KRAS mutation-induced EndMT of brain arteriovenous malformation is mediated through the TGF-β/BMP-SMAD4 pathway

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ABSTRACT

Objective Somatic KRAS mutations have been identified in the majority of brain arteriovenous malformations (bAVMs), and subsequent in vivo experiments have confirmed that KRAS mutation in endothelial cells (ECs) causes AVMs in mouse and zebrafish models. Our previous study demonstrated that the KRASG12D mutant independently induced the endothelial-mesenchymal transition (EndMT), which was reversed by treatment with the lipid-lowering drug lovastatin. However, the underlying mechanisms of action were unclear.

Methods We used human umbilical vein ECs (HUVECs) overexpressing the KRASG12D mutant for Western blotting, quantitative real-time PCR, and immunofluorescence and wound healing assays to evaluate the EndMT and determine the activation of downstream pathways. Knockdown of SMAD4 by RNA interference was performed to explore the role of SMAD4 in regulating the EndMT. BAVM ECs expressing the KRASG12D mutant were obtained to verify the SMAD4 function. Finally, we performed a coimmunoprecipitation assay to probe the mechanism by which lovastatin affects SMAD4.

Results HUVECs infected with KRASG12D adenovirus underwent the EndMT. Transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signalling pathways were activated in the KRASG12D-mutant HUVECs and ECs in BAVM tissue. Knocking down SMAD4 expression in both KRASG12D-mutant HUVECs and ECs in BAVM tissues inhibited the EndMT. Lovastatin attenuated the EndMT by downregulating p-SMAD2/3, p-SMAD1/5 and acetylated SMAD4 expression in KRASG12D-mutant HUVECs.

Conclusions Our findings suggest that the KRASG12D mutant induces the EndMT by activating the ERK-TGF-β/BMP-SMAD4 signalling pathway and that lovastatin inhibits the EndMT by suppressing TGF-β/BMP pathway activation and SMAD4 acetylation.

INTRODUCTION

Brain arteriovenous malformations (bAVMs) constitute a congenital cerebrovascular disease caused by abnormally dilated vascular tangles and are characterised by a lack of capillary beds, which are important for direct arterial-venous communication.1 BAVMs may lead to devastating haemorrhages, which account for approximately one-third of primary intracerebral haemorrhages in young adults.2 Clinical treatments mainly include neurosurgery, embolisation and stereotactic radiotherapy.3 However, the management of large bAVMs, particularly those located in eloquent cortex areas, remains challenging.

In previous research, somatic KRAS-activating mutations were identified in endothelial cells (ECs) of human sporadic bAVMs.4 5 Subsequent in vivo experiments confirmed that KRAS mutations could drive abnormal vascular morphology and AVMs in mice and zebrafish models in ECs.6 7 In our previous study, we found endothelial-mesenchymal transition (EndMT) of cells in BAVM tissue, and KRAS mutation induced the

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Somatic KRAS mutations have been identified in the majority of brain arteriovenous malformations (bAVMs). Meanwhile, the KRASG12D mutant independently induced the endothelial-mesenchymal transition (EndMT), which was reversed by treatment with the lipid-lowering drug lovastatin. However, the underlying mechanisms of action were unclear.

WHAT THIS STUDY ADDS

⇒ KRAS mutation induced EndMT through the transforming growth factor beta (TGF-β)/bone morphogenetic protein (BMP)-SMAD4 pathways.

⇒ Lovastatin reversed KRAS mutation-induced EndMT by inhibiting the TGF-β/BMP pathways and SMAD4 acetylation.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study implies that SMAD4 may be a potential target by which to inhibit the development of bAVMs with a KRAS mutant and lovastatin (an FDA-approved drug) might alleviate the development of bAVMs and reduce the risk of bAVM rupture, which need further animal studies and clinical trials.
EndMT. Nevertheless, the mechanism by which KRAS mutants trigger the EndMT is still unclear.

In the EndMT, ECs lose their endothelial markers (von Willebrand factor (VWF) and VE-Cadherin) and obtain mesenchymal or myofibroblastic phenotype to express mesenchymal cell products (N-Cadherin and SLUG). As the EndMT contributes to many diseases, pharmacological modulation of an important signalling pathway for EndMT is prove to be effective therapy. Since the logical modulation of an important signalling pathway contributing to many diseases, pharmaco-therapy for bAVMs.

METHODS AND MATERIALS

HUVECs culture and treatment

HUVECs cell lines were purchased from ScienCell (#8000, ScienCell) and cultured in endothelial cell medium (ECM, #1001, ScienCell); no HUVECs cultured after the tenth passage (P10) were used. Inhibitors of MAPK (U0126, 20 μM, Selleck), transforming growth factor beta (TGF-β) (SB525334, 20 μM, Selleck), bone morphogenetic protein (BMP, LDN193189, 0.5 μM, Selleck) andLovastatin (S2061, 10 μM, Selleck) were dissolved with Dimethyl sulfoxide (DMSO) and added directly to the ECM.

Cell culture of primary bAVM ECs

AVM surgical specimens were obtained from the Department of Neurosurgery at Beijing Tiantan Hospital, Capital Medical University. The ECs of the bAVMs were isolated and cultured according to previously described method. Briefly, the tissue was washed with PBS. After cutting into small cubes, tissue was incubated by 0.1% collagenase (Sigma, USA) at 37°C for 15 min. Predigested tissue was triturated with a 2 ml pipette and filtered through a 100 μm cell strainer (BD, USA). After centrifugation of the cell suspension (300 g, 3 min), the cells were washed and resuspended in ECM. ECs were isolated using anti-CD31 Dynabeads (#130-091-935, Miltenyi Biotec).

DNA extraction and whole-exome sequencing

To isolate genomic DNA from primary ECs, commercially available kits (QIAGEN Gentra Puregene) were used following established protocols. First, DNA degradation and contamination were monitored on 1% agarose gels. Second, the DNA concentration was quantitated by Qubit DNA Assay Kit (Illumina, USA) using Qubit V.2.0 Fluorometer (Invitrogen, USA) using Qubit DNA Assay Kit. Using an Agilent SureSelect Human All Exon V6 kit (Agilent Technologies, USA), we generated sequencing libraries. The index-coded samples were clustered by cBot Cluster Generation System using an Illumina HiSeq PE Cluster Kit. After that, the DNA libraries were sequenced on an Illumina HiSeq platform, and 150 bp paired-end reads were produced. The generated data were mapped to the UCSC hg19 human genome reference with Burrows-Wheeler Aligner software using the default parameters. Picard was used to mark duplicates, and SAMtools was used to sort the alignments by coordinates. Median coverage was estimated by calculating the depth of autosomal common single-nucleotide polymorphism loci using the multivoc function in bedtools. Finally, three cases of ECs with KRAS G12D mutation confirmed by WES were randomly selected for subsequent in vitro experiments.

Droplet digital PCR

Approximately 10 ng of fresh-frozen template DNA was used in each reaction. The analysis was performed on a QX200 droplet digital PCR (ddPCR) system (Bio-Rad Laboratories, Hercules, California, USA). A variant was considered positive when the sample displayed at least four positive droplets.

Adenovirus-mediated gene expression

Adenovirus encoding KRAS isoform B (G12D) was designed, constructed and produced by Beijing Syngen-Tech. All adenoviruses were amplified in Ad293 cells, purified using the freeze-thaw method followed by CsCl
density gradient centrifugation. For transient transduction, HUVECs were infected with adenovirus encoding KRASG12D or an EGFP control at a multiplicity of infection of 20 for 48 hours. Finally, the cells were grown in ECM until confluent.

**Short interfering RNA (siRNA) transfection**

siRNA directed against SMAD4 was used for the knockdown experiments. ECs were transfected with siRNAs using Lipo3000 (Invitrogen). After transfection for 48 hours, the cells were harvested for subsequent mRNA or protein expression analysis. siRNA was synthesised with the following sequences: SMAD4, 5’-CGGCUUUUUGACAGAGUATT-3’ and the negative control, 5’-UAACUCGUAGAAAACGCGTT-3’.

**Immunohistochemistry**

The histological sections obtained from KRASG12D mutant bAVMs were obtained from our previous study. Briefly, bAVM tissue slices were incubated with primary antibody against p-SMAD2/3 (1:200, #8828; CST) or p-SMAD1/5 (1:200, #9516; CST) overnight. In the next day, slices were incubated with secondary antibody (25°C, 1 hour) and horseradish peroxidase–labelled streptavidin (25°C, 30 min). After washing with Tris buffer for three times, sections were stained with 3,3’-diaminobenzidine, and nuclei were counterstained with haematoxylin. We used a positive fluorescence microscope (Zeiss Axio Scope A1) to acquire images.

**Immunofluorescence**

For immunofluorescent staining, HUVECs were grown on glass coverslips until confluence, and then 4% paraformaldehyde was used to fix HUVECs. After permeabilising with 0.3% Triton X-100 and blocking with 10% goat serum, HUVECs were incubated with primary antibodies against VE-Cadherin (1:400, #2500, CST), VWF (1:400, #65707, CST), N-Cadherin (1:200, #13116, CST) and SLUG (1:400, #9585, CST) at 4°C overnight and to Goat Anti-Rabbit IgG H&L (1:500,150077, Abcam) and Goat Anti-Rabbit IgG H&L (1:500, ab150080, Abcam). DAPI staining was used to label cell nuclei. All glass coverslips were imaged using an inverted OLYMPUS IX71 microscope.

**Quantitative real-time PCR**

Total RNA from HUVECs was isolated using TRizol reagent (Invitrogen, USA). cDNA was reversely transcribed and amplified using a PrimeScript RT reagent Kit (Takara). PCR was performed using TB Green Premix Ex Taq (Takara) with a QuantStudio 3 System (Applied Biosystems). The relative quantification was determined with 2-ΔΔCt method, and GAPDH was considered as the internal reference gene for normalisation. All primer sequences used in this study are shown in online supplemental table S2.

**Coimmunoprecipitation and western blot analyses**

HUVECs were lysed with radioimmunoprecipitation assay lysis buffer (Sigma, USA), and a bicinechonic acid (BCA) protein assay kit (Sigma, USA) was used to detect protein concentration. For Western blot, different samples with an equal amount of protein (20 µL) were loaded into sodium dodecyl sulfate–polyacrylamide gel electrophoresis for electrophoresis and followed by transferring to a polyvinylidene fluoride transfer membrane. The electrochemiluminescence (ECL) reagent was used for visualisation of protein signals, and the grey values of bands were quantified with ImageJ (National Institutes of Health (NIH) Image, Bethesda, MD). The detailed information of antibodies used are as follow: anti-VE-Cadherin (1:1000, ⁣#2500, CST), anti-VWF (1:1000, #65707, CST), anti-N-Cadherin (1:1000, #13116, CST), anti-SLUG (1:1000, #9585, CST), anti-SMAD4 (1:1000, #46535, CST), anti-ERK1/2 (1:1000, #4695, CST), anti-p-ERK1/2 (1:2000, #4370, CST), anti-AKT (1:1000, #4685, CST), anti-p-AKT (1:2000, #4060, CST), anti-P38 (1:1000, #8690, CST), anti-p-P38 (1:1000, #4511, CST), anti-p-SMAD2/3 (1:1000, 8828, CST), anti-SMAD2/3 (1:1000, 8685, CST), anti-p-SMAD1/5 (1:1000, 9516, CST), anti-SMAD1/5 (5/9 (1:1000, ab80255, Abcam), anti-β-catenin (1:1000, 8480, CST), anti-SIRT1 (1:1000, 8469, CST), Acetylated-Lysine Antibody (1:1000, 9441, CST), anti-GAPDH (1:1000, #5174, CST).

Coimmunoprecipitation was carried out by using a Protein A/G immunoprecipitation kit (Solorbio). HUVEC lysates were incubated with an anti-SMAD4 antibody (1:200, #46535, CST), followed by incubation with Pierce Protein A/G magnetic beads. The protein eluates were blotted with anti-SMAD4 and anti-acetylated lysine antibodies (1:1000, #9441, CST).

**Wound healing assay**

Wound healing assays were performed using an Ibidi culture insert. Treated HUVECs (700000 cells/mL) were suspended in ECM, and 70 µL of cell suspension aliquots were pipetted into each chamber of the cell culture insert.

When the cell density reached more than 90% confluence, the culture insert was gently removed. Meanwhile, ECM was replaced with serum-free medium. Images were photographed using an inverted-phase microscope (IX51, OLYMPUS, Japan) 12 hours after a scratch was made in the cell monolayer. The reduction in the wounded area was measured with NIH ImageJ software (V.1.52a) and reported as a percentage.

**Statistical analysis**

Statistical analyses were accomplished using GraphPad Prism V.8.00 and R V.3.0. Statistical differences between two groups were analysed by using the unpaired Student’s t-test, and those of differences among multiple groups of data were analysed by one-way analysis of variance.

**RESULTS**

The KRASG12D-mutant induces the EndMT of HUVECs

Transcriptome differences between KRASG12D mutant HUVECs (n=3) and control HUVECs (n=3) were evaluated by comparing the RNA-Seq expression profiles.
Differentially expressed EndMT-related genes were defined as genes with an expression fold change $>1.50$ or $<0.67$ and a $P \leq 0.05$, and 53 genes were identified (online supplemental table S1). Compared with the control group, the expression of endothelial markers in the mutation group was significantly decreased, and the mesenchymal markers were increased (figure 1A).

After infection with KRAS-mutant adenovirus, the mRNA levels of VWF and CDH5 were reduced, and those of CDH2 and SLUG were increased (online supplemental figure S1A). To further confirm that the EndMT in HUVECs was induced by KRAS mutation, we examined, in protein level by western blot analysis, the endothelial and mesenchymal markers. The results showed that the expression of VE-Cadherin and VWF was significantly downregulated, and N-Cadherin and SLUG were upregulated (figure 1B, online supplemental figure S2). Further investigation of cellular behaviour found that HUVECs infected with KRAS$^{G12D}$ adenovirus showed enhanced migration compared with the control HUVECs, and the cell morphology changed from polygonal to elongated shape, which is similar to mesenchymal cell phenotype. (figure 1C). Immunofluorescence staining revealed decreased expression of VE-Cadherin and increased expression of SLUG in HUVECs after treatment with KRAS$^{G12D}$ adenovirus (figure 1D). These results...
suggested that the KRAS<sub>G12D</sub> mutant induced the EndMT of HUVECs.

**Activation of the ERK-TGF-β/BMP signaling pathway, but not the Wnt or notch signaling pathway, contributes to KRAS mutant-induced EndMT**

KRAS is an effector molecule that is expressed downstream of activated receptor tyrosine kinases and activates several pathways including the RAF-MEK-ERK pathway and the PI3K-AKT pathway. In this study, the phosphorylation of ERK1/2, AKT, and p38 MAPK was examined in HUVECs infected with KRAS<sub>G12D</sub> adenovirus. The increased level of ERK1/2 phosphorylation was observed in mutant KRAS expressing HUVECs, but not in AKT or p38 signalling pathways (figure 2A, online supplemental figure S3). U0126, a specific ERK inhibitor, inhibited the downregulation of endothelial marker expression, the upregulation of mesenchymal marker expression and the reduced cell mobility of KRAS<sub>G12D</sub>-mutant HUVECs (figure 2B–C, online supplemental figure S1B and S4). Our findings suggested that KRAS<sub>G12D</sub> mutant specifically activates the MAPK-ERK pathway in ECs.

Previous articles reported that the main pathways regulating the EndMT include the TGF-β, BMP, Notch and Wnt signalling pathways. To determine which pathway downstream of KRAS regulates the EndMT, we focused on several key mRNAs and proteins related to the aforementioned signalling pathways. The TGF-β superfamily consists of more than 50 structurally related ligands that belong to three major subfamilies: TGF-β, BMPs and Activins. SMAD2 and 3 have been reported to be specifically activated by activin/nodal and TGF-β receptors, meanwhile SMAD1, 5 and 8 are activated by BMP type I receptors, and it can simultaneously regulate the TGF-β and BMP signalling pathways. We hypothesised that SMAD4 might be a core molecule in the KRAS-induced EndMT. To confirm the regulatory role of SMAD4 on the EndMT, we investigated changes in endothelial and mesenchymal markers expression in KRAS<sub>G12D</sub> mutant HUVECs in which SMAD4 was silenced. The gene knockdown efficiency of SMAD4 was confirmed by RT-qPCR (online supplemental figure S10). As displayed in figure 4A and online supplemental figure S11, N-Cadherin and SLUG expression was decreased, and that of VE-Cadherin and VWF was increased in cells with SMAD4 knocked down. Similarly, the immunofluorescence results were consistent with the Western blot results (online supplemental figure S12A). As shown in online supplemental figure S12B, SMAD4 knockdown in ECs partially reversed the fibroblast-like phenotype acquisition induced by KRAS<sub>G12D</sub>, and the mobility of the cells was inhibited (n=9).

Primary ECs isolated from 3 bAVM surgical samples harbouring KRAS<sub>G12D</sub> somatic mutations were detected by WES. Through WES, we averaged 114×–217×coverage across the exome and obtained ≥10× coverage and ≥50× coverage for 95%–100% and 76%–85%, respectively (figure 4B). The results showed that all three cases of bAVM had KRAS<sub>G12D</sub> mutation in the endothelium (figure 4C, online supplemental figure S13). ddPCR also confirmed the presence of KRAS<sub>G12D</sub> mutation in bAVM primary cells.

In primary AVM ECs harbouring the KRAS<sub>G12D</sub> mutant, the expression of mesenchymal markers was decreased significantly after SMAD4 knockdown, but that of endothelial markers remained unchanged (figure 4D). The statistical analysis results are shown in online supplemental figure S14. This result may be explained by the fact that ECs undergoing the EndMT were excluded during CD31+ magnetic bead sorting, and therefore, only ECs that partially exhibited mesenchymal behaviour were cultured. The results indicated that knockdown of SMAD4 expression inhibits the progression of EndMT in vitro.
Lovastatin attenuates the KRAS<sup>G12D</sup>-induced EndMT by inhibiting TGF-β/BMP pathway activation and SMAD4 acetylation

Lovastatin, as a lipid-lowering drug, inhibits the EndMT. In this study, we found that lovastatin was able to restore the expression of VE-Cadherin and VWF that had been downregulated by KRAS<sup>G12D</sup> and that lovastatin reversed the upregulation of N-Cadherin and SLUG that had been induced by KRAS<sup>G12D</sup> (Figure 5A, online supplemental figure S15). The results of immunofluorescence also confirmed that lovastatin reversed EndMT (online supplemental figure S16A). Moreover, lovastatin inhibited...
the increase in cell migration caused by the KRAS mutant (online supplemental figure S16B, n=9). In AVM ECs, lovastatin only reversed mesenchymal marker N-Cadherin and SLUG but not endothelial markers (online supplemental figure S16C).

To further investigate the mechanism by which lovastatin affects KRAS\textsuperscript{G12D}-induced EndMT, we treated HUVECs infected with KRAS\textsuperscript{G12D} adenovirus with lovastatin for 48 hours. First of all, we examined the effect of lovastatin on the TGF-\(\beta\) and BMP signalling pathways and found that lovastatin inhibited SMAD2/3 and SMAD1/5 phosphorylation (figure 5B, online supplemental figure S17). Then, we tested the effect of lovastatin on SMAD4, and the results showed no change in total SMAD4 expression (figure 5C, online supplemental figure S18).

A previous study has shown that TGF-\(\beta\)1 is able to increase acetylation level of SMAD4 to induce EndMT; SIRT1 inhibits this progress through deacetylating SMAD4.\textsuperscript{23} As reported, SIRT1 is a nuclear NAD\textsuperscript{+}-class III histone deacetylase, which influence their stability, transcriptional activity and translocation.\textsuperscript{27} SIRT1 reportedly negatively regulated TGF-\(\beta\) pathway by targeting SMAD4,\textsuperscript{28} and statins increased SIRT1 levels in ECs.\textsuperscript{29} We hypothesised that this might be the molecular mechanism by which lovastatin inhibits EndMT induced by KRAS\textsuperscript{G12D}. As shown in figure 5C and online supplemental figure S18, lovastatin increased the level of SIRT1 expression in HUVECs. Furthermore, we performed a coimmunoprecipitation assay to test the effect of lovastatin on SMAD4 acetylation. Our results showed that
the acetylation levels of SMAD4 were upregulated in the KRASG12D-mutant HUVECs and that lovastatin reduced the acetylation level of SMAD4 induced by KRAS G12D (figure 5D, online supplemental figure S19). Taken together, these results suggested that lovastatin inhibits the KRASG12D-induced EndMT by inhibiting TGF-β/BMP pathway activation and SMAD4 acetylation.

DISCUSSION

In the current study, we found that KRAS mutation induces the EndMT by activating the ERK-TGF-β/BMP-SMAD4 pathway. Furthermore, we demonstrated that lovastatin inhibits the EndMT by inhibiting TGF-β/BMP pathway activation and SMAD4 acetylation.

The EndMT plays a crucial role in the occurrence and development of cardiovascular and cerebrovascular diseases, and the reversal of the EndMT is becoming a promising treatment modality. In bAVM tissue cells, the EndMT is not only a pathogenic mechanism, it is also closely associated with bAVM bleeding. Our previous study demonstrated that the KRASG12D mutant independently induced the EndMT. However, the mechanism by which KRAS mutations lead to the EndMT remains unclear. Previous studies reported that KRAS mutations lead to the activation of a wide range of downstream signaling pathways, including the TGF-β, BMP, Notch and Wnt pathways. In previous research, the activation of any of these pathways is independently capable of inducing the epithelial-mesenchymal transition (EMT) or EndMT. In our study, we found that KRAS mutation induces the EndMT through the TGF-β/BMP-SMAD4 pathways but not the Wnt or Notch pathways and that knockdown of SMAD4 can reverse the EndMT process. Our results imply that SMAD4 may be a potential target by which to inhibit the development of bAVM with a KRAS mutant, an idea worthy of further research.
Lovastatin is an Food and Drug Administration (FDA)-approved drug that has been used widely as a lipid-lowering medication and has also been reported to reverse the EndMT; the mechanism of lovastatin action might involve the suppression of oxidative stress and inhibited TGF-β1/Smad signaling pathway activation. Our previous study demonstrated that lovastatin inhibits the EndMT caused by a KRAS mutant. In the current study, we found that lovastatin has the ability to inhibit the TGF-β/BMP pathway and decrease Smad4 acetylation to attenuate the KRAS<sub>G12D</sub>-induced EndMT. In summary, lovastatin, as a novel pharmacological target, may alleviate the development of hAVMs and reduce the risk of hAVM rupture; therefore, further exploration of lovastatin treatment in animal studies and clinical trials is needed.

**Contributors** HX and RH designed the study and contributed to the writing of the study. HX, SZ, JZ and QH contributed to conducted experiments and data analysis. ZY, JW and YS contributed to bioinformatics analysis and data statistics. HL, YJ and JW analysed, interpreted the data and revised the manuscript for intellectual content. SW and YC designed the study and revised the manuscript for intellectual content.

**Figure 5** Lovastatin attenuates the KRAS<sup>G12D</sup>-induced EndMT by inhibiting TGF-β/BMP pathway activation and Smad4 acetylation. (A) Western blot of mesenchymal markers N-cadherin and Slug and endothelial markers VWF and VE-cadherin in HUVECs overexpressing KRAS<sup>G12D</sup>, HUVECs overexpressing KRAS<sup>G12D</sup> with lovastatin and control HUVECs (CTRL). Results from one representative experiment out of three are shown. (B) Western blot showing the expression of p-SMAD2/3 and p-SMAD1/5 in HUVECs overexpressing KRAS<sup>G12D</sup>, HUVECs overexpressing KRAS<sup>G12D</sup> and treated with lovastatin and control HUVECs (CTRL). Results from one representative experiment out of three are shown. (C) Western blot of Smad4 and SIRT1 in KRAS<sup>G12D</sup> HUVECs, KRAS<sup>G12D</sup> HUVECs treated with lovastatin and CTRL. Results from one representative experiment out of three are shown. (D) Immunoprecipitation of Smad4 acetylation afterlovastatin treatment. Results from one representative experiment out of three are shown. EndMT, endothelial-mesenchymal transition; HUVECs, human umbilical vein endothelial cells; VWF, von Willebrand factor.
YC provided overall oversight of the research, and acted as the guarantor who accepted full responsibility for the work and the conduct of the study, had access to the data, and controlled the decision to publish.

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