

LACC1 contributes to inflammation and cognitive disorder after stroke *via* the AMPK/NLRP3 pathway

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The current study aimed to investigate the effects of LACC1 on cognitive disorder due to stroke, as well as its underlying mechanism. LACC1 promoted inflammation and aggravated cognitive impairment in a mouse model of stroke. In an *in vitro* model of stroke, inhibition of LACC1 reduced inflammation and ROS-induced oxidative stress by activating AMP-activated protein kinase (AMPK) expression and suppressing NLRP3 expression. Furthermore, our studies revealed that inhibition of AMPK activity reduced the effects of si-LACC1 on cognitive disorder in mice after stroke *via* the AMPK/NLRP3 pathway. AMPK activation also reduced the effects of LACC1 on inflammation and ROS-induced oxidative stress *via* the NLRP3 pathway in the *in vitro* model that we evaluated. Our study suggests that LACC1-aggravated inflammation causes cognitive impairment after stroke *via* the AMPK/NLRP3 pathway, which may provide a new therapeutic target for stroke and other neurological diseases and their associated complications. In sum, we identified an important role and regulatory mechanism for LACC1 in maintaining stroke-induced cognitive disorder *via* the AMPK/NLRP3 pathway.

Key words: LACC1, cognitive disorder, stroke, AMPK, NLRP3

INTRODUCTION

Executive disorders and slowed actions are the most prevalent impairments in stroke patients including those who suffer cerebral hemorrhage, cerebral venous thrombosis (CVT) and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Ajoalabady et al., 2021; Hokazono 2021). In the cognitive domain of executive function, rule deduction and shifting are impaired in 20% to 30% of patients, verbal fluency and action speed are impaired in 33% to 60% and an overall impairment is observed in 41% of patients. The few studies that have focused on the behavioral domain of executive function concentrated on apathy, which

was found in 20% to 40% of stroke patients and 40% of CADASIL patients (English et al., 2021; Enomoto et al., 2021; Zhuo et al., 2021). Inflammation and related immunological responses are key factors in the pathophysiology of stroke (Anrather and Iadecola, 2016). The immune system is involved in brain injury and exerts an immunosuppressive effect at the injury site, which attenuates the body's resistance and can cause lethal bacterial infections that threaten the prognosis and life of stroke patients (Chamorro et al., 2016; Dong et al., 2019).

Adenosine 5'-monophosphate-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that plays a vital role in regulating the homeostasis of cell energy metabolism through-

out the body (Duan et al., 2019). Under normal physiological conditions, AMPK can promote brain development and regulate neuronal polarization (Wang et al., 2018). In ischemic stroke, the upregulation of AMPK can attenuate oxidative stress, inhibit neuroinflammation, regulate neuronal autophagy and apoptosis, improve mitochondrial function, inhibit glutamate excitotoxicity and promote neovascularization (Grisi et al., 2021). AMPK can promote functional recovery in ischemic stroke, primarily through drug therapy, physical therapy and receptor targeted therapy (Pfeiffer et al., 2021).

The oxidative respiratory chain is damaged during cerebral ischemia, which can produce reactive oxygen species (ROS) (Alishahi et al., 2019). Thioredoxin can bind to interactive protein, which can be inhibited by thioredoxin oxidoreductase (Feng et al., 2020). ROS facilitate the binding of thioredoxin-interactive protein to the NLRP3 receptor, thereby activating NLRP3 (Gao et al., 2017; Hong et al., 2019; Pu et al., 2019).

Laccase domain-containing 1 (LACC1), formerly known as C13orf31, is a genetic variant of laccase containing domain 1, including the common coding single nucleotide polymorphism Ile254val (Lahiri et al., 2017). LACC1 is associated with leprosy, inflammatory bowel disease and juvenile arthritis (Assadi et al., 2016; Szymanski and Ombrello, 2018; Skon-Hegg et al., 2019), but only a limited number of studies have examined the functional consequences of LACC1 protein and its variants in mammals (Szymanski and Ombrello, 2018; Huang et al., 2019; Rabionet et al., 2019). The current study is aimed at investigating the effects of LACC1 on cognitive disorder post-stroke and its mechanisms.

METHODS

Animals

C57BL/6J mice, LACC1^{-/-} mice and wild type (WT) mice underwent transient middle cerebral artery occlusion (tMCAO) and were anaesthetized using isoflurane. The proximal common carotid artery and the external carotid artery were ligated, and a standardized silicon rubber-coated 6.0 nylon monofilament (Docol Corp., Redlands, CA) was inserted and advanced via the right internal carotid artery to occlude the origin of the right middle cerebral artery (MCA). The intraluminal suture was left in situ for 60 min.

This study was approved and supervised by the Ethics Committee of the First Hospital of Hebei Medical University. Significant efforts were made in order to

minimize both the number of animals used as well as their respective suffering.

Sample acquisition, RNA extraction and qPCR validation

TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) was applied to extract the total RNA from patient samples, mice, cell lines according to the manufacturer's instructions. Next, cDNA was synthesized using a TaqMan microRNA reverse transcription (RT) kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) and then amplified via TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Afterwards, mRNA was quantified as cDNA synthesized by a high-capacity cDNA RT kit (Thermo Fisher Scientific) and amplified using SYBR-Green-PCR-Master-Mix and gene-specific primers (both from Thermo Fisher Scientific). The relative expression was calculated by the 2^{-ΔΔCt} method.

siRNA transfection and immunofluorescence staining

Cells were washed twice with cold PBS and then treated with ice-cold RIPA lysis buffer. The protein concentrations of cell lysates were determined by the BCA Assay Kit (Thermo Fisher). Proteins were separated via 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (BIO-RAD Laboratories). The membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated with LACC1 (ab108597, 1:1000, Abcam), AMPK (ab79885, 1:1000, Abcam), p-AMPK (ab92701, 1:1000, Abcam), NLRP3 (ab263899, 1:1000, Abcam) and β-actin (ab115777, 1:10000, Abcam) primary antibodies at 4°C overnight. The membranes were incubated with the secondary antibody: horseradish peroxidase (HRP) combined goat anti-rabbit second antibody (1:10000; Cell Signaling) or goat anti-mouse second antibody (1:10000, Abclonal, Wuhan, Hubei, China) at room temperature for 2 h. Protein banding was determined by ECL assay and evaluated by ImageJ (NIH, Bethesda, Maryland, USA).

Enzyme-linked immunosorbent assay (ELISA)

CAT, SOD, ROS, MDA, GSH, IL-1β, IL-6, INF-γ, IL-10 and IL-22 levels in cultured supernatants or serum samples were quantified using an ELISA kit according to the manufacturer's instructions. Subsequently, the

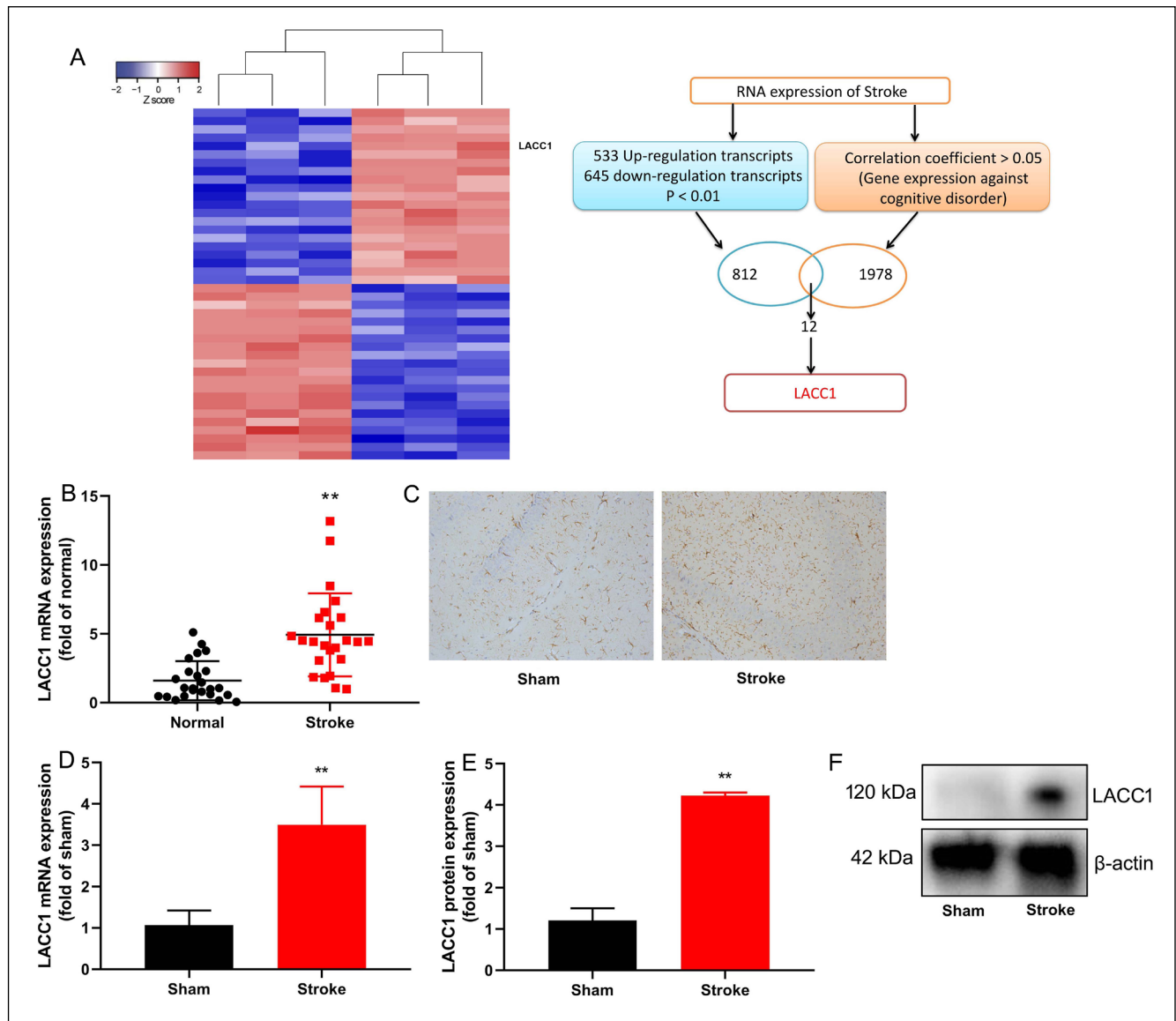


Fig. 1. LACC1 expression in a model of stroke-induced cognitive disorder. Heat map (A), serum mRNA of LACC1 (B) in patients with stroke; LACC1 protein expression (immunohistochemistry, C); brain tissue showing LACC1 mRNA expression (D); brain tissue showing LACC1 protein expression (E); LACC1 protein expression by western blot (F) in mice after stroke. Normal, normal volunteers group; Stroke, patients after stroke; Sham, sham control group; Stroke, mice after stroke. **p<0.01 compared with normal volunteers group or sham control group.

optical density at 450 nm was determined with a microplate reader (Bio-Rad).

Immunofluorescence analysis

Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Coverslips were washed with PBS and incubated 5 min with 0.1% Triton X-100 following fixation. Coverslips were washed again with PBS and, after 20 min incubation with 5% BSA for 1 h, coverslips were stained overnight at 4°C with LACC1 (1:100,

Abcam), p-AMPK (1:100, Abcam). Coverslips were incubated with secondary antibodies: goat antimouse - Alexa Fluor 488 (Sigma-Aldrich; 1:200), and goat anti-rabbit - Alexa Fluor 555 (Sigma-Aldrich; 1:200). Images were captured with a Nikon titanium inverted fluorescence microscope (Nikon, Tokyo, Japan).

Cell cultivation and transfection

BV2 microglia and N2a cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone Compa-

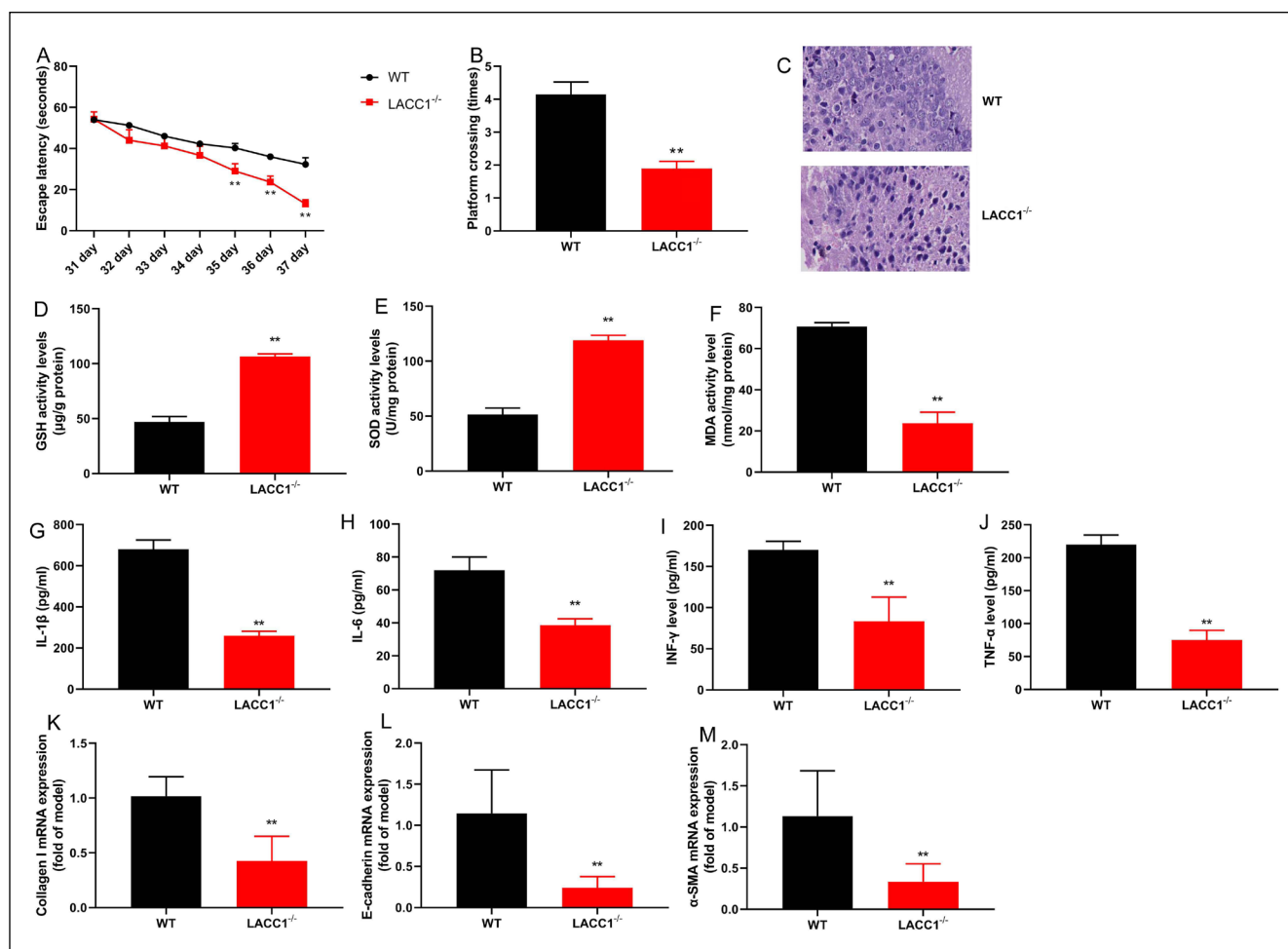


Fig. 2. LACC1 aggravated cognitive disorder of Stroke by the regulation of inflammation and oxidative stress. Escape latency (A), platform-crossing times (B), HE staining (C), GSH (D), SOD (E) and MDA levels (F); IL-1 β (G), IL-6 (H), INF- γ (I) and TNF- α (J); collagen I (K), E-cadherin (L) and α -SMA (M) mRNA expression in brain tissue. WT mice after stroke; LACC1^{-/-}, LACC1^{-/-} mice after stroke. ** $p < 0.01$ compared with WT mice after stroke.

ny, Logan, UT, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C with 5% CO₂. Afterwards, chemically synthesized LACC1 mimic, mimic negative control (NC), si-LACC1, and si-NS (Guangzhou RiboBio Co., Ltd, Guangzhou, Guangdong China) were instantaneously transfected into cells in strict accordance with the kit instructions of the Lipofectamine™ 2000 kit (ThermoFisher Scientific, Waltham, MA, USA). After the transfection of 48 h, BV2 cells were cultured DMEM without glucose and FBS and N₂ was inflated for 5 min prior to administration. Then, BV2 cells were cultured with 1% O₂, 5% CO₂ and 92% N₂ at 37°C for 24 h. RNA expression was measured by quantitative real-time polymerase chain reaction (qRT-PCR) 48 h after the transfection.

Next, LACC1 mimic, mimic negative control (NC), si-LACC1, and si-NS (Guangzhou RiboBio Co., Ltd, Guangzhou, Guangdong China) were instantaneously

transfected into BV2 cells in strict accordance with the kit instructions of the Lipofectamine™ 2000 kit (ThermoFisher Scientific, Waltham, MA, USA) for 24 h. Then, AMPK agonist (2 mM of buformin hydrochloride) or AMPK inhibitor (10 nM dorsomorphin) was added into BV2 cells for 24 h. BV2 cells were induced into vitro model.

Statistical analysis

The results are presented as mean \pm standard deviation. GraphPad Prime 8.0 (GraphPad Software, San Diego, CA, USA) was employed to perform data analysis. The Student's t-test was used for analyzing comparisons between two groups. One-way analysis of variance (ANOVA) was used for comparing different groups and Tukey's multiple comparisons test for pairwise com-

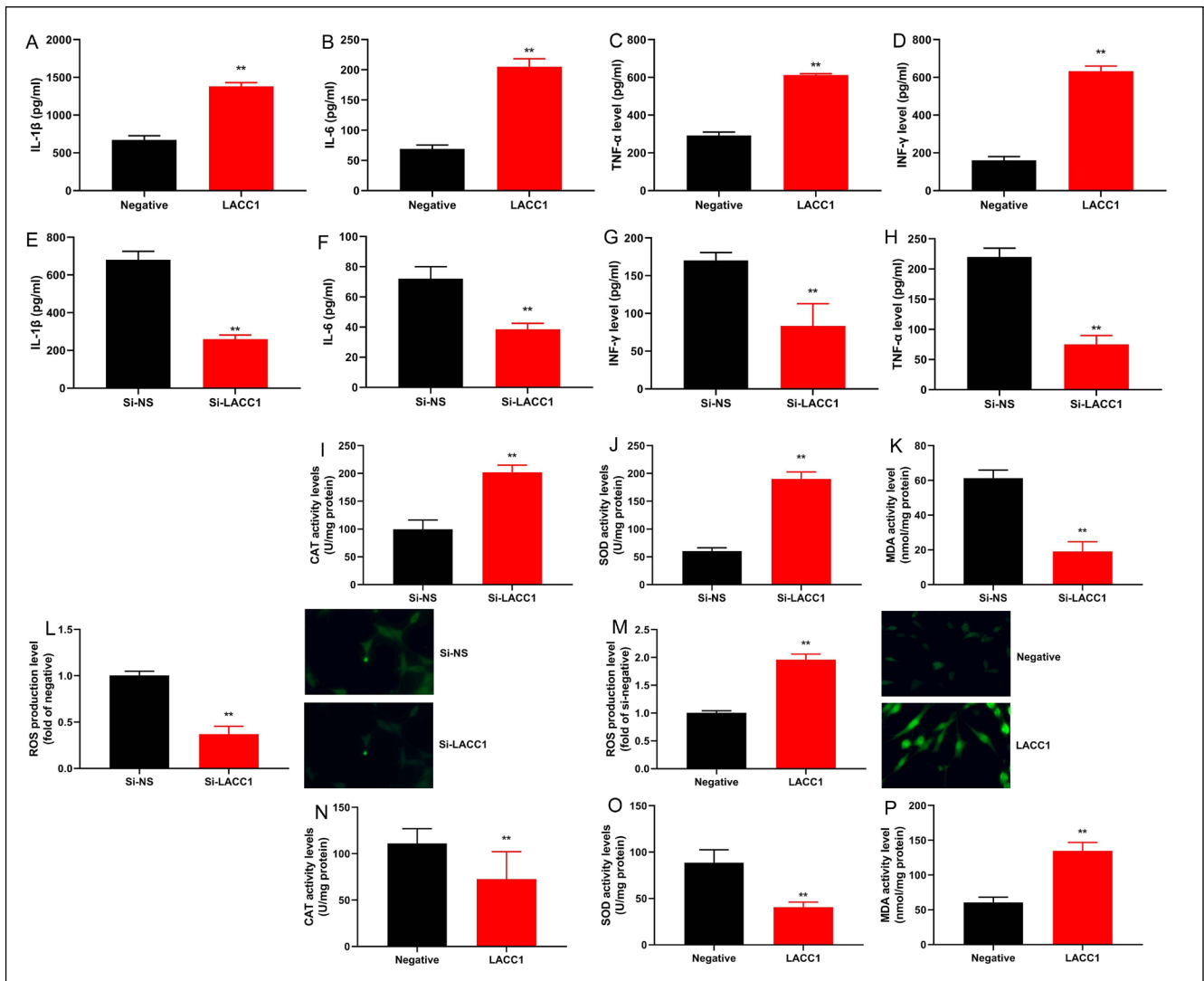


Fig. 3. LACC1 regulated inflammation and oxidative stress in an *in vitro* model of stroke. IL-1 β (A), IL-6 (B), INF- γ (C) and TNF- α (D) in an *in vitro* model of overexpression of LACC1; IL-1 β (E), IL-6 (F), INF- γ (G) and TNF- α (H) in an *in vitro* model of downregulation of LACC1; CAT (I), SOD (J), MDA (K) and ROS production levels (L) in an *in vitro* model of downregulation of LACC1; CAT (M), SOD (N), MDA (O) and ROS production levels (P) in an *in vitro* model of overexpression of LACC1. Negative, negative group; LACC1, overexpression of LACC1 group; Si-NS, si-negative group; Si-LACC1, downregulation of LACC1 group. ** $p < 0.01$ compared with negative group or si-negative group.

parisons after ANOVA. Differences were considered significant at values of $P < 0.05$.

RESULTS

LACC1 expression in model of stroke-induced cognitive disorder

To evaluate gene expression in stroke-induced cognitive disorder, we detected changes in expression levels using microarray analysis (Fig. 1A). LACC1 mRNA ex-

pression was up-regulated in brain tissue from patients with stroke-induced cognitive disorder (Fig. 1B). Immunohistochemical data showed LACC1 expression in brain tissue in the stroke model were higher than in the sham group (Fig. 1C). LACC1 mRNA and protein expression in mice with stroke-induced cognitive disorder was also increased in brain tissue samples (Fig. 1D-E).

We next investigated the function of LACC1 in mice after stroke. The time of escape latency, platform-crossing times and number of nerve cells were reduced in LACC1^{-/-} mice after stroke as compared with WT mice after stroke (Fig. 2A-C). GSH and SOD activity levels in-

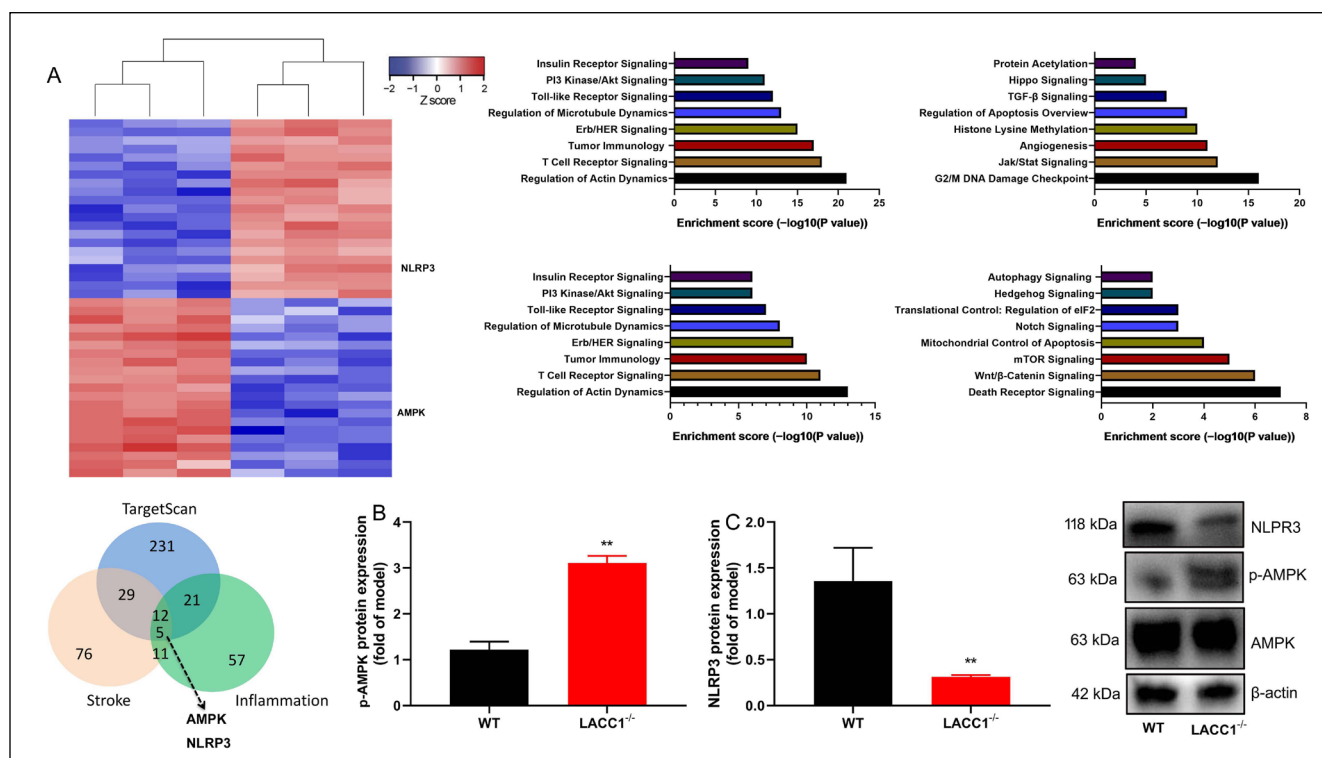


Fig. 4. LACC1 aggravated inflammation to cause cognitive disorder *via* the AMPK/ NLRP3 pathway in mice after stroke. Heat map (A), AMPK and NLRP3 protein expression (B and C). WT, WT mice after stroke; LACC1^{-/-}, LACC1^{-/-} mice after stroke. ** $p < 0.01$ compared with WT mice after stroke.

creased and MDA activity was reduced in LACC1^{-/-} mice after stroke as compared with WT mice after stroke (Fig. 2D-F). Additionally, IL-1 β , IL-6, INF- γ , TNF- α , collagen I, E-cadherin and α -SMA mRNA expression was reduced in brain tissue in LACC1^{-/-} mice after stroke as compared with WT mice after stroke (Fig. 2D-M).

Next, the study investigated the effects of LACC1 in an *in vitro* model of stroke. Overexpression of LACC1 increased IL-1 β , IL-6, INF- γ and TNF- α levels in the *in vitro* model (Fig. 3A-D). Downregulation of LACC1 resulted in a decrease in IL-1 β , IL-6, INF- γ and TNF- α levels *in vitro* (Fig. 3E-H). Downregulation of LACC1 increased CAT and SOD levels, and inhibited MDA levels and ROS production levels *in vitro* model (Fig. 3I-L). Downregulation of LACC1 reduced CAT and SOD activity levels and increased MDA levels and ROS production *in vitro* (Fig. 3M-P).

LACC1 aggravated inflammation to cause cognitive disorder after stroke *via* the AMPK/NLRP3 pathway

We next wanted to identify the mechanism of LACC1 in cognitive disorder after stroke using microarray anal-

ysis (Fig. 4A). The expression of p-AMPK protein levels were increased, and NLRP3 protein expression was suppressed in brain tissue of LACC1^{-/-} mice after stroke as compared with WT mice after stroke (Fig. 4B-C).

Next, downregulation of LACC1 suppressed LACC1 and NLRP3 protein expression and induced p-AMPK protein expression in the *in vitro* stroke model (Fig. 5A-C). Immunofluorescence showed that overexpression of LACC1 reduced the expression of p-AMPK in the *in vitro* stroke model (Fig. 5D), while the overexpression induced LACC1 and NLRP3 protein expression and suppressed p-AMPK protein expression (Fig. 5E-G).

To clarify the relationship between AMPK/NLRP3 pathway and the function of LACC1 in a model of stroke, an AMPK agonist (2 mM of buformin hydrochloride) or AMPK inhibitor (10 nM dorsomorphin) was used to regulate the activity of p-AMPK *in vitro* by regulating LACC1. AMPK agonism induced p-AMPK protein expression, suppressed NLRP3 protein expression, reduced IL-1 β and MDA levels, increased SOD levels and inhibited ROS production in the *in vitro* model by inducing LACC1 expression (Fig. 6A-F). AMPK inhibition reversed the effects of si-LACC1 on the induction of p-AMPK protein expression and SOD activity, the sup-

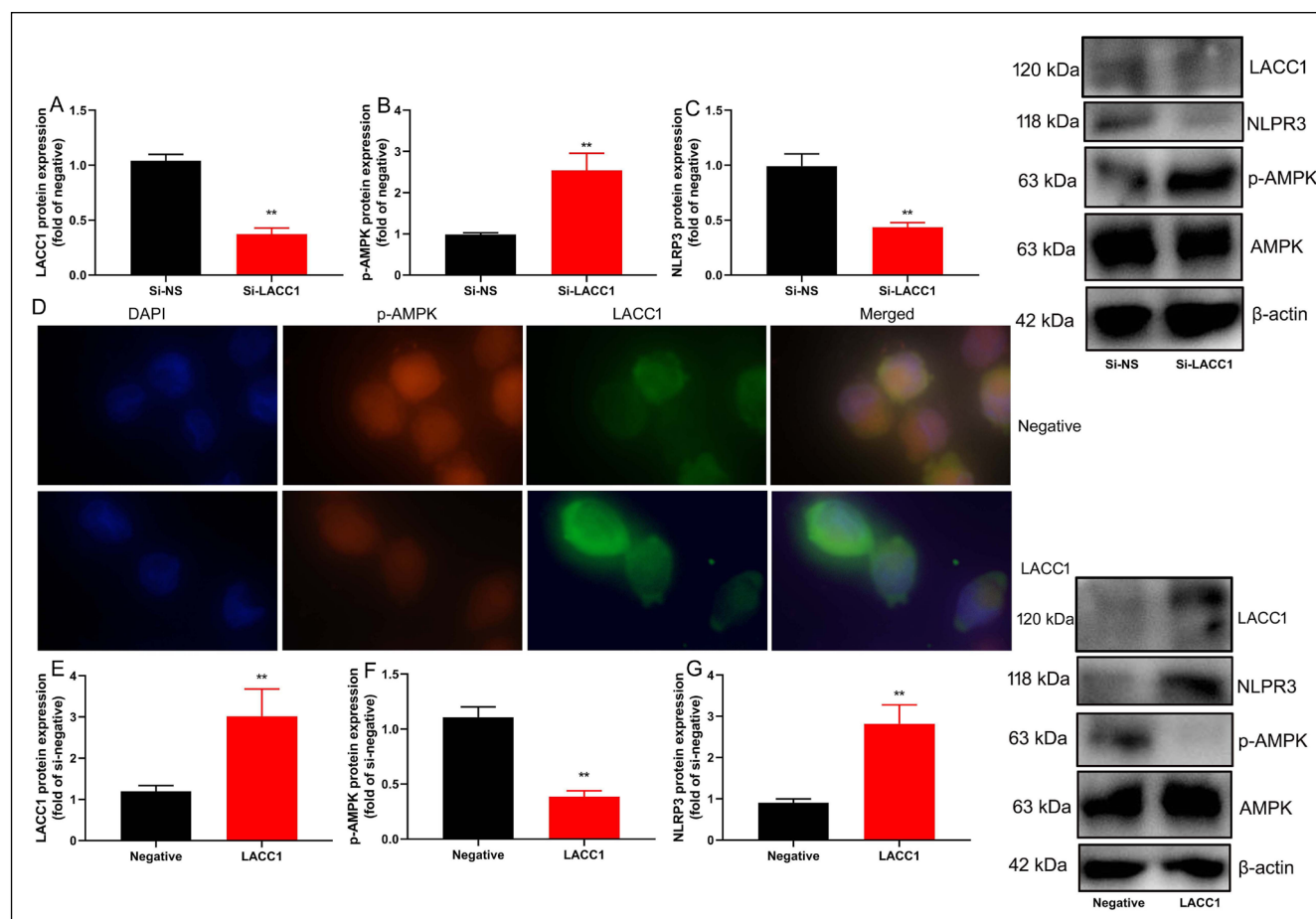


Fig. 5. LACC1 regulated the AMPK/ NLRP3 pathway in an *in vitro* model of stroke. LACC1, AMPK, NLRP3 protein expression (A, B and C) in an *in vitro* model of LACC1 overexpression; LACC1 and p-AMPK expression in an *in vitro* model of LACC1 overexpression (D); LACC1, AMPK, NLRP3 protein expression (E, F and G) in an *in vitro* model of LACC1 downregulation. Negative group; LACC1, overexpression of LACC1 group; Si-NS, si-negative group; Si-LACC1, downregulation of LACC1 group. ** $p < 0.01$ compared with negative group or si-negative group.

pression of NLRP3 protein expression and IL-1 β levels, the inactivation of MDA and on the production of ROS in the *in vitro* model (Fig. 6G-L).

DISCUSSION

LACC1 promoted inflammation and aggravated cognitive disorder in a mouse model of stroke. In the *in vitro* model of stroke, the inhibition of LACC1 reduced inflammation and ROS-induced oxidative stress via the activation of AMPK expression and the suppression of NLRP3 expression. Furthermore, our studies reveal that inhibition of AMPK activity reduced the effects of si-LACC1 on cognitive disorder in mice after stroke via the AMPK/NLRP3 pathway. Enhancement of AMPK activity also reduced the effects of LACC1 on inflammation and ROS-induced oxidative stress in the *in vitro* model via the NLRP3 pathway.

Stroke patients may have cognitive impairment, causing decreased self-care ability, decreased work ability and impaired social function and mental health, which seriously affect quality of life and survival time in patients (Hulde 2021; Li et al., 2021). This study showed that LACC1 mRNA expression was up-regulated in brain tissue from patients with stroke-induced cognitive disorder. Meanwhile, LACC1 mRNA and protein expression in mice with stroke-induced cognitive disorder was also increased in brain tissue samples. Omarjee et al. (2021) showed that LACC1 deficiency was linked to juvenile arthritis. Our study extends LACC1's role to potentially contributing to the occurrence and development of diseases of stroke-induced cognitive disorder.

Inflammation and the related immunological response are key factors in the pathophysiology of stroke (Jin et al., 2013; Lamberts et al., 2019; Parikh et al., 2020). In the current study, we showed that LACC1 ag-

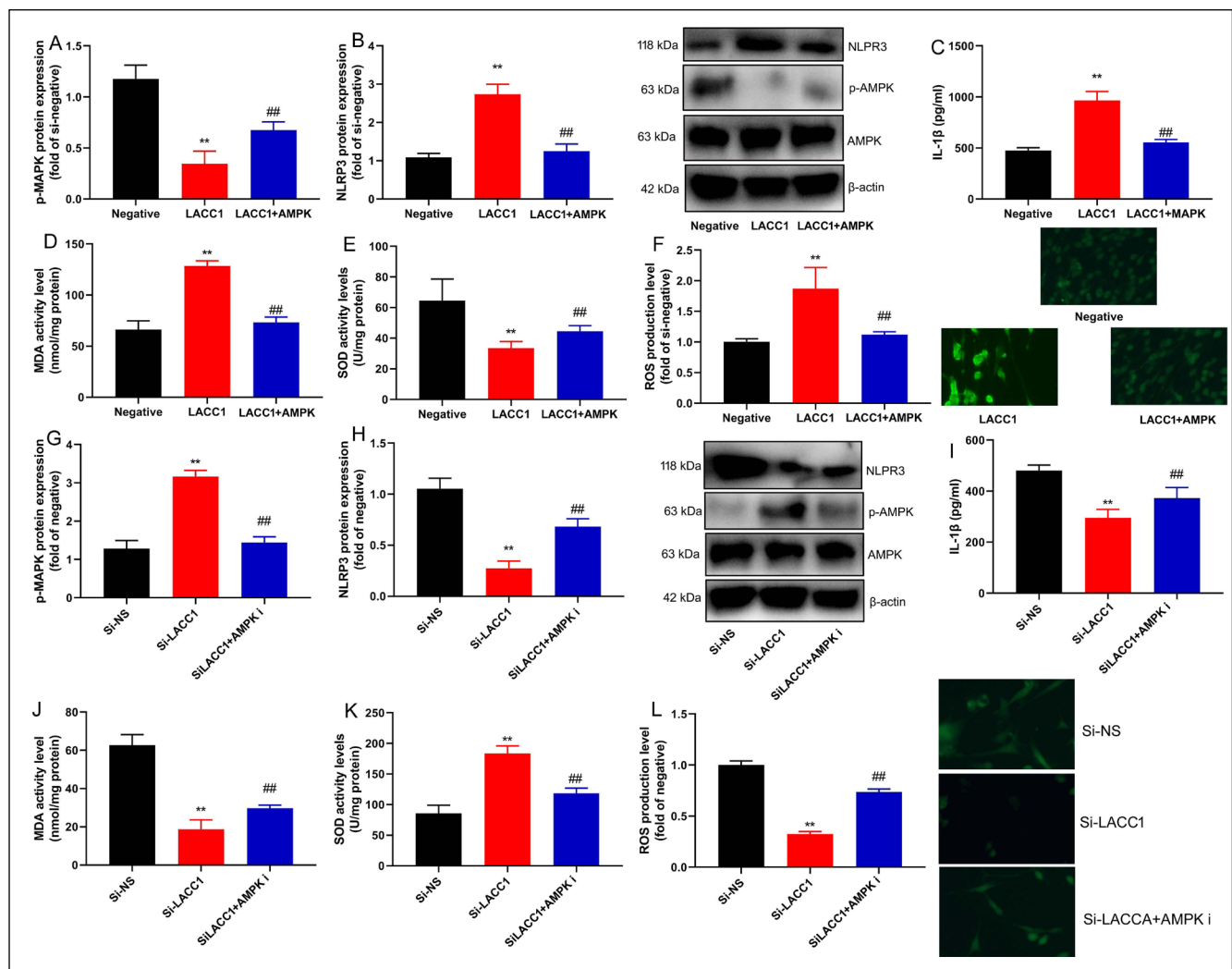


Fig. 6. LACC1 regulated the AMPK/ NLRP3 pathway in a model of stroke. AMPK and NLRP3 protein expression (A and B), IL-1 β (C), MDA (D), SOD (E), and ROS production (F) in an *in vitro* model of LACC1 and AMPK overexpression; AMPK and NLRP3 protein expressions (G and H), IL-1 β (I), MDA (J), SOD (K), and ROS production (L) in an *in vitro* model of LACC1 and AMPK downregulation. Negative, negative group; LACC1, overexpression of LACC1 group; LACC1+AMPK, overexpression of LACC1 and AMPK group; Si-NS, si-negative group; Si-LACC1, downregulation of LACC1 group; Si-LACC1+AMPK i, downregulation of LACC1 and AMPK inhibitor group. ** $p < 0.01$ compared with negative group or si-negative group; ## $p < 0.01$ compared with overexpression of LACC1 group or downregulation of LACC1 group.

gravated cognitive disorder due to stroke by the promotion of inflammation and oxidative stress. Skon-Hegg et al. (2019) showed that LACC1 regulates inflammation in mice with arthritis, supporting that LACC1 promoted inflammation and aggravated cognitive disorder phenotypes after stroke in our model. Therefore, our results appear to be in accordance with previous studies.

AMPK is called as the sensor and regulator of energy balance and plays an important role in the development of the nervous system (Wang et al., 2019). AMPK also plays a protective role in the occurrence and development of ischemic stroke, such as inhibiting oxidative stress, cell apoptosis, mitochondrial

dysfunction, glutamate excitotoxicity, neuroinflammation and promoting autophagy (Yu et al., 2020; Li et al., 2021; Tang et al., 2021). We also found that overexpression of LACC1 induced LACC1 and NLRP3 protein expression and suppressed p-AMPK protein expression in an *in vitro* model of stroke. Omarjee et al. (2021) showed that LACC1 deficiency is linked to juvenile arthritis *via* the regulation of AMPK, which is similar to our results.

NLRP3 inflammasomes increase the production and secretion of IL-1 β and IL-18 precursors through a series of mechanisms to mediate the death of cerebral ischemic neurons and glial cells (Li et al., 2018; Luo et

al., 2019). Moreover, NLRP3 can mediate the apoptosis and pyroptosis of cerebral ischemic neurons and glial cells through diverse effects of caspase-1 (Pu et al., 2020). Our results demonstrate that LACC1 aggravated inflammation to cause cognitive disorder after stroke via the AMPK/NLRP3 pathway. Additionally, these results showed that LACC1 suppressed p-AMPK protein expression and reduced NLRP3 in a model of stroke-induced cognitive disorder. Additionally, these results showed that LACC1 suppressed p-AMPK protein expression and reduced NLRP3 in a model of stroke-induced cognitive disorder.

In summary, we identified an important role and regulatory mechanism for LACC1 in maintaining stroke-induced cognitive disorder via the AMPK/NLRP3 pathway, which may provide a new therapeutic target for stroke or other neurological diseases and their associated complications.

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