted with the approval of the medical board of Quzhou People's Hospital. Informed consent was obtained prior to the collection of samples and subsequent analysis.

Participant population

From June 2017 to January 2019, the department of neurology at Quzhou People's Hospital admitted 66 patients with UA (34 males, 32 females). These patients had symptoms such as headache, vomiting, ptosis, or diplopia, but were otherwise normal. Another 69 patients with RA (35 males, 34 females) were included in this study during the same period. According to a previous study [19], the main inclusion criteria were: 1) Cerebral CT scan, digital subtraction angiography (DSA), neuroimaging studies, and the presence of blood or yellowing of lumbar cerebrospinal fluid to diagnose aSAH. CT was to assess whether an intracranial aneurysm has ruptured. DSA was mainly to determine the aneurysm's location and morphology. 2) Acute patients with severe nausea, headache, vomiting. 3) Excluding SAH not caused by intracranial aneurysms, such as vascular malformation or arterial dissection. 4) Patients with parenchymal haemorrhage or intracranial hematoma were also excluded. What is more, 68 patients (32 males, 36 females) with age-matched normal pressure hydrocephalus in the same period served as the controls. And the inclusion criteria were: 1) Cerebral CT confirmed ventricular amplification but maintained normal intracranial pressure. 2) Need for cerebrospinal fluid shunt surgery. 3) No recurrence of cardiovascular and cerebrovascular diseases, such as stroke and meningitis. The clinicopathological features of the subjects were listed in Table I.

Collection of CSF

Participants' 4 ml CSF samples were collected through a lumbar catheter and placed in sterile tubes. And immediately, the cells and cell debris were removed by centrifugation. The supernatant was immediately stored at -80°C for subsequent analysis.

Isolation and identification of exosomes

Based on previous studies [3], exosomes were isolated from CSF using the Total Exosome Isola-

tion Reagent (4484453, Thermo Fisher Scientific). The isolated exosomes were then identified by Western-blot and extracts were confirmed as exosomes.

Western-blot analysis

Lysis of exosome microspheres with protein lysates containing protease inhibitors. And its concentration is determined by BCA assay Kit. The proteins were then added to SDS-PAGE gels for separation and subsequently transferred to PVDF membranes at a voltage of 90V for 110 min. 5% of bovine serum white was blocked. Subsequently incubated overnight at 4°C with primary antibodies of anti-CD9 (ab92726, Abcam), anti-HSC70 (PA5-27337, Thermo Fisher Scientific), and anti-TSG101 (sc-7964, Santa Cruz Biotechnology). TBST was washed 3 times for 10 min each time and incubated with a secondary antibody at room temperature for 2 h. TBST was washed another three times, followed by a chemiluminescence assay to detect the protein expression level.

Real time quantitative polymerase (RT-qPCR) reaction

The miRcury exosome isolation kit (76743, Qia-gen) isolates exosomal miRNAs from CSF. The column purification was then performed through the miRcury RNA isolation kit. TRIzol was added into cells to isolate total RNA. Then RNA was detected in the NanoDRop spectrophotometer to detect the concentration and purity of the eluted RNA. Complementary DNA (cDNA) was synthesized by the miRcute Plus miRNA First-Strand cDNA synthesis Kit (KR201-02, Tiangen Biotech) or FastKing gDNA Dispelling RT SuperMix Kit (KR118-02, Tiangen Biotech). miRcute Plus miRNA qPCR (SYBR Green) kit (KR211, Tiangen Biotech) or SuperReal PreMix Plus (SYBR Green) kit (FR205, Tiangen Biotech) and perform the amplification reaction on ABMI PRISM 7000 to quantitatively analyse RNA. $2^{-\Delta\Delta C_t}$ method normalized the relative expression of miR-152-3p and PTEN to U6 or GAPDH. The sequences of primer were the following: miR-152-3p forward 5'-TCGGCAGGTCAGTGCATGACAGAA-3', reverse 5'-CTCAACTGGTGTGTCGTGGA-3'; PTEN forward 5'-TCCCAGACATGACAGCCATC-3'; reverse 5'-TGCTTTGAATCCAAAAACCTTACT-3'; U6 forward 5'-CTCGCTTCGGCAGCACA-3'; reverse 5'-AACGCTTACGAATTTGCGT-3'; GAPDH forward 5'-AATGTGTCCGTCGTGGATCTGA-3'; reverse 5'-GATGCCTGCTTACCACCTTCT-3'. The thermocycling conditions

Table 1. Basic clinical information of the subjects

Parameters	Controls (n = 68)	UA (n = 66)	RA (n = 69)	P-value
Gender				0.858
Male	32	34	35	
Female	36	32	34	
Age (years)				0.170
> 61	38	29	41	
≤ 61	30	37	28	
Hypertension				0.421
Yes	40	45	47	
No	28	21	22	
Smoking				0.484
Yes	30	30	37	
No	38	36	32	
Drinking				0.754
Yes	32	35	36	
No	36	31	33	
Aneurysm size (cm)				0.021
≤ 5		38	29	
> 5		28	40	

used were as follows: 95°C for 10 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for the 30s.

Culture and treatment of human vascular smooth muscle cells (HVSMCs)

HVSMCs were purchased from BeNa Culture Collection and grew in an incubator with moderate humidity of 37°C and 5% CO₂ supplemented with DMEM medium with 10% FBS and 1% penicillin/streptomycin. miR-152-3p mimic, miR-152-3p inhibitor, mimic NC, or inhibitor NC were obtained from RiboBio and transfected into HVSMCs with the help of the transfected reagent of Lipofectamine 3000. The RT-qPCR was performed 48 h after transfection to verify the transfection efficiency.

Cell proliferation analysis

The transfected HVSMCs were inoculated into each well of a 96-well plate at 1×10^4 cells/100 μ l. Then 10 μ l Cell Counting Kit (CCK-8, GK10001, Beyotime) reagent was added to the well every 24 h after cell attachment, mixed gently, and placed in the incubator for incubation for 1 h. The changes in cell proliferation were then assessed by detecting the optical density at 450 nm.

Cell migration analysis

After transfection 48 h, HVSMCs were re-suspended in DMEM containing 1% FBS and inoculated into the upper part of the Transwell chamber at a concentration of 1×10^4 cells/200 μ l. The Transwell was then transferred to an incubator for 16 h to allow complete migration. After PBS washing, the unmigrated cells in the upper compartment were removed with a cotton swab. 4% paraformaldehyde was fixed for 10 min, and 0.1% crystal violet was stained for 20 min. The number of cells in 10 fields of view was counted under the microscope.

Luciferase reporter assay

Three online prediction databases TargetScan, miRDB, and miRWalk, were analysed for potential miR-152-3p targets, and PTEN was identified as containing potential binding sites for miR-152-3p. The plasmid containing the predicted miR-152-3p binding site (WT-PTEN) or mutated binding site (MUT-PTEN) were subcloned into the luciferase vector pmirGLO to construct the recombinant plasmid. And they were co-transfected with miR-152-3p mimic or inhibitor or NC by Lipofectamine 3000. After 2 days, luciferase activity was assessed by a dual-luciferase assay system (E1910, Promega Corporation) and standardized to Renilla luciferase activity.

Statistical analysis

Continuous variables were presented as mean \pm standard deviation (SD) after 3 independent biological experiment replication. And two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to analyse statistical differences between multiple groups. Categorical variables were carried out using χ^2 tests. SPSS 23.0 and GraphPad prism 6.0 analysed the data and plotted the table and figure. Logistics regression analysis of risk factors from UA to RA was performed. Receiver operating characteristic (ROC) curves were plotted and the area under the curve (AUC) was calculated to assess the diagnostic accuracy of SCF exosomal miR-152-3p. $P < 0.05$ was considered statistically significant.

Results

General characteristics of the patients included

A total of 203 subjects were included in the present research, of which 66 patients with UA (34 males, 32 females), 69 patients with RA (35 males, 34 females), and 68 patients with hydrocephalus (32 males, 36 females). Demographic statistics were recorded in Table I. And the difference between the participants was only in the size of the intracranial aneurysm ($p < 0.05$). The results indicate that the studied sample was representative.

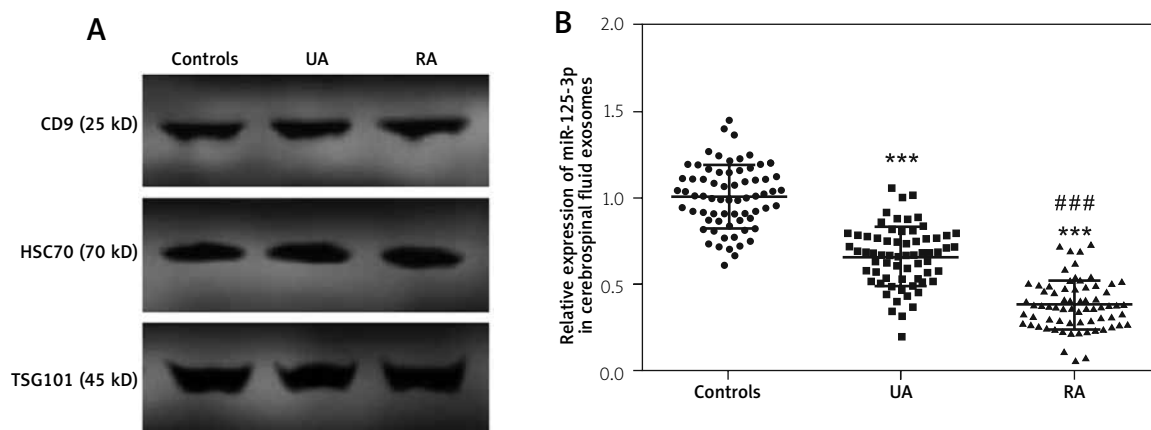


Fig. 1. Exosome miR-152-3p levels from cerebrospinal fluid (CSF) were detected. **A)** Western-blot verified the expression of exosome-specific markers and they were positive. **B)** The levels of CSF exosomal miR-152-3p in patients with hydrocephalus, UA, and RA were investigated by real time quantitative polymerase reaction (RT-qPCR). *** $p < 0.05$ vs. patients with hydrocephalus; ### $p < 0.05$ vs. UA. UA – unruptured intracranial aneurysms, RA – ruptured intracranial aneurysms.

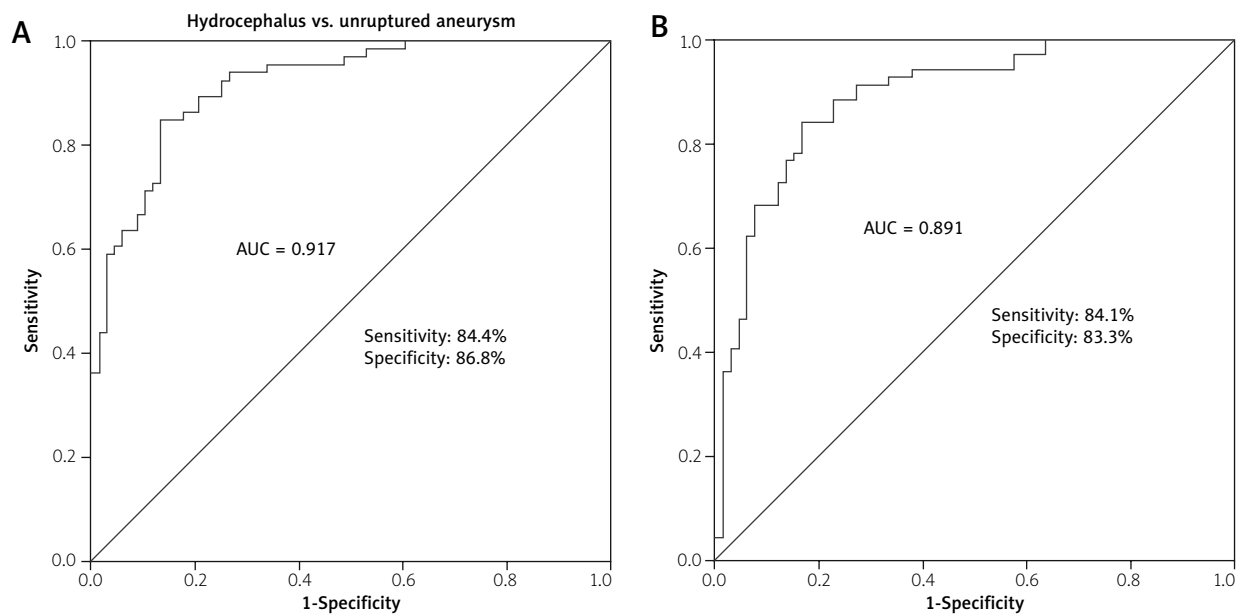


Fig. 2. Receiver operating characteristic curve (ROC) was used to evaluate the clinical predictive value of exosome miR-152-3p. **A)** The exosome miR-152-3p can significantly identify UA patients from hydrocephalus patients. **B)** Exosome miR-152-3p can typically predict the occurrence of SAH in intracranial aneurysm patients.

Exosome miR-152-3p was reduced in CSF from RA patients

Western blotting was performed to examine the exosome-specific markers CD9, HSC70, and TSG101, which were all positive (Fig. 1A). The association of exosome miR-152-3p with the intracranial aneurysm was then evaluated. RT-qPCR showed that exosomal miR-152-3p was markedly lower in patients with intracranial aneurysms compared with patients with hydrocephalus, among which RA patients were significantly lower than UA patients ($p < 0.05$, Fig. 1B). This suggests that low expression of miR-152-3p may play a crucial role in the progress of intracranial aneurysm.

Clinical value of exosome miR-152-3p in predicting SAH in patients with intracranial aneurysm

After identifying the differences in exosome miR-152-3p expression levels between UA and RA patients, we attempted to explore its predictive value in SAH. ROC results showed that exosome miR-152-3p could significantly distinguish UA patients from patients with hydrocephalus, with an AUC

Table II. Relationship between different variables and the development of unruptured aneurysm into the ruptured aneurysm

Characteristics	OR	95% CI	P value
MiR-152-3p	0.039	0.015-0.106	< 0.001
Gender	0.718	0.286-1.801	0.480
Age (years)	1.825	0.734-4.539	0.196
Hypertension	1.128	0.434-2.933	0.804
Smoking	1.812	0.714-4.599	0.211
Drinking	0.872	0.349-2.178	0.770
Aneurysm size	2.701	1.045-6.980	0.040

of 0.917, specificity of 84.80%, and sensitivity of 86.8%, respectively (Fig. 2A). What is more, exosome miR-152-3p was able to identify RA patients with SAH from UA patients. The AUC was 0.891, the sensitivity was 84.1% and the specificity was 83.3% (Fig. 2B). Subsequently, logistic regression analysis revealed that miR-152-3p (OR = 0.039, 95% CI = 0.015-0.106, $p < 0.001$) and aneurysm size (OR = 2.701, 95% CI = 1.045-6.890, $p = 0.040$, Table II) were an independent predictors of UA progression to RA. The above experimental results confirmed the predictive value of exosome miR-152-3p for the occurrence of SAH.

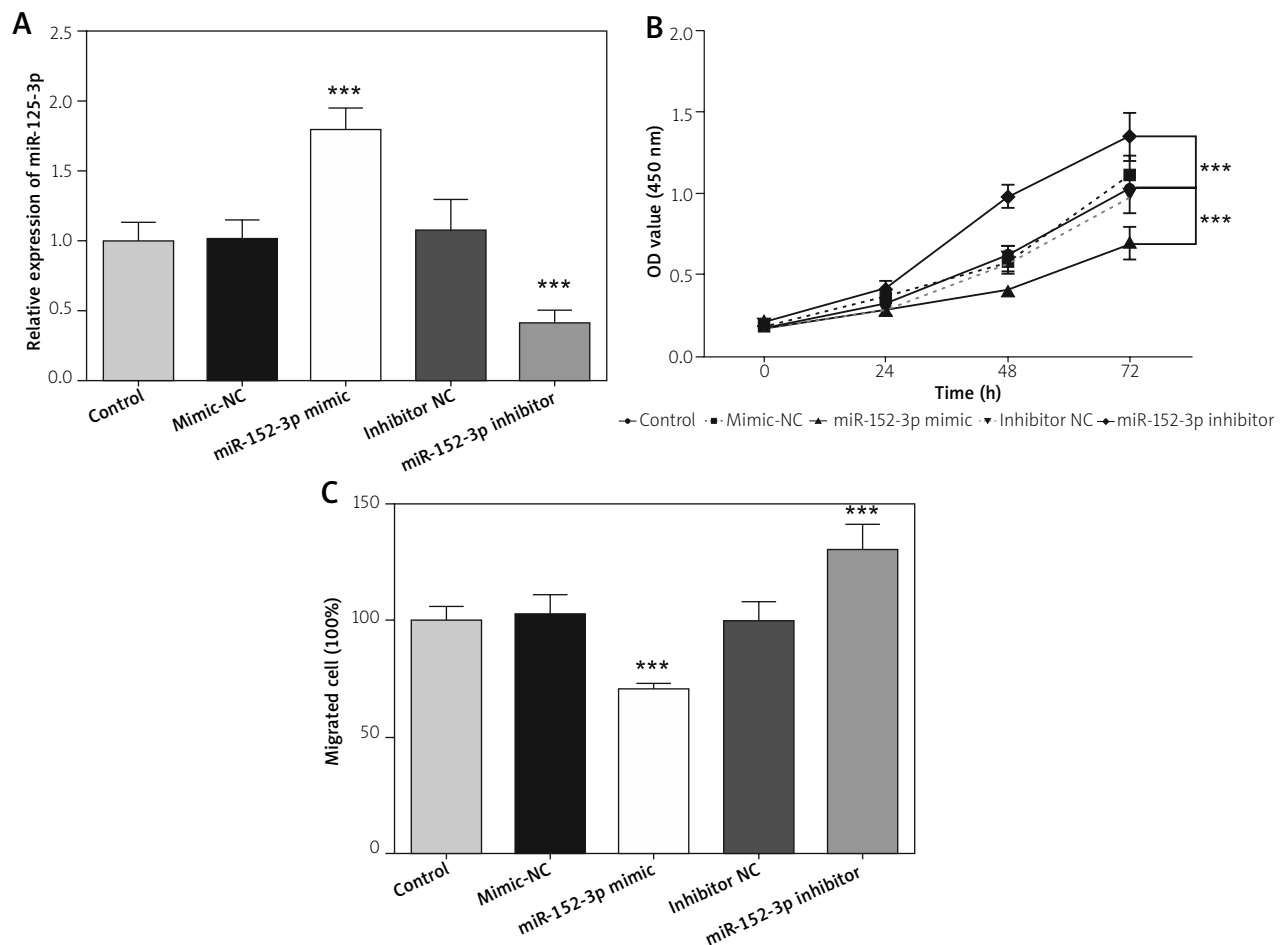


Fig. 3. Effects of miR-152-3p on cell proliferation and migration after transfection with miR-152-3p mimic or inhibitor. **A)** The miR-152-3p mRNA level after transfection in HVSMCs was analysed by real time quantitative polymerase reaction (RT-qPCR). **B)** Cell Counting Kit-8 (CCK-8) has detected the proliferation of HVSMCs. **C)** Transwell assay detected the regulation of miR-152-3p level on cell migration. *** $p < 0.05$ vs. control.

miR-152-3p inhibited the proliferation and migration of HVSMCs

As described in previous studies [7], VSMCs trigger stress and injury changes in response to blood pressure by accelerating cell migration and proliferation. Therefore, to investigate the effects of miR-152-3p on cell proliferation and migration, we transfected miR-152-3p mimic and inhibitor in HVSMCs and RT-qPCR confirmed that it was typically up- or down-regulated ($p < 0.05$, Fig. 3A). CCK-8 and Transwell assays confirmed that elevated miR-152-3p remarkably inhibited the proliferation and migration of HVSMCs, while decreased miR-152-3p significantly promoted their characteristics ($p < 0.05$, Fig. 3B, C). The above results suggest that miR-152-3p may be involved in the

pathological process of the intracranial aneurysm by regulating the proliferation and migration of HVSMCs.

miR-152-3p directly targets the PTEN gene in HVSMCs

Online database analysis of miR-152-3p could be combined with the 3'UTR of PTEN (Fig. 4A). Luciferase reporter assays assessed the target relationship between miR-152-3p and PTEN. The miR-152-3p mimic significantly decreased luciferase activity of WT-PTEN compared to the control, while miR-152-3p inhibitor typically increased the luciferase activity. However, there was no significant effect on MUT-PTEN luciferase activity ($p < 0.05$, Fig. 4B). And miR-152-3p elevation in HVSMCs remarkably inhibited

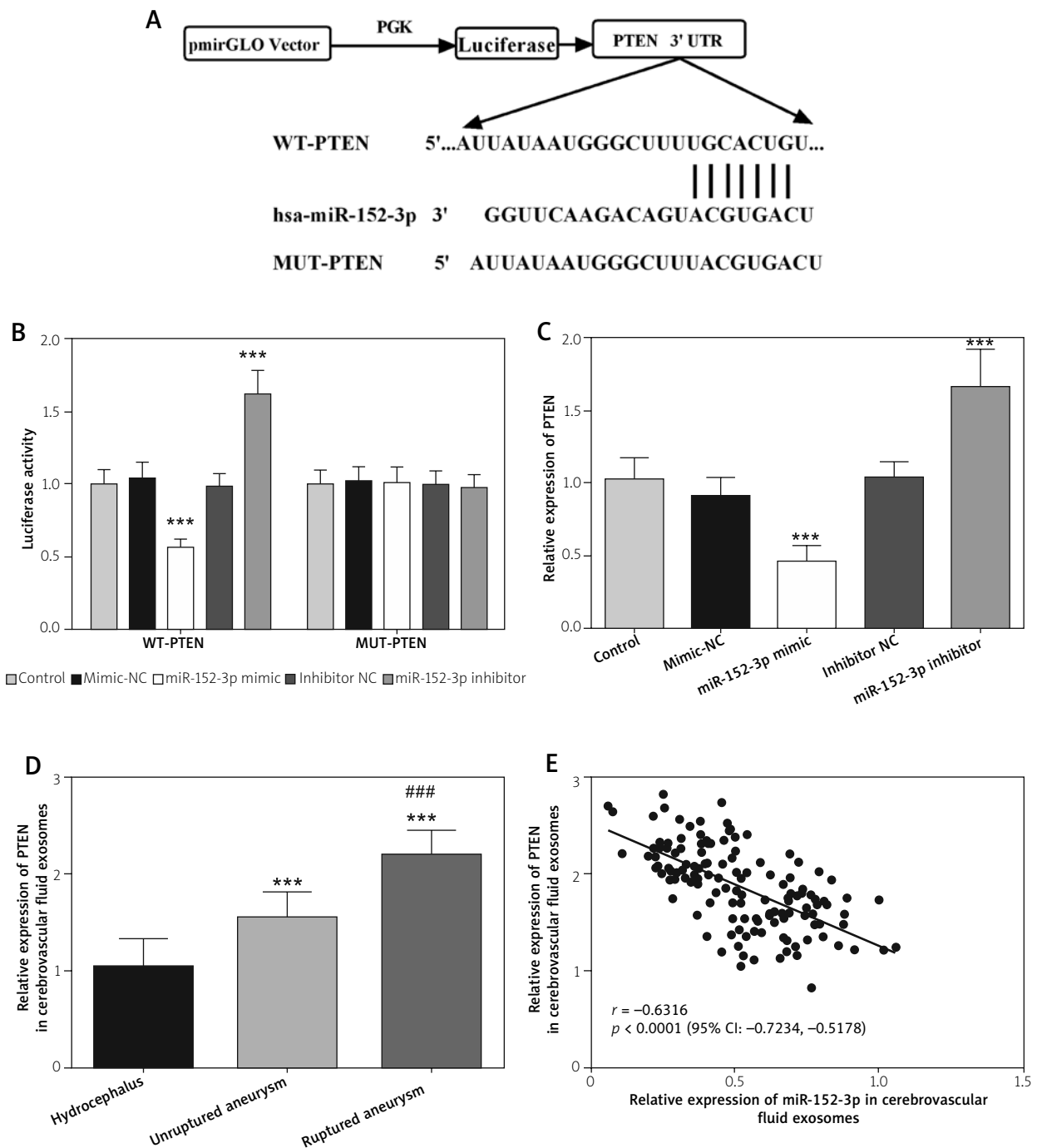


Fig. 4. miR-152-3p directly targets the PTEN gene in human vascular smooth muscle cells (HVSMCs). **A**) Predicted binding sites between miR-152-3p and PTEN mRNA 3'UTR. **B**) miR-152-3p mimic suppressed the luciferase activity of the WT-PTEN 3' UTR reporter. **C**) Elevated miR-152-3p inhibited PTEN mRNA levels in HVSMCs. **D**) CSF-derived exosome PTEN was typically elevated in patients with intracranial aneurysms. **E**) The level of miR-152-3p in the exosome was negatively correlated with PTEN in intracranial aneurysm patients. *** $p < 0.05$ vs. control or hydrocephalus patients; ### $p < 0.05$ vs. unruptured aneurysm.

PTEN mRNA ($p < 0.05$, Fig. 4C). More importantly, PTEN was significantly elevated in CSF-derived exosomes from patients with intracranial aneurysm ($p < 0.05$, Fig. 4D), which negatively correlated with miR-152-3p in intracranial aneurysm patients ($p < 0.05$, Fig. 4E).

Discussion

In the current study, we found that the level of exosome miR-152-3p in RA patients was typically lower than that in hydrocephalus and UA patients. Moreover, exosome miR-152-3p can significantly predict the occurrence of SAH in intracranial aneurysm patients. PTEN was the direct target gene of miR-152-3p, which was remarkably elevated in CSF-derived exosomes and negatively correlated with miR-152-3p levels. Afterwards, miR-152-3p was involved in the dysfunction of HVSMCs by regulation proliferation and migration, and this may be achieved through PTEN.

miRNA is an important regulator of gene expression regulation, and changes in its levels will typically affect the progression of the disease. MiR-152-3p is located on human chromosome 17q21.32 and its regulation has been described in some diseases. For instance miR-152-3p is involved in the progression of rectal cancer cells by regulating their proliferation and migration [21]. Its regulation can protect neurons from oxygen-glucose deprivation injury [27]. miR-152-3p is also associated with inflammatory response and apoptosis in acute kidney injury [11]. In addition, circulating miRNAs have been proved to be clinical biomarkers of diseases due to their high conservatism including diagnosis, prediction, and prognosis. miR-152-3p is a potential biomarker for predicting in-stent restenosis in coronary artery disease [12]. miR-152-3p was positively correlated with the degree of renal injury, suggesting that diabetic nephropathy was diagnosed with increased proteinuria [5]. Because exosomes can carry proteins, miRNAs, and lipids and release them into the peripheral circulation system, exosomal miRNAs from patients are more suitable as disease biomarkers than traditional miRNAs [13]. Therefore, exosome miRNAs have recently attracted a lot of attention. Such an exosome miR-146a is a biomarker for the diagnosis of acute coronary syndromes [8]. The low expression level of serum-derived exosome miR-152-3p in patients with acute ischemic stroke is associated with its progression [18]. Remarkably, studies have

analysed the differentially expressed CSF-derived miRNAs in SAH and non-SAH patients, and the signature down-regulation of miR-152-3p among miRNAs has been identified [19]. miR-152-3p is enriched in CSF and can be used as a biological tool for the early diagnosis of Parkinson's disease [4].

An intracranial aneurysm is an abnormal dilatation of intracranial arteries, which is usually caused by damage to the internal elastic layer and destruction of the media, resulting in a decrease in the structural integrity of the local arterial wall [9]. However, RA is serious and fatal, and it is also the main cause of SAH [16]. Due to the asymptomatic nature of UA and the rapid occurrence of RA, timely screening and intervention are the major challenges in the current RA research. At present, the commonly used clinical screening methods are brain CT and DSA accidentally discovering and identifying intracranial aneurysm and its progress and predicting the SAH [22]. Therefore, it is necessary to find suitable biomarkers.

In our study, we analysed the levels of CSF-derived exosome miR-152-3p in hydrocephalus and intracranial aneurysm patients and confirmed that the levels in intracranial aneurysm patients were markedly reduced. More importantly, the CSF-derived exosome miR-152-3p was lower in RA patients than in UA patients, suggesting that reduced miR-152-3p may be involved in the progression of intracranial aneurysm. Therefore, we further analysed its predictive value for SAH induced by RA. Exosome miR-152-3p could not only recognize UA from patients with hydrocephalus but also predict SAH in the intracranial aneurysm, showing high predictive value. What is more CSF-derived exosome miR-152-3p and aneurysm size were independent predictors of progression from UA to intracranial aneurysm.

After confirming the clinical predictive value of miR-152-3p in the intracranial aneurysm, we attempted to explore the potential mechanism of miR-152-3p affecting vascular rupture in an intracranial aneurysm *in vitro*. As most intracranial aneurysm occurs mostly in the arterial ring between the lower part of the brain and the base of the skull [23]. And the vascular VSMCs are the main cells in the arteries to maintain vascular plasticity in response to vascular injury, hypertension, and other physiological and pathological diseases [1]. CCK-8 and Transwell assay confirmed that increased miR-152-3p remarkably inhibited the proliferation and migration of HVSMCs, while decreased miR-152-3p significant-

ly promoted their characteristics. Previous studies have confirmed that the occurrence of intracranial aneurysms promotes the excessive proliferation and migration of HVSMCs, which is consistent with the results that our clinical underexpression prompts miR-152-3p. Our results suggest that miR-152-3p may be involved in the progression of the intracranial aneurysm by affecting the proliferation and migration of HVSMCs.

MiR-152-3p targeting PTEN is involved in cell apoptosis and inflammatory damage in Alzheimer's disease [26]. miR-152-3p targeting PTEN modulates the progression of non-small cell lung cancer [28]. miR-152-3p induced downregulation of PTEN delayed wound healing in diabetic foot ulcers [24]. Our study confirmed that PTEN was the target gene of miR-152-3p in the intracranial aneurysm, and the significant increase in CSF-derived exosome, and negatively correlated with the level of miR-152-3p. Moreover, miR-152-3p negatively regulated the mRNA level of PTEN in HVSMCs.

It is undeniable that the levels of several miRNAs may be altered when a vascular aneurysm ruptures. However, given the importance of miR-152-3p in cerebrovascular disease, we only targeted and examined the expression of miR-152-3p and its clinical role, and the failure to comprehensively focus on other miRNAs is a potential limitation of the present study, which will be addressed in our subsequent studies.

In conclusion, our study confirmed that the exosome miR-152-3p derived from CSF is a good predictor of SAH and its level is involved in the dysfunction of HVSMCs.

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Disclosure

The authors report no conflict of interest.

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