Guo *et al. Biology Direct* (2024) 19:76
https://doi.org/10.1186/s13062-024-00522-w

Biology Direct

RESEARCH Open Access

Proteomics and digital subtraction angiography approaches reveal CDH18 as a potential target for therapy of moyamoya disease

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Abstract
Moyamoya disease, characterized by basal cerebral artery obstruction, was studied for differential protein expression to elucidate its pathogenesis. Proteomic analysis of cerebrospinal fluid from 10 patients, categorized by postoperative angiography into good and poor prognosis groups, revealed 46 differentially expressed proteins. Notably, cadherin 18 (CDH18) was the most significantly upregulated in the good prognosis group. In addition, the expression of cadherin 18 (CDH18) and phenotypic transformation-related proteins were measured by qRT-PCR and western blot. The effects of CDH18 in vascular smooth muscle cells were detected by CCK-8, EdU, transwell and wound healing assays. The overexpression of CDH18 in vascular smooth muscle cells (VSMCs) was found to inhibit proliferation, migration, and phenotypic transformation. These findings suggest CDH18 as a potential therapeutic target in moyamoya disease.

Keywords Moyamoya disease, Proteomics, Digital subtraction angiography, CDH18

Introduction

Moyamoya disease is a rare cerebrovascular disorder characterized by progressive stenosis of the terminal portions of the internal carotid arteries and compensatory capillary collaterals [1, 2]. Moyamoya disease may lead to cerebral ischemia, cognition impairment or cerebral infarction, significantly impacting health and quality of life [3]. At present, the etiology of moyamoya disease remains unlear, but it may be associated with genetic factors, infections, immune responses, and vasculitic damage [4]. The diagnosis of moyamoya disease primarily relies on imaging techniques, particularly cerebral angiography. Common treatment approaches for moyamoya disease include rehabilitation therapy, pharmacological interventions and surgical revascularization [5]. However, the effectiveness of treatment can be influenced by various factors. Identifying viable therapeutic targets is essential for enhancing the efficacy of treatments for moyamoya disease.

Proteins are extensively involved in a multitude of cellular processes and play an essential role in maintaining normal physiological functions and health. Abnormal expression of many proteins is associated with the occurrence and progression of diseases

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[6]. Proteomics allows for rapid and comprehensive screening of differentially expressed proteins, which aids in uncovering the underlying mechanisms of disease development and in identifying effective biomarkers [7, 8]. In addition, proteomics technology has been extensively applied in the fields of drug discovery and development. By comparing the differences in protein expression levels in cells, tissues or organisms under normal and pathological conditions, as well as before and after drug treatment, it can help identify disease-related biomarkers and assess the therapeutic effects and the relationship between drug structure and activity [9, 10]. With ongoing technological advancements and in-depth research, proteomics is poised to offer more precise and effective tools for the prevention, diagnosis and treatment of diseases.

Cadherin 18 (CDH18), a type II classical cadherin, originates from the complete membrane protein cadherin superfamily that mediates calcium-dependent cell-cell adhesion [11]. CDH18 is primarily expressed in the central nervous system and is involved in the development of axons [12]. In glioma, CDH18 is downregulated and it represses the progression of glioma cells and enhances chemoresistance [13]. Additionally, CDH18 may exert an antitumor effect in gastric cancer by regulating the PI3K/AKT pathway [14].

Table 1 The detailed baseline data and clinical features of patients with moyamoya disease

Patient number	Age	Gender	Past medical history	Postoperative smoke vessels (1: rare; 2: Dense)
A1	44	female	none	1
B1	31	male	none	2
A2	29	female	none	1
A3	49	female	none	1
B2	51	male	none	2
B3	50	female	hypertension	2
A4	48	female	none	1
A5	47	male	none	1
B4	39	male	none	2
B5	53	female	none	2

Moreover, CDH18 inhibits the differentiation of stem cells into vascular smooth muscle cells (VSMCs) and serves as a biomarker in fetal epicardium [15]. However, the function of CDH18 in moyamoya disease has not been studied.

In this research, we conducted a proteomics analysis to screen for differentially expressed proteins between patients with good and poor prognosis in the context of moyamoya disease, and identified CDH18 as a specific biomarker for the disease. The cellular activity of VSMCs is implicated in the progression of moyamoya

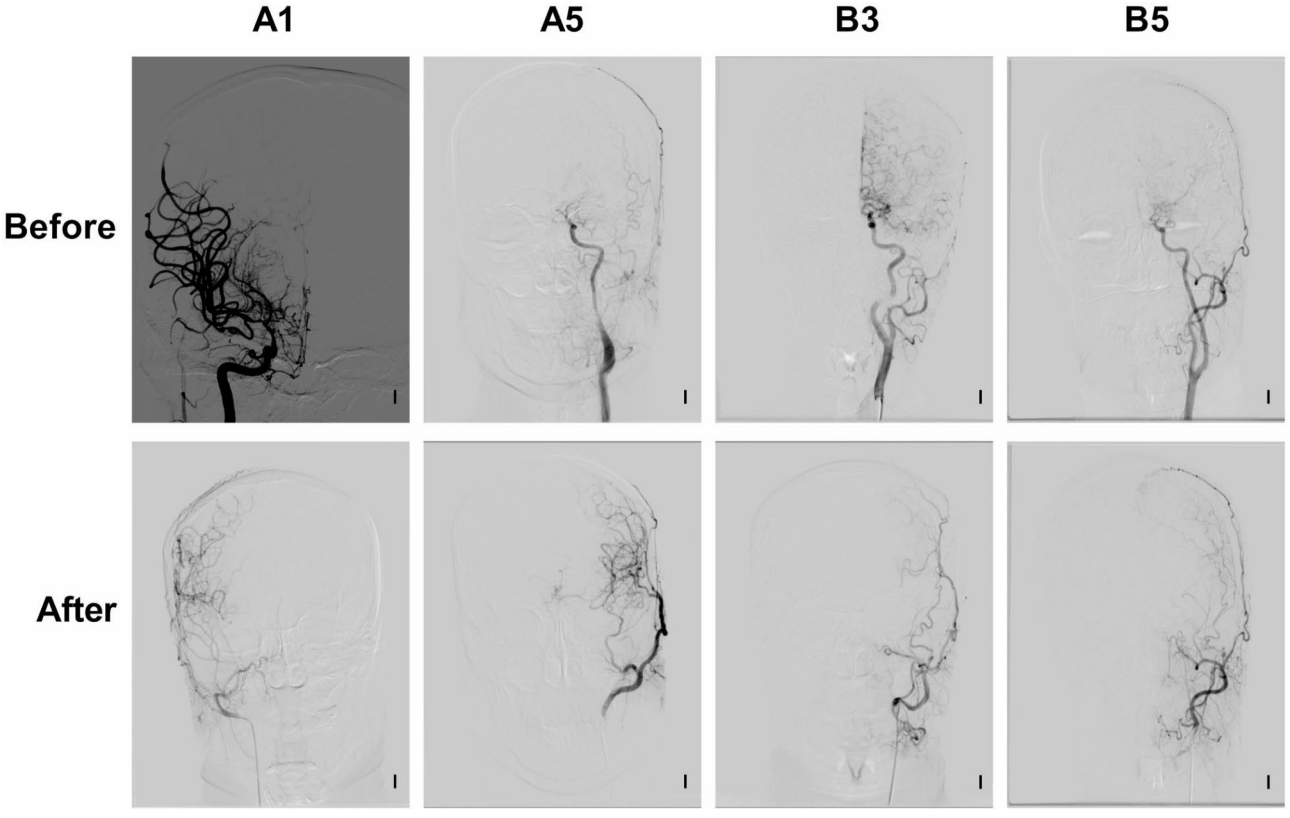

Fig. 1 The images of DSA of moyamoya disease patients before and after treatment. Scale bar: 10 mm

disease, and platelet-derived growth factor (PDGF) mediates the behavior of VSMCs, which is associated with the pathogenesis of various cardiovascular diseases [16–18]. Here, we explored the effects of CDH18 on VSMCs and PDGF-BB induced VSMCs, revealing the potential roles of CDH18 in the pathophysiology of moyamoya disease.

Materials and methods

Clinical sample

Ten patients diagnosed with moyamoya disease were included in the study, and cerebrospinal fluid samples were collected from each patient. Prior to treatment, all patients exhibited the same severity of illness. They all received identical treatments, which included cerebral revascularization surgery and aspirin therapy. Postoperatively, based on the density of smoky vessels observed (sparse or dense), patients were categorized into two groups: a good prognosis group (Group A, $n=5$) and a poor prognosis group (Group B, $n=5$). This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all participants. Detailed baseline data and clinical characteristics of the patients are presented in Table 1.

Mass spectrometry (MS) analysis

After extraction, the protein concentration was measured using a Bradford assay kit (Beyotime, Shanghai, China). The proteins were then digested with trypsin and loaded onto a C18 desalting column. Impurities were washed away using 0.1% formic acid (FA), after which the peptides were eluted with 70% acetonitrile (ACN) and collected. The peptides were separated using a 2 h ACN gradient in 0.1% FA at a flow rate of 600 nL/min. A Q Exactive HF-X mass spectrometer and Nanospray Flex™ (NSI) ionization source were utilized. The proteins were identified using Proteome Discovery 2.4 software with the Homo sapiens species database.

Quantitative real-time PCR (qRT-PCR)

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then reverse-transcription conducted using a cDNA Synthesis Kit (Takara, Tokyo, Japan). QPCR was carried out with SYBR Green (Takara) and specific primers (Table 1). Relative expression levels were assessed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies, including anti-CDH18, anti-OPN, anti-VIM, anti-α-SMA and anti-GAPDH (Abcam, Cambridge, UK). Subsequently, the membranes were incubated with the corresponding secondary antibody. The blots were visualized using enhanced chemiluminescence (Beyotime).

Cell culture and treatment

Human VSMCs (Procell, Wuhan, China) were cultured in DMEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco) at 37 °C in an atmosphere of 5% CO_2. Cell transfection was performed using Lipofectamine 3000 (Invitrogen) with either a pcDNA (control) or a CDH18 overexpression vector. For PDGF-BB stimulation, VSMCs were exposed to PDGF-BB at a concentration of 20 ng/mL for 24 h.

Cell counting kit 8 (CCK8)

After treatment, VSMCs were seeded in a 96-well plate. 10 μL of CCK-8 solution (Solarbio, Beijing, China) was added to each well. Following a 2-hour incubation, the absorbance at 450 nm was measured using a microplate reader.

5-Ethynyl-2′-deoxyuridine (EdU) assay

An EdU kit (RiboBio, Guangdong, China) was utilized to evaluate cell proliferation. VSMCs were incubated with a 50 μM EdU labeling solution for 3 h. Subsequently, the cells were fixed with 4% formaldehyde for 30 min, followed by staining with the 1 × Apollo® reaction cocktail for an additional 30 min. DAPI was applied for counterstaining of cell nuclei. Ultimately, the number of EdU-positive cells was determined using a fluorescent microscope.

Transwell

VSMCs with 200 μL serum-free medium were added into the upper transwell chamber (Corning, New York, Madison, USA). The lower chamber was added with 500 μL serum medium. After 48 h, the migrated cells were fixed and stained by crystal violet. The cells were imaged and counted under microscope.

Wound healing assay

VSMCs were seeded in 6-well plates and cultured in serum-free medium. Then the cells were scratched by a 10 μL pipetting gun head. At 0 h and 24 h, the scratched distance was taken under a microscope.

Statistical analysis

Data were presented as mean ± SD and analyzed by GraphPad Prism. The difference was compared by t test or one-way ANOVA. $P<0.05$ indicated significant differences.

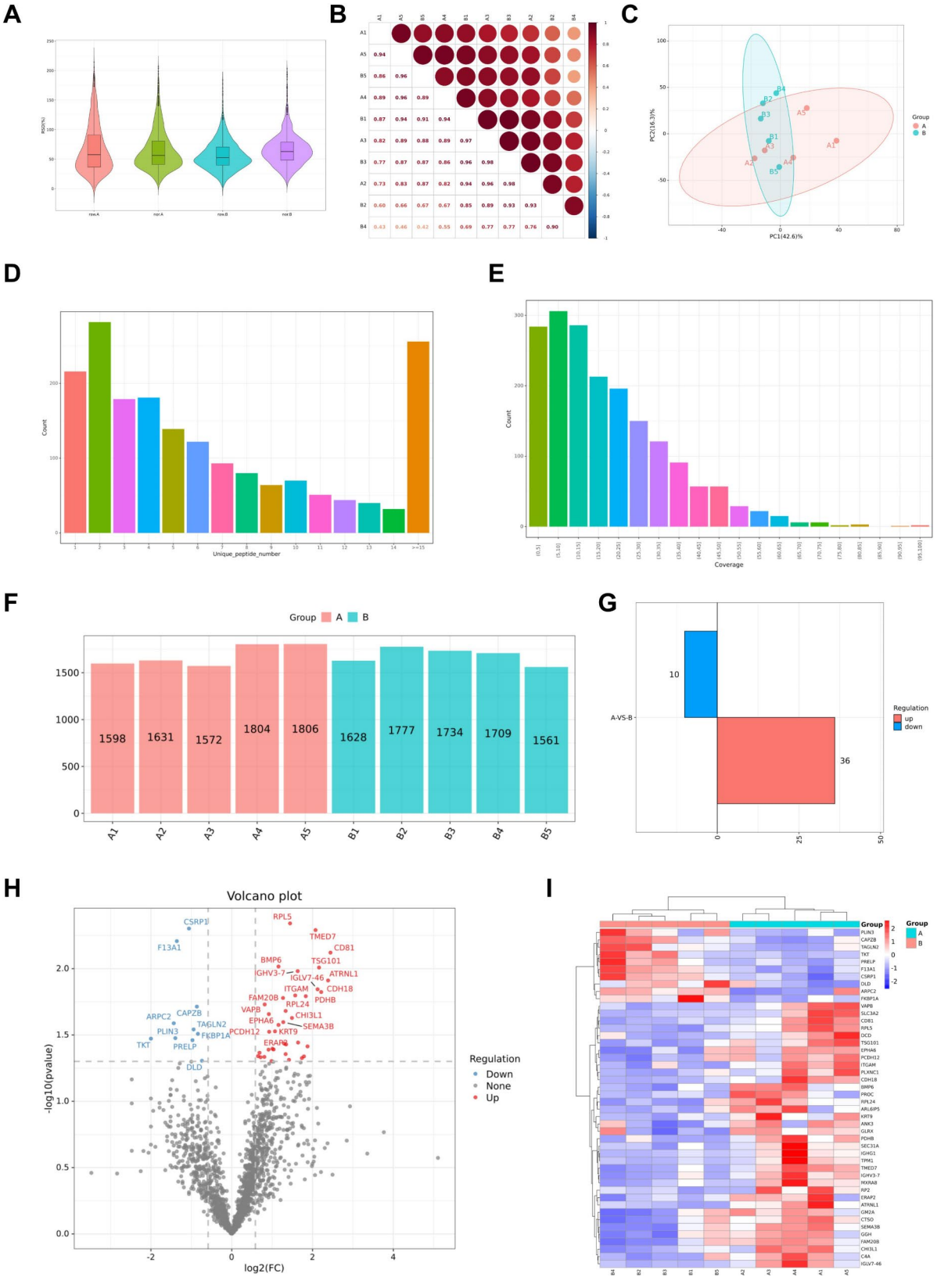

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Protein quality control, protein identification, quantitative results and differential expression analysis of moyamoya disease samples. **(A)** RSD distribution. The horizontal axis represents the sample group, and the vertical axis represents the corresponding RSD value. **(B)** Sample correlation analysis heat map. The horizontal and vertical axes represent samples, respectively. The color indicates the correlation coefficient. The red color in the color scale indicates the higher the positive correlation, and the blue color indicates the higher the negative correlation. **(C)** Differential protein principal component analysis heat map. The horizontal axis represents the first principal component interpretation, and the vertical axis represents the second principal component interpretation. Dots represent experimental samples, and colors represent different groups. **(D)** Unique peptide distribution. The horizontal axis is the number of unique matching peptides for each protein, and the vertical axis is the number of proteins. **(E)** Protein coverage analysis. The horizontal axis is the percentage range of protein coverage, and the vertical axis is the number of proteins. **(F)** Statistics on the number of identified proteins in the ten samples. **(G)** Statistics of the number of differentially expressed proteins. **(H)** Volcano map of differentially expressed proteins. The X-axis represents the difference multiple of the differential protein (log2 value), the Y-axis represents the p value (-log10 value), the gray represents the non-significant difference, the red represents the up-regulated proteins, and the blue represents the down-regulated proteins. By default, the names of the top 20 differentially expressed proteins are displayed in order of P-value significance, or all of them if there are less than 20. **(I)** Differentially expressed proteins cluster heat map. The X-axis represents the samples for cluster analysis, the Y-axis represents the differentially expressed proteins, and the color represents the expression amount after conversion. The more red the color, the higher the expression, and the more blue the color, the lower the expression. The left side and the top side respectively represent the clustering analysis of row and column data by clustering algorithm

Results

Imaging characteristics of patients with moyamoya disease after cerebral revascularization

After cerebral revascularization, five patients had a good therapeutic effect with few smoky vessels, and five patients had a poor therapeutic effect with dense smoky vessels. According to the therapeutic effect, the patients were divided into a good prognosis group (Group A, $n=5$) and a poor prognosis group (Group B, $n=5$) (Table 1). Digital subtraction angiography (DSA) of patients with moyamoya disease was conducted. The images before and after treatment showed that the therapeutic effect was better in patients in group A than that in Group B (Fig. 1).

Protein expression profile of cerebrospinal fluid in patients with moyamoya disease

To investigate the factors influencing the therapeutic effect of patients with moyamoya disease, the proteomics based on MS was performed with the cerebrospinal fluid of patients. To begin with, the quality of proteomic data was analyzed. The relative standard deviation (RSD) values of Group A and B were similar (Fig. 2A). As shown in pearson correlation coefficient heat map, the correlation of different samples was high in the same group (Fig. 2B). In addition, the principal components of differential proteins were analyzed, and the result indicated that the samples within the same group were clustered, and there was some dispersion of the samples between the two groups (Fig. 2C). The unique peptide distribution was showed in Fig. 2D, and it displayed that the number of unique matching peptides was less than 10 in most proteins. Protein coverage analysis showed that the protein coverage is mostly less than 10% (Fig. 2E).

Subsequently, the differentially expressed proteins between the two groups were analyzed. The numbers of identified proteins in ten samples were showed in Fig. 2F. Compared with group B, ten downregulated and thirty-six upregulated proteins were identified in Group B (Fig. 2G-I). The top 10 significantly upregulated differentially expressed proteins were listed in Table 2.

CDH18 was highly expressed in moyamoya disease patients with good prognosis

Further, the mRNA expression of the top 10 upregulated proteins was detected by qRT-PCR. Consistent with the proteomic data, these genes were upregulated in Group A, and CDH18 was the most significantly upregulated gene (Fig. 3A). Besides, the western blot assay manifested that CDH18 protein level was higher in group A than that in group B (Fig. 3B). These results suggested that CDH18 might play important role in moyamoya disease.

Overexpression of CDH18 inhibited proliferation, migration and phenotypic transformation in VSMCs

To export the role of CDH18 in moyamoya disease, its overexpression vector was transfected into VSMCs and PDGF-BB-induced VSMCs. The mRNA and protein expression was upregulated by CDH18 overexpression vector (Fig. 3C and D). CCk8 and EdU assays proved that cell proliferation was induced by PDGF-BB, and inhibited by CDH18 in VSMCs and PDGF-BB-induced VSMCs (Fig. 3E and F). In addition, cell migration was assessed by transwell and wound healing assays. The results indicated that cell migration of VSMCs was induced by PDGF-BB, while CDH18 overexpression repressed cell migration of VSMCs (Fig. 4A and B). The expression of OPN, VIM and α-SMA was measured to evaluate phenotypic transformation, and the data indicated that PDGF-BB induced phenotypic transformation of VSMCs, which was suppressed by CDH18 (Fig. 4C). These results revealed that CDH18 inhibited PDGF-BB-induced VSMCs proliferation, migration and phenotypic transformation.

Table 2 Top 10 significantly upregulated differentially expressed proteins in the cerebrospinal fluid of moyamoya disease patients

Gene Symbol	Protein IDs	log2(FC)	*p* Value
CD81	P60033	2.4436066514756147	<0.05
ATRNL1	Q5VV63	2.3840498067951597	<0.05
CDH18	Q13634	2.2141248053528475	<0.05
TSG101	Q99816	2.160274831408593	<0.05
IGLV7-46	A0A075B6I9	2.124328135002202	<0.05
TMED7	Q9Y3B3	2.0738202332916713	<0.05
PROC	P04070	1.875780063068488	<0.05
PDHB	P11177	1.831877241191673	<0.05
ARL6IP5	O75915	1.7865963618908067	<0.05
IGHG1	P01857	1.7355221772965377	<0.05

Discussion

To data, the knowledge of the pathogenesis of moyamoya disease is very limited. It has been proved that RNF213 was related to the pathogenesis of moyamoya disease, and silencing RNF213 promoted the progression of moyamoya disease [19, 20]. In addition, the circular RNAs, long noncoding RNAs, and microRNAs profile in patients with moyamoya disease were also analyzed [21–23]. Identification of biomarkers in moyamoya disease has attracted great interest, which has been studied via RNA-sequencing and transcriptome analysis [24, 25]. A previous study analyzed the differentially expressed proteins in serum of moyamoya disease patients and healthy volunteers through proteomics, which elucidated FLNA and ZYX were upregulated in moyamoya disease, and facilitated endothelial cell proliferation and angiogenesis [26]. Wu et al. identified apolipoprotein E as a diagnostic marker by combining proteomic data and GEO database, and revealed that cholesterol metabolism might participate in the pathogenesis of moyamoya disease [27]. In our study, we found that the therapeutic effects of moyamoya disease patients were different, despite patients received the same treatment. Therefore, we performed proteomics to identify the biomarkers on the therapeutic effect of moyamoya disease patients. Compared with the poor prognosis group, ten downregulated and thirty-six upregulated proteins were identified in good prognosis group. Among the top 10 upregulated proteins, CDH18 was the most significantly upregulated gene.

CDH18 has been reported to be a tumor-suppressor in a variety of cancers [13, 14]. In addition, CDH18 is also involved in heart development, and might be diagnostic marker of congenital heart disease [15, 28]. Nevertheless, the function of CDH18 in cerebrovascular disease has not been studied. Our data showed the high expression level of CDH18 in moyamoya disease patients with good prognosis, implying that CDH18 might participate in the progression of moyamoya disease. Thus, we explored the function of CDH18 in VSMCs.

Abnormal cell proliferation and migration of VSMCs are significant causes of intima thickening in cerebrovascular disease [29]. The phenotypic transformation is closely related to cell activity of VSMCs and accelerates vascular hyperplasia [30]. Meanwhile, in the pathological state, some cytokines such as PDGF were release, which further induced VSMCs proliferation, migration and phenotypic transformation [31]. Consistent with this, our experiments also exhibited the promotion of PDGF-BB on proliferation, migration and phenotypic transformation of VSMCs, while CDH18 overexpression repressed these effects. These results suggested CDH18 might act as inhibitor in moyamoya disease progression, and upregulated CDH18 might elevate the therapeutic effect of patients via inhibiting the progression of moyamoya disease.

However, our research still has some shortcomings. Firstly, the sample size is small, and a large sample size helps to obtain more accurate data. Proteomics with a large number of samples is difficult to achieve due to the expensive cost, and we will detected the expression of CDH18 and other differentially expressed proteins in more clinical sample to verify our results. Secondly, whether other pathways mediate the therapeutic effect of moyamoya disease is unknown, which needs to be further analyzed and studied. Thirdly, the role of CDH18 in animal model of moyamoya disease has not been explored. Fourthly, the molecular mechanism of CDH18 in moyamoya disease remains unclear. These will be the focus of our further research.

In conclusion, our study identified CDH18 as a biomarker on the therapeutic effect of moyamoya disease, and verified the function of CDH18 in PDGF-BB-induced VSMCs, which might contribute to the treatment of patients with moyamoya disease.

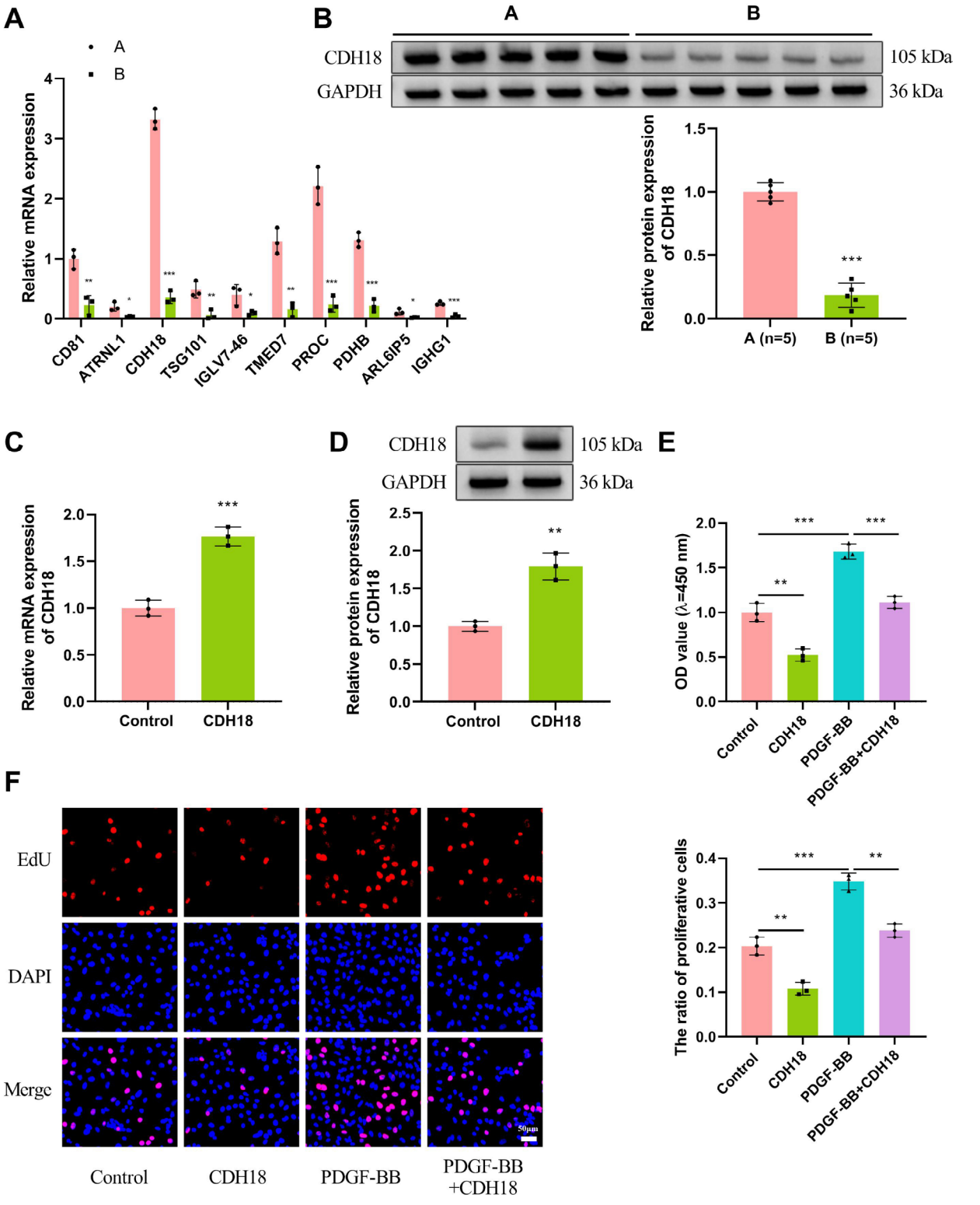

Fig. 3 Overexpression of CDH18 inhibited proliferation of VSMCs. **(A)** The mRNA expression of the top 10 upregulated proteins was detected by qRT-PCR. **(B)** The expression of CDH18 was detected by western blots. (**C** and **D**) The mRNA and protein expression of CDH18 was detected by qRT-PCR and Western blot in VSMCs transfected with pcDNA (control group) or CDH18 overexpression vector (CDH18 Group). (**E** and **F**) Cell proliferation was assessed by CCK8 and EdU assays in VSMCs transfected with pcDNA (control group) or CDH18 overexpression vector (CDH18 Group) or combined PDGF-BB treatment (PDGF-BB group and PDGF-BB + CDH18 Group). $^{**}P < 0.01$; $n = 3$. Scale bar: 50 μm

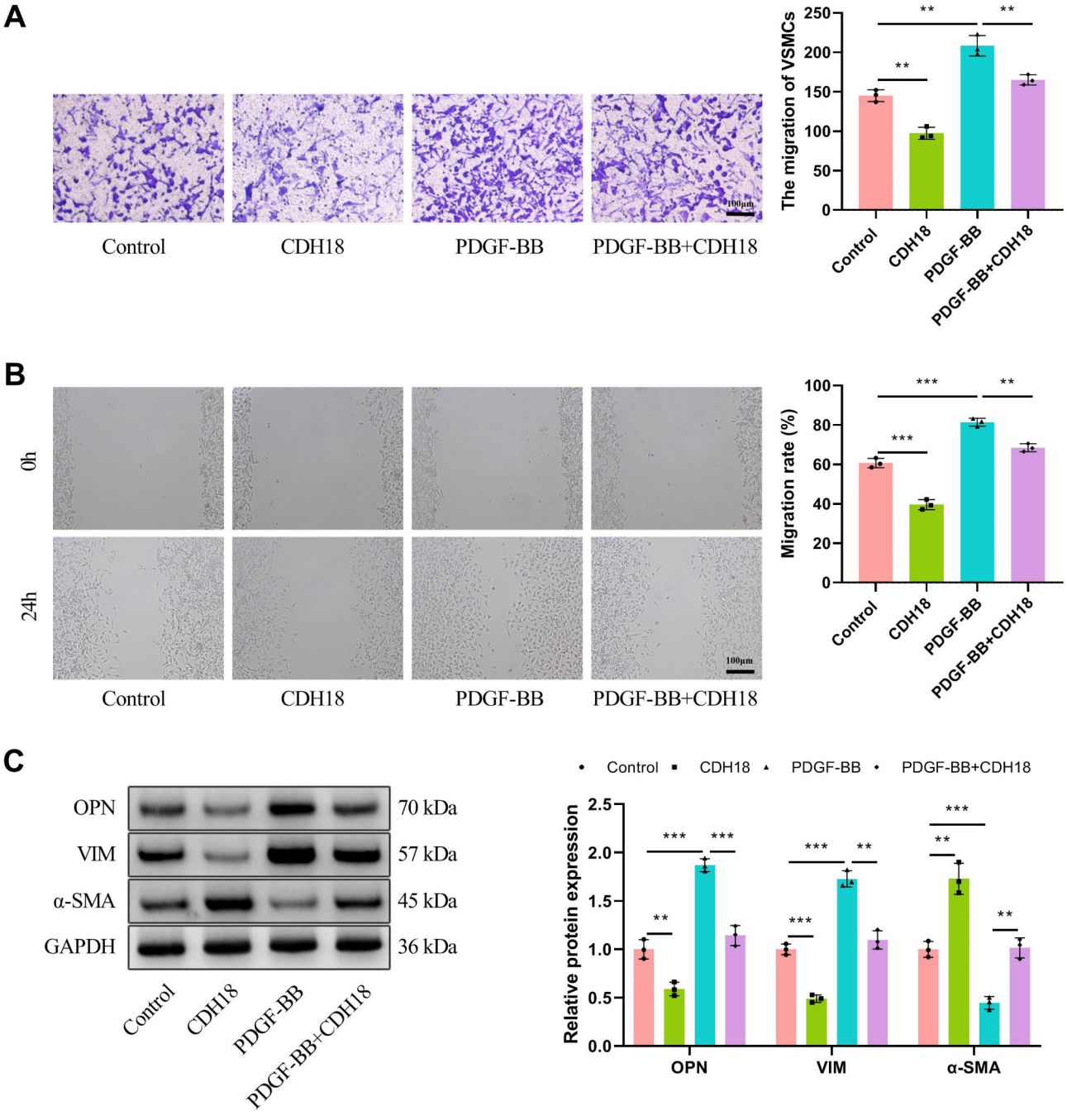

Fig. 4 Overexpression of CDH18 repressed VSMCs migration and phenotypic transformation. VSMCs were transfected with pcDNA (control group) or CDH18 overexpression vector (CDH18 Group) or combined PDGF-BB treatment (PDGF-BB group and PDGF-BB + CDH18 Group). (**A** and **B**) Cell migration was assessed by transwell and wound healing assays. **(C)** The expression of phenotypic transformation-related proteins was detected by western blot. $^{**}P < 0.01$; $n = 3$. Scale bar: 100 μm

Acknowledgements
None.

Funding
None.

Author contributions
Dong Guo conducted the experiments and drafted the manuscript. Yang Dong collected and analyzed the data, prepared figures. Hongbin Li contributed the methodology, operated the software and edited the manuscript. Hongwei Li and Bo Yang designed and supervised the study. All authors reviewed the manuscript.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consents were acquired from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
The authors declare that they have no conflicts of interest to be declared.

Received: 30 May 2024 / Accepted: 28 August 2024
Published online: 05 September 2024

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