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ORIGINAL RESEARCH

LncRNA THRIL Functions as a Marker for Carotid Artery Stenosis and Affects the Biological Function of Human Aortic Endothelial Cell

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Purpose: Carotid artery restenosis (CAS) is a leading contributor to cerebrovascular diseases and one of the leading causes of death in the world. The purpose of this study was to assess the predictive efficiency of long non-coding RNA (lncRNA) TNFalpha-and hnRNP L-related immunoregulatory lncRNA (THRIL) and its association with the pathogenesis of CAS.

Patients and Methods: The expression of THRIL was determined in patients with asymptomatic CAS and human aortic endothelial cell (HAEC) models induced by oxidized low-density lipoprotein (ox-LDL). The receiver operating characteristic (ROC) curve and Kaplan–Meier (K-M) drawings were constructed to predict the risk of poor prognosis in patients with CAS. The cell proliferation, death rate, and inflammation were detected by 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), flow cytometry, and enzyme-linked immunosorbent assay (ELISA) assays.

Results: The relative expression of THRIL was elevated in patients with asymptomatic CAS. The findings of ROC curve indicated that THRIL had a predictive possibility on CAS. K-M finding and Cox regression analysis showed that the expression of THRIL and the degree of CAS were independent risk factors for poor prognosis in patients with CAS. THRIL was up-expressed in HAECs induced by ox-LDL. Down-regulation of THRIL could promote the proliferation of HAECs, inhibit cell apoptosis, and restrict cell inflammation.

Conclusion: THRIL was a diagnostic and prognostic biomarker in CAS and played an important role in regulating the proliferation, apoptosis, and inflammation of HAECs induced by ox-LDL.

Keywords: THRIL, carotid artery restenosis, prognosis, diagnosis, proliferation

Introduction

Carotid artery stenosis (CAS) has attracted much attention because of its influence on cerebral blood supply, and 90% of CAS is caused by atherosclerosis.[1,2] CAS can cause intracranial ischemia, and produce corresponding clinical symptoms, such as dark vision, blurred hemiplegia, and aphasia.[3] Morbidity and mortality are high, and patients usually lose their self-care ability, thus bringing huge social and economic burdens. CAS mostly occurs at the bifurcation of carotid artery, especially in the bulbous of carotid artery.[4] The main pathological changes include vascular endothelial cell damage, inflammatory cell infiltration, abnormal lipid deposition, foam cell formation, and atherosclerotic plaque formation.[5] The risk factors of CAS factors include obesity, hypertension, hyperlipidemia, smoking, chronic infection, and genetic factors. Many patients with CAS often have no obvious clinical symptoms, while acute ischemic symptoms, such as sudden stroke and hemiplegia, are often caused by carotid plaques or thrombus on the surface of plaque, leading to intracranial arteriovenous ischemia.[6] Therefore, it is of great clinical significance to study the stability of carotid plaque for preventing serious complications.

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Long non-coding RNA (lncRNA) is more than 200 nucleotides (nts) in length and does not encode protein.[7] The biological characteristics of lncRNA are more complicated than microRNA (miRNA), and there are various ways to regulate the expression of target genes and proteins, so it is difficult to study its mechanism of action and pathway.[8] With the deepening of research, the role of lncRNA in vascular diseases has been gradually discovered. Many lncRNAs are involved in the regulation of the occurrence and progress of CAS, and their main functions include regulating the proliferation, apoptosis, adhesion, and migration of cells. LncRNA RhabdoMyoSarcoma 2-associated Transcript (RMST) is a highly expressed RNA in CAS patients and it has accuracy in distinguishing CAS patients.[9] SNHG14 is another lncRNA researched in restenosis, which can regulate cell proliferation through binding miR-145.[10] Abnormality of lncRNA THRIL expression is observed in several disorders that are associated with CAS. In a study of mice fed with a high-fat diet, silenced THRIL may militate myocardial impairment in coronary heart disease via regulating miR-424.[11] In a model of brain ischemia-reperfusion injury, the knockdown of THRIL assuages the neurological lesion by regulating miR-24-3p.[12] Nonetheless, the potential impacts of THRIL in CAS were not reported before.

In this observation, patients with CAS were collected and the expression of THRIL was evaluated in these patients. In addition, the receiver operating characteristic (ROC) curve, Kaplan–Meier (K-M), and COX regression were carried out to assess the clinical significance of THRIL. A human aortic endothelial cell (HAEC) model was established by oxidizing low-density lipoprotein (ox-LDL). The impacts of THRIL on cell proliferation, apoptosis, and inflammation were further calculated for analysis of the mechanism.

Material and Methods

Recruitment of Volunteers

Eighty-eight subjects who were admitted to the Affiliated Hospital of Weifang Medical University from October 2015 to February 2017 were selected. The diagnostic criteria of asymptomatic CAS patients were as follows: diagnosed CAS by Doppler ultrasound; no nervous system defects, such as transient ischemic attack (TIA) and cerebral infarction caused by CAS in the past 6 months. The control group consisted of 81 cases of normal physical examination in the same period. All these control individuals were examined by Doppler ultrasound, and the internal carotid artery was less than 20%. This study was performed in line with the principles of the Declaration of Helsinki. And approval was from Ethics Committee of Affiliated Hospital of Weifang Medical University. All participants were informed of the purpose and content of this study and voluntarily signed the written informed consent to use the collected data, blood samples, and regular follow-up information.

Exclusion and Inclusion Criteria

Inclusion criteria of CAS patients were as follows: (1) no previous history of cerebrovascular disease, only the early clinical manifestations of dizziness or mild headache; (2) asymptomatic patients with stenosis over 50% on ultrasonic technique examination; and (3) written informed consent form. Patients with recent intracranial hemorrhage, malformation or aneurysm of other intracranial vessels, mental illness, malignant tumors, severe cardiac, renal insufficiency, or thyroid dysfunction were excluded. A flowchart of patients included in this study was shown in Figure 1.

The inclusion criteria of the control group were as follows: (1) No previous history of cerebrovascular disease; (2) Cervical color ultrasound excluded CAS; (3) signed informed consent. The exclusion criteria of the control group were as follows: (1) Brain diseases, such as hydrocephalus, dementia, brain tumor, Parkinson's disease, brain trauma, intracranial infection, and acute cerebrovascular disease; (2) Suffering from mental illness; (3) Malignant tumor; and (4) Cerebrovascular examination revealed severe stenosis, malformation or aneurysm of other intracranial vessels.

Specimen Separation

The fasting venous blood was taken for about 3 mL, and after centrifugation at 3000 r/min for 10 minutes, the supernatant was placed in the refrigerator at −70°C to be tested.

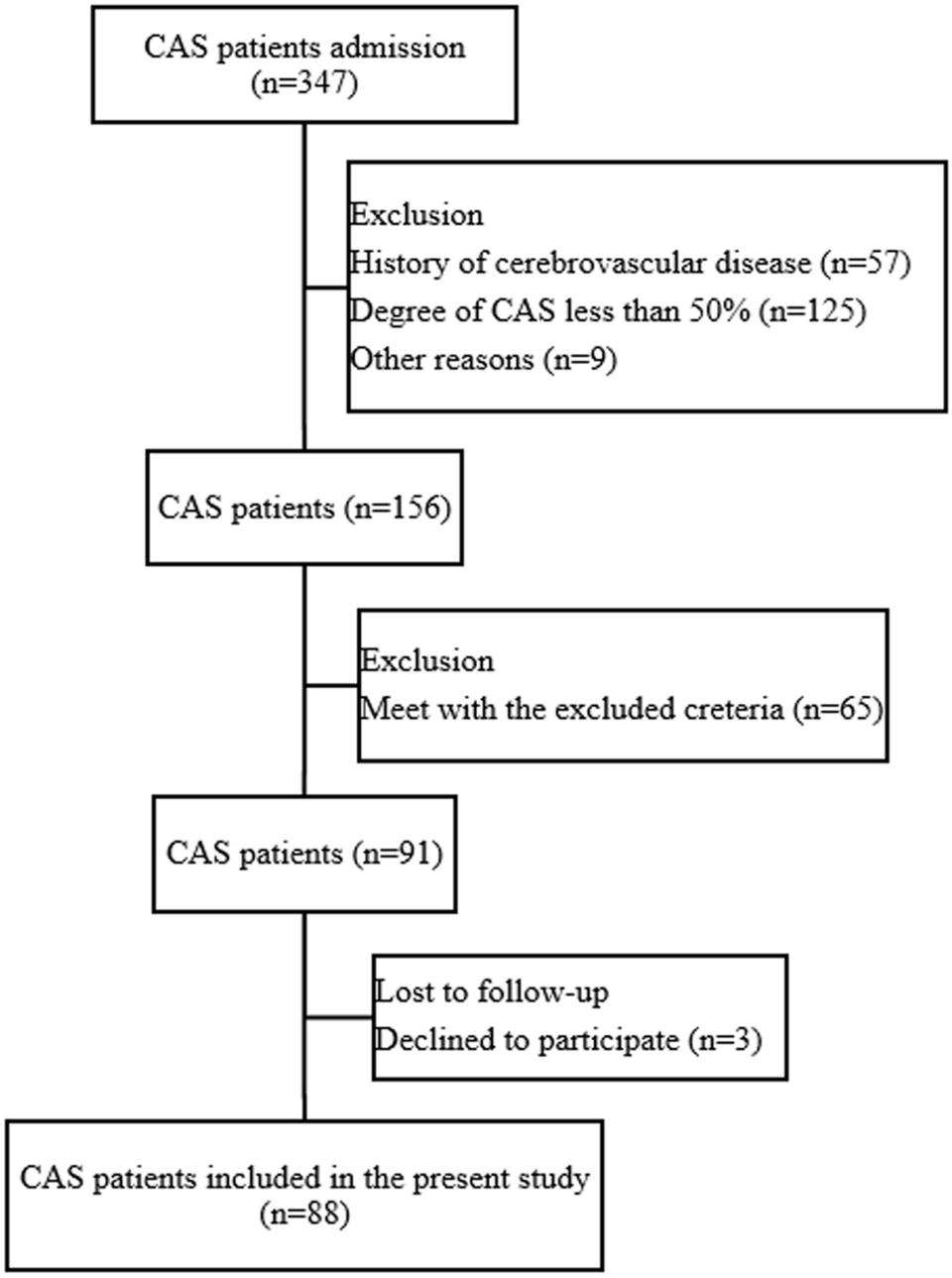

Figure 1 A flowchart of included patients.

Implement of Five-Year Follow-Up

All patients with CAS received a five-year follow-up. The occurrence of TIA, stroke, and sudden death caused by CAS was recorded as an endpoint event.

Cell Source and Management

HAECs were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HAECs were inoculated into DMEM medium containing 10% fetal bovine serum and 1% penicillin streptomycin (KetGEN, Jiangsu, China). The cells were cultured in an incubator at 37°C and 5% CO_2 and the medium was changed every 2–3 days. The cells in the ox-LDL group were treated with 100 mg/L ox-LDL,[13] the cells in the ox-LDL + small interference negative control (si-NC) group were treated with ox-LDL for 24 h after transfection with si-NC, and the

cells in the ox-LDL + si-THRIL group were treated with ox-LDL after transfection with THRIL sequences. When HAECs grew to 80% fusion, they were induced with ox-LDL for 24 h. HAECs were inoculated into a 6-well plate with 5×10^4 cells per well, and Lipofectamine^{TM} 3000 was used to transfect obtained sequences according to the instructions. The sequences of si-THRIL and its controls were purchased from the GenePharm company (Shanghai, China).

Detection of THRIL Expression by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA of HAECs and serum was extracted by adding TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA, USA), and then complementary (cDNA) was obtained by reverse transcription (TaKaRa, Tokyo, Japan) with total RNA as template. The qRT-PCR reaction system (TaKaRa, Tokyo, Japan) was mixed with the obtained cDNA and amplified on the Applied Biosystems 7300 PCR system. The relative expression of each gene was calculated according to the $2^{-\Delta\Delta CT}$ method.

Experimental Operation on Cells

The cells of each group were inoculated in a 96-well plate. After being cultured for 72 hours, 20 μL of 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Beyotime, Shanghai, China) was added to each well. After 4 hours of culture, 150 μL of dissolved dimethyl sulfoxide (DMSO) was added to shake until the crystal was fully dissolved. The optical density of each hole at 490 nm was detected by an enzyme labeling instrument.

The cells treated in each group were inoculated on a 6-well plate and incubated in a 37°For 48 hours and then digested with trypsin. The cell suspension was collected and precipitated by centrifugation. 200 μL binding buffer, 5 μL Annexin V-FITC, and 5 μL propidium iodide (PI) were added. Cells were incubated in the absence of light at room temperature, and the apoptosis of cells in each group was detected by flow cytometry.

The expression of interleukin (IL)-6 and IL-1β was detected by enzyme linked immunosorbent assay (ELISA) reagent (mlbio, Shanghai, China). The standard and samples were added to the coating hole of IL-6 and IL-1β antibodies. The IL-6 and IL-1β primary antibody working solutions were added, respectively. The plate was incubated for 1 hour and an enzyme-labeled second antibody working solution was added for 30 minutes. The data were measured using an enzyme labeling instrument. The concentration of IL-6 and IL-1β was calculated according to the standard curve and regression equation.

Statistical Analysis

Statistical Package for the social sciences (SPSS) 26.0 software and GraphPad were used for statistical analysis. Count data were expressed as n/% and calculated using the chi-square test. When measuring data conforms to the normal distribution, they were analyzed using a *t*-test. The ROC of the tested samples was drawn to determine the diagnostic value of THRIL value. The K-M curve and Cox regression methods were applied to manifest the prognostic significance of THRIL. One-way analysis of variance test (ANOVA) was used for calculating comparison among groups. $P < 0.05$ indicated statistical significance.

Results

Comparison Between Baseline Data Groups

The general data of sex and age between the two groups were similar and there is no statistical difference (Table 1, $P > 0.05$), so the two groups were comparable. The number of hypertension in CAS group was raised in comparison with control group (Table 1, $P < 0.05$). The mean degree of CAS was 66.19 ± 8.74% for all CAS patients (Table 1).

Comparison of Serum THRIL Levels Between the Two Groups

The expression level of serum THRIL in CAS patients was increased in comparison with control volunteers (Figure 2, $P < 0.001$). This abnormality of THRIL expression suggested that CAS is associated with elevated levels of THRIL.

Table 1 Clinical Statistical Information of Recruited Individuals

Parameter	Controls (n = 81)	CAS (n = 88)	*P* value
Age (years)	62.68 ± 10.27	63.31 ± 9.46	0.680
Gender (male, %)	44, 54.32	44, 50.00	0.574
Smoking (n, %)	24, 29.63	33, 37.50	0.280
Diabetes mellitus (n, %)	29, 35.81	31, 35.23	0.938
Hypertension (n, %)	37, 45.68	56, 63.64	0.019
BMI (kg/m^2)	23.10 ± 2.76	23.36 ± 2.61	0.531
LDL-C (mmol/l)	2.88 ± 0.64	2.81 ±0.62	0.443
HDL-C (mmol/l)	1.29 ± 0.24	1.32 ± 0.24	0.411
TC (mmol/l)	4.91 ± 0.91	5.14 ± 0.64	0.055
TG (mmol/l)	1.46 ± 0.37	1.48 ± 0.34	0.731
Degree of CAS (%)	/	66.19 ± 8.74	/

Abbreviations: CAS, carotid stenosis; BMI, body mass index; LDL, low density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; TG, triglycerides.

The Distinguishable Value of THRIL on CAS from Controls

As expressed in Figure 3, the sensitivity of the ROC curve was 85.18%, specificity was 84.09%, and the AUC was 0.904 at the cut-off value of 1.24. The serum THRIL expression could predict CAS patients from the control cohort.

The Predictive Value of THRIL in the Risk Degree of End-Point Events in Patients with CAS

All patients were included in two different groups according to the average expression of THRIL. The 5-year overall outcome of the high expression of THRIL group was poorer in comparison with the low expression of THRIL group (Figure 4, $P = 0.009$).

Cox regression method analyzed all indicators and evaluated their prognostic value for CAS patients. Serum THRIL expression (hazard ratio = 0.222, 95% CI = 0.069–0.713, P = 0.011) and degree of CAS (hazard ratio = 0.293, 95% CI = 0.100–0.855, P = 0.025) were both independent markers in predicting risk degree of end-point events (Table 2).

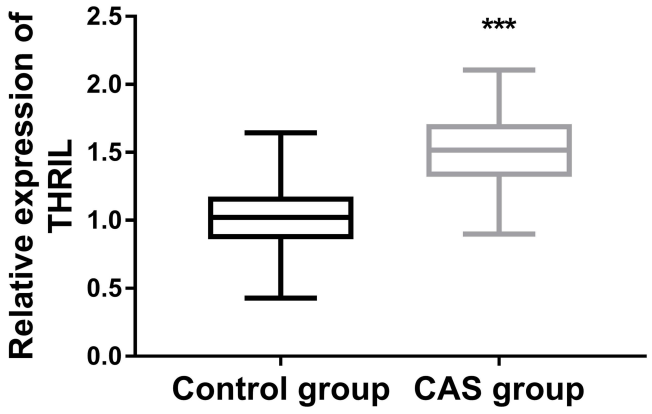

Figure 2 An enforced expression of THRIL in CAS patients. ***$P < 0.001$.

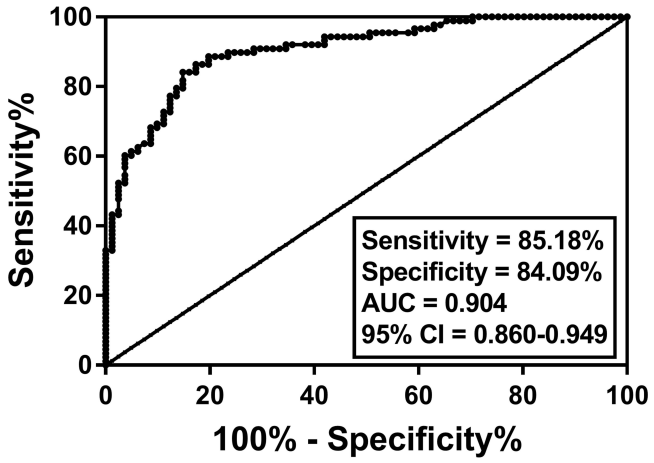

Figure 3 The AUC of THRIL for predicting poor prognosis in patients with asymptomatic CAS was 0.904.

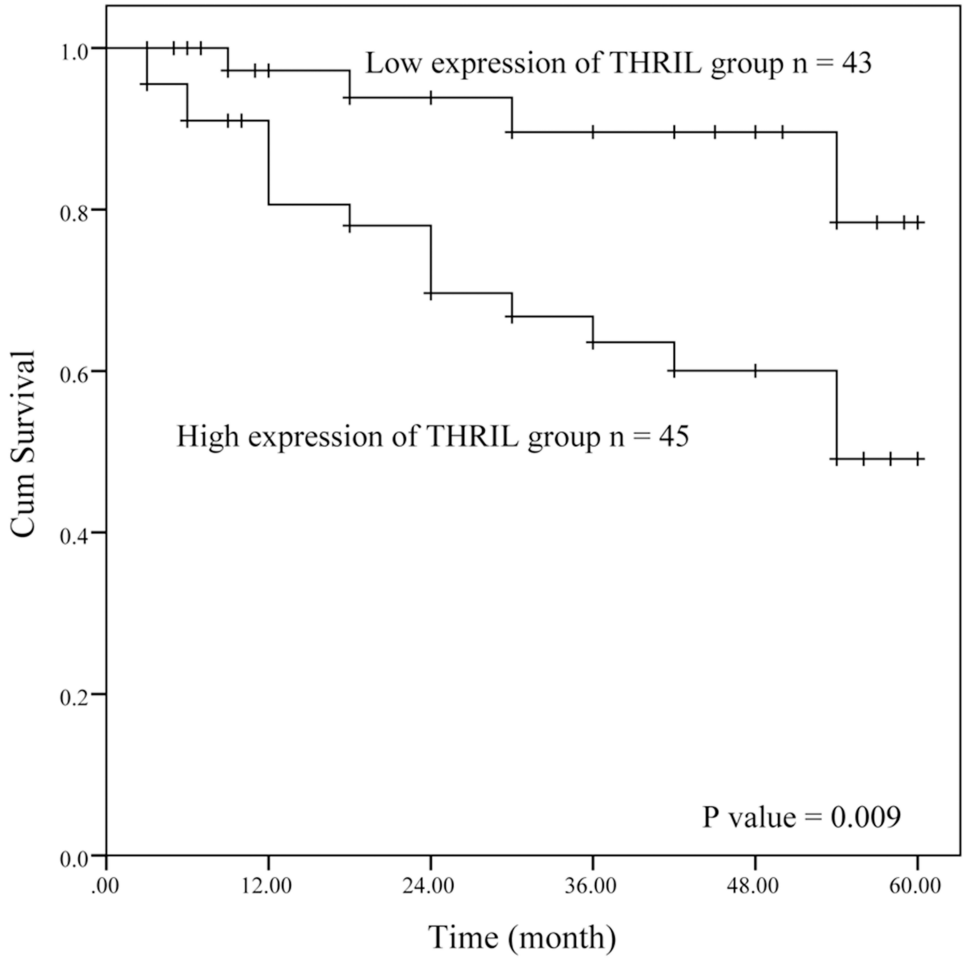

Figure 4 The K-M curve estimated the prognostic value of THRIL.

Elimination of THRIL Abrogated Ox-LDL Impairment on HAECs

The expression of THRIL was increased in HAECs treated with ox-LDL, while the si-THRIL inhibited the overexpression of THRIL in the OX-LDL group (Figure 5A, $P < 0.001$). This outcome certified the level of

Table 2 Cox Regression Multivariate Analysis for Overall Survival in CAS

Parameter	Hazard Ratio	95.0% CI		*P* value
		Lower	Upper	
THRIL expression	0.222	0.069	0.713	0.011
Age (years)	1.068	0.343	3.330	0.910
Gender (male, %)	1.106	0.409	2.989	0.843
Smoking (n, %)	1.436	0.510	4.041	0.493
Diabetes mellitus (n, %)	0.997	0.360	2.761	0.996
Hypertension (n, %)	0.868	0.316	2.388	0.784
BMI (kg/m^2)	1.125	0.416	3.047	0.816
LDL-C (mmol/l)	1.411	0.521	3.824	0.499
HDL-C (mmol/l)	1.511	0.546	4.183	0.427
TC (mmol/l)	0.764	0.261	2.238	0.624
TG (mmol/l)	1.068	0.350	3.255	0.909
Degree of CAS (%)	0.293	0.100	0.855	0.025

Abbreviations: CAS, carotid stenosis; BMI, body mass index; LDL, low density lipoprotein; HDL, high-density lipoprotein; TC, total cholesterol; TG, triglycerides.

THRIL was changed with the ox-LDL treatment and transfection of si-THRIL exerted inhibitory function on gene expression.

After 72-hour incubation, the proliferation of the ox-LDL group was inhibited, and silenced THRIL prevented the decrease of cell proliferation (Figure 5B, $P < 0.01$). Additionally, the ox-LDL management promoted cell apoptosis, but the decreased THRIL expression reversed the adverse impact of ox-LDL (Figure 5C, $P < 0.001$).

Absence of THRIL Repressed Inflammatory Indicators

The expression of IL-6 and IL-1β represented the inflammatory situation in HAECs. As shown in Figure 5D, treatment of ox-LDL damaged the inflammatory balance by elevating IL-6 and IL-1β expression ($P < 0.001$). The interference of THRIL had an inhibitory effect on inflammatory disorder (Figure 5D, $P < 0.001$).

Discussion

Stroke is the second leading cause of death in the world and poses a great threat to public health. Studies have shown that CAS is an important reason for ischemic stroke, so asymptomatic CAS can be used as a breakthrough in the screening of stroke in high-risk population.[14] Clinically, the degree of asymptomatic CAS < 70% is generally not regarded as an absolute indication of surgery.[15] However, it is worth noting that even if the degree of CAS does not meet the surgical standards, serious complications such as ischemic stroke may occur. Once ischemic stroke occurs, local brain tissue may be necrotic due to ischemia and hypoxia, and the mortality and disability are very high.[16] Early intervention of CAS can reduce the risk of ischemic stroke to some extent. Thus, it is vital to research indicators for CAS patients.

Non-coding RNA is a study hotspot in CAS development. An increase of miR-186-5p expression is observed in CAS patients, indicating its association with CAS progression.[17] In patients with in-stent restenosis, the expression lncRNA CDKN2B-AS1 is elevated and the expression of miR-143-3p is lessened, which provides that the abnormal expression of CDKN2B-AS1 and miR-143-3p may be risks of CAS.[18] THRIL can participate in the occurrence of hematological diseases in a variety of ways. In myocardial infarction, the expression of THRIL is increased and it can aggravate the impairment via negative regulation of miR-99a.[19] An increase of THRIL expression is observed in coronary heart

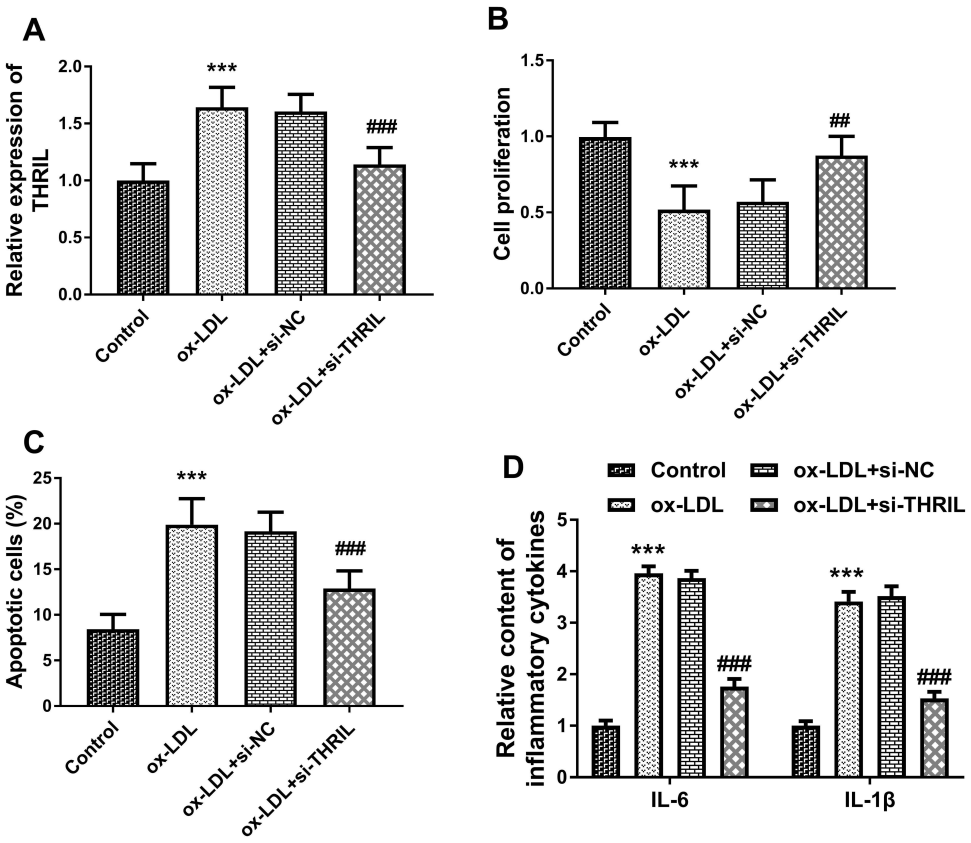

Figure 5 (**A**) The si-THRIL reversed the up-regulation of THRIL in the ox-LDL group. (**B-D**) THRIL regulated the cell proliferation, apoptosis, and inflammation steered by ox-LDL. ***$P < 0.001$, in comparison with control cells; ###$P < 0.01$, ####$P < 0.001$, in comparison with ox-LDL group.

disease, and its interference can recover the damage caused by a high-fat diet in mice models.[11] This current observation found that THRIL expression was increased in CAS patients, emphasizing that THRIL might be implicated in CAS progression.

The clinical significance of non-coding RNA on CAS has been reported by investigators. MiR-27b, miR-637, and miR-19a-3p are diagnostic biomarkers and possible factors for predicting the risk of cerebral ischemic events.[20–22] RMST is highly expressed in CAS patients and the ROC curve indicates that it is a useful indicator in screening CAS patients.[9] Considering the overexpression of THRIL in patients with ACS, the predictive possibility was estimated. THRIL has a high area under the curve (AUC) value of ROC curve, corroborating that THRIL could serve as a predictor for differentiating CAS patients. Moreover, the prognostic possibility was reckoned in light of the expression of THRIL in CAS patients. As a result, patients with high expression of THRIL had a high risk of suffering a bad overall outcome, indicating THRIL might be an indicator for patients with CAS. The Cox regression method manifested that THRIL was an independent biomarker in prognosing the outcome of CAS patients. In addition, the degree of CAS was also an independent risk factor in the development of CAS, however, several well-described indicators were not associated with the survival outcome. This might be due to the included asymptomatic ACS patients and the small sample size.

The pathogenesis of carotid atherosclerosis is a complicated process in which the dysfunction of vascular smooth muscle cells and endodermal cells plays important roles. In endothelial progenitor cells of coronary atherosclerotic heart disease, the cell viability and autophagy are injured, and the THRIL inhibits the viability and accelerates autophagy of cells via AKT pathway and FUS.[23] In diabetes retinopathy, THRIL can regulate the proliferation and migration of microvascular endothelial cells injured by high glucose, providing that THRIL may correlate with the aberration of endothelial cells.[24] The levels of THRIL in the HAECs treated by ox-LDL were increased, indicating the ox-LDL treatment induced injured influence in HAECs. The proliferation and apoptosis of HAECs were impaired by ox-LDL, while the interference of THRIL reversed the adverse impacts of ox-LDL, summarizing that THRIL is linked to the

mechanism of CAS. As for the inflammation, THRIL may expedite the pro-inflammatory cytokines.[25,26] A proinflammatory function of THRIL is found in osteoarthritis by the evidence that THRIL promotes IL-6 expression.[27] Interestingly, this literature discerned that decreased THRIL expression inhibited the inflammatory disorder evoked by ox-LDL, providing that THRIL exerted promoting role in inflammation.

Conclusion

This study documented that the expression of THRIL was increased in patients with ACS and HAECs treated with ox-LDL. Detection of the expression of THRIL had high efficacy in distinguishing CAS patients and predicting the outcome of these patients. THRIL could inhibit the proliferation of HAECs, and accelerate apoptosis and inflammation, thus aggravating the influence of ox-LDL.

Disclosure

The authors report no conflicts of interest in this work.

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