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·**Original Article**·

A conserved region in the 3′ untranslated region of the human *LIMK1* gene is critical for proper expression of *LIMK1* at the post-transcriptional level

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ABSTRACT

LIM kinase 1 (LIMK1), a cytosolic serine/threonine kinase, regulates actin filament dynamics and reorganization and is involved in neuronal development and brain function. Abnormal expression of LIMK1 is associated with several neurological disorders. In this study, we performed a conservation analysis using Vector NTI (8.0) software. The dual-luciferase reporter assay and real-time quantitative RT-PCR were used to assess the protein and mRNA levels of the reporter gene, respectively. We found that a region ranging from nt +884 to +966 in the human *LIMK1* 3′ untranslated region (UTR) was highly conserved in the mouse *Limk1* 3′ UTR and formed a structure containing several loops and stems. Luciferase assay showed that the relative luciferase activity of the mutated construct with the conserved region deleted, pGL4-hLIMK1-3U-M, in SH-SY5Y and HEK-293 cells was only ~60% of that of the wild-type construct pGL4-hLIMK1-3U, indicating that the conserved region is critical for the reporter gene expression. Real-time quantitative RT-PCR analysis demonstrated that the relative *Luc2* mRNA levels in SH-SY5Y and HEK293 cells transfected with pGL4-hLIMK1-3U-M decreased to ~50% of that in cells transfected with pGL4-hLIMK1-3U, suggesting an important role of the conserved region in maintaining *Luc2* mRNA stability. Our study suggests that the conserved region in the *LIMK1* 3′ UTR is involved in regulating *LIMK1* expression at the post-transcriptional level, which may help reveal the mechanism underlying the regulation of *LIMK1* expression in the central nervous system and explore the relationship between the 3′-UTR mutant and neurological disorders.

Keywords: LIM kinase 1; three-prime untranslated region; post-transcriptional regulation; RNA secondary structure

INTRODUCTION

LIM kinase 1 (LIMK1) is a cytosolic serine/threonine kinase that phosphorylates and inactivates cofilin, regulating actin filament dynamics and reorganization[1]. In neurons, actin filament dynamics and reorganization are essential for controlling growth-cone motility and morphology and for determining the direction and speed of neurite extension, so LIMK1 is thought to be involved in neuronal development and brain function[2,3].

LIMK1 is expressed predominantly in both the

peripheral and central nervous systems[4,5]. LIMK1-knockout mice exhibit significant abnormalities in spine morphology and synaptic function and show altered fear responses and spatial learning[3]. The loss of LIMK1 induces nervous system impairment and is implicated in Williams syndrome, a mental disorder with profound deficits in visuospatial cognition[6-8]. *LIMK1* promoter C(-187)T single nucleotide polymorphism decreases gene transcription *in vitro* and is significantly associated with intracranial aneurysms[9]. Therefore, the accurate expression of LIMK1 is essential for normal brain function.

Three-prime untranslated regions (3′ UTRs) play an important role in regulating gene expression at the post-transcriptional level. The 3′ UTRs are involved in mRNA 3′ end formation and polyadenylation[10], and also in mRNA stability/degradation, nuclear export, subcellular localization and translation efficiency[11,12]. These various regulatory roles are carried out by *cis*-acting elements including microRNA (miRNA) targets in the 3′ UTRs that interact with diverse *trans*-acting factors including miRNAs in a given cellular state and environment[13,14]. Mutations in the 3′ UTR elements influence the expression of specific genes at the level of translation, resulting in abnormal development and various diseases[11,12,15]. A recent study showed that a brain-specific miRNA, miR-134, inhibits *LIMK1* translation by interacting with the miRNA target in the *LIMK1* 3′ UTR, and negatively regulates the size of dendritic spines—postsynaptic sites of excitatory synaptic transmission[16].

In this study, an evolutionarily-conserved region was predicted in the 3′ UTRs of *LIMK1* transcripts using cross-species comparison. Then the human *LIMK1* 3′ UTR was cloned into a firefly luciferase expression construct to characterize the *LIMK1* 3′ UTR *in vitro*.

MATERIALS AND METHODS

LIMK1 3′-UTR Sequence Data and Conservation Analysis

The human and mouse *LIMK1* 3′ UTRs were accessed at http://www.ncbi.nlm.nih.gov/ (NCBI Accession Nos: NM_002314 and NM_010717, respectively). Conservation analysis was performed based on a previous study[17]. The sequences were considered to be highly conserved if the average absolute complexity was ≥8.0 and the alignment length ≥50 bp.

RNA Secondary Structure Prediction

The sequences of human *LIMK1* 3′ UTR and its mutant (conserved region deleted) were subjected to secondary structure prediction using the minimum free energy (MFE) method implemented on the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The graphical output of MFE plain structure drawing was used for visual comparison between the wild-type and mutation sequences.

Vector Construction

Human genomic DNA was extracted and purified from peripheral blood with QuickGene DNA Whole Blood Kit L (Fuji Photo Film Co., Ltd) using the Automatic Nucleic Acid Isolation System (QuikGene-610L, Fujifilm). This study was approved by the institutional review board of the Second Affiliated Hospital of Guangzhou Medical University. The *LIMK1* 3′-UTR sequence was amplified from the genomic DNA with primers P1 and P2 (P1: 5′-TATACGCGTCCACTCAGCTGCCCCTGTC-3′; P2: 5′-TATGTCGACGACCTGGGTGGGGAGTACG-3′). PCR amplification was performed by denaturation at 94°C for 5 min followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, and 4 min at 72°C, with a final extension step of 10 min at 72°C. The PCR product was visualized by ethidium bromide staining of agarose gel, and purified using the QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). The purified product was cloned into the pGL4.13 vector (Promega, Madison, WI) at the *Mlu* I and *Sal* I sites to produce the recombinant luciferase construct pGL4-hLIMK1-3U in which the 3′ UTR sequence was located directly downstream of the luciferase translation stop codon.

Site-directed Mutagenesis

Using a KOD-Plus-Mutagenesis Kit (Toyobo, Osaka, Japan), the mutated construct pGL4-hLIMK1-3U-M (conserved region deleted) was generated according to the manufacturer's instructions. In brief, the inverse PCR amplifications were performed using mutation primers P3 and P4 for deleting the conserved region (P3: 5′-CAGGGGCTTGGGATTTTATTTTTGTGGCGG-3′; P4: 5′-GGGGCATGAGGAGGGGCAGGGAG-3′). PCR amplification was initiated by denaturation at 94°C for 2 min followed by 8 cycles of 10 s at 98°C, 30 s at 65°C, and 10 min at 68°C. The PCR products were digested by *Dpn* I. Finally, self-ligation of PCR products was performed by

reaction with *T4* ligase and then transformed into competent *E. coli* cells. The mutant construct pGL4-hLIMK1-3U-M was purified and confirmed by DNA sequencing.

Cell Culture, Transfection and Luciferase Assay

The human neuroblastoma SH-SY5Y cell line and the human embryonic kidney HEK-293 cell line were purchased from ATCC (Manassas, VA). Cell culture, the transfection procedure, luciferase assay, and data analysis were performed as in our previous reports[18,19]. Cells in 24-well plates were transfected with 100 ng of test plasmids and 2.5 ng of pRL-TK plasmids (as an internal control reporter vector; Promega) using Lipofectamine 2000. After 24-h transfection, the cells were harvested to assay the activities of firefly luciferase and *Renilla* luciferase using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was detected using a GloMax 20/20 luminometer (Promega). Transfection efficiency and cell number were evaluated based on the co-transfected *Renilla* luciferase values and the total cellular protein of each plate. The relative activity (firefly/*Renilla*) of the wild-type vector in each cell line was normalized to 100%.

Real-Time Quantitative RT-PCR

Total RNA was extracted and purified using a GeneJET RNA Purification Kit (Thermo Fisher Scientific, Ottawa, ON). The total RNA sample was treated with RNase-free DNase I before cDNA synthesis. First-strand cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Fisher Scientific). RT-PCR amplification was performed using Maxima SYBR Green qPCR Master Mix (Thermo Scientific). RT-PCR amplification mixtures (25 μL) contained 100 ng template cDNA, 2× SYBR Green I Master Mix buffer (12.5 μL) (Thermo Scientific) and 300 nmol/L primer pairs (P5 and P6 for *Luc2*: P5: 5′-TGTACACCTTCGTGACTTCCC-3′; P6: 5′-TGACTGAATCGGACACAAGCG-3′; P7 and P8 for *β-actin*: P7: 5′-GCTGTATTCCCCTCCATCGTG-3′; P8: 5′-TTGTAGAAGGTGTGGTGCCAG-3′). One-step real-time RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Kit (Thermo Scientific) and the Rotor-Gene Q real-time thermocycler (Corbett Research, Australia). The cycling conditions comprised 10-min polymerase activation at 95°C and 30 cycles at 95°C for 15 s and 60°C for 60 s. All PCR efficiencies were >95%. The *Luc2* mRNA levels were measured and normalized to the expression level of *β-actin*. All measurements were performed in triplicate and the experiments were repeated at least twice. Rotor-Gene software (version 1.7; Qiagen) results were exported into Microsoft Excel for further analysis.

RESULTS

The sequences of human and mouse *LIMK1* 3′ UTRs were aligned with Vector NTI software to identify conserved regions. According to the evaluation standard (average absolute complexity ≥8.0 and alignment length ≥50 bp) of the conserved regions described in Material and Methods, a region ranging from nt +884 to +966 in the human *LIMK1* 3′ UTR was highly conserved in the mouse *Limk1* 3′ UTR (Fig. 1). To determine whether the conserved region affects its

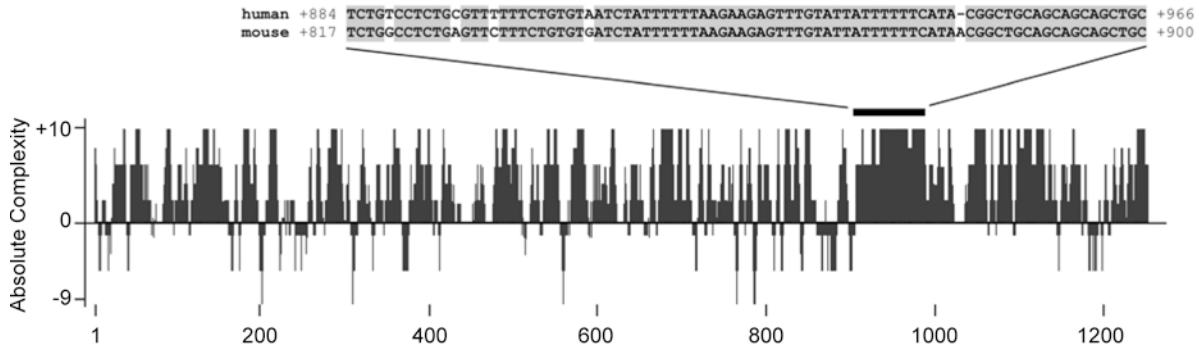

Fig. 1. Conservation analysis of the *LIMK1* 3′ UTR between human and mouse. The black bar indicates a highly-conserved region. The absolute complexity on the y-axis was expressed as the sum of all pairwise residue substitution scores at a given alignment position normalized by the number of pairs in the alignment. A higher y value indicates higher sequence conservation. The x-axis indicates the distance (base-pairs, bp) from the translation stop codon.

mRNA structure, we performed the secondary structure prediction using the *LIMK1* 3′ UTR and its mutated sequence (conserved region deleted) and found that the conserved region formed a structure containing several loops and stems (Fig. 2).

To study the role of the conserved region in regulating gene expression at the post-transcriptional level, we generated two recombinant luciferase constructs pGL4-hLIMK1-3U and pGL4-hLIMK1-3U-M (Fig. 3A, B). The pRL-TK construct containing a *Renilla*-expression cassette was used as an

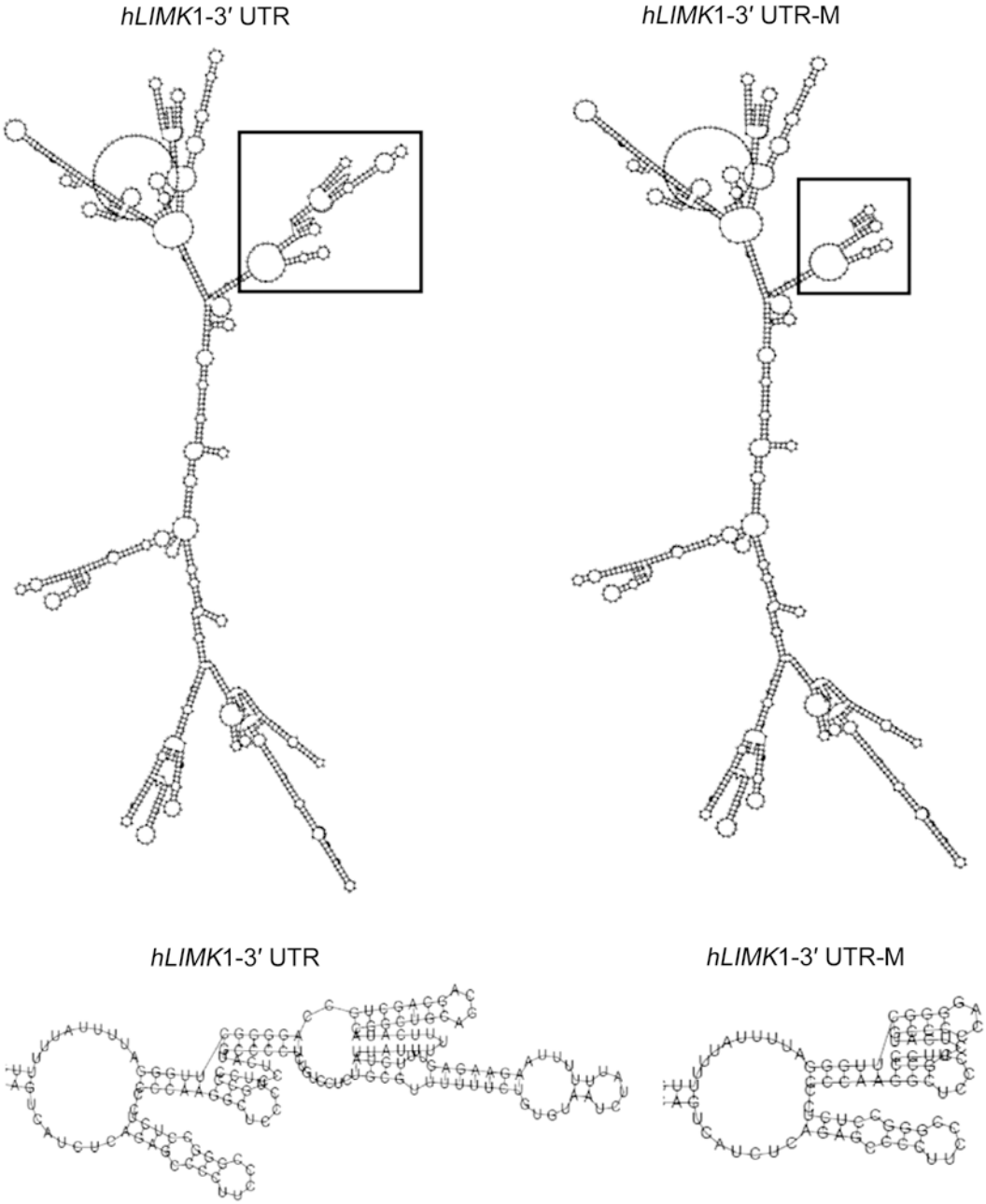

Fig. 2. Secondary RNA structure prediction of the hLIMK1-3′ UTR and its mutant hLIMK1-3′ UTR-M (conserved region deleted). The structures in boxes show that the conserved region forms five loops and five stems (bottom row).

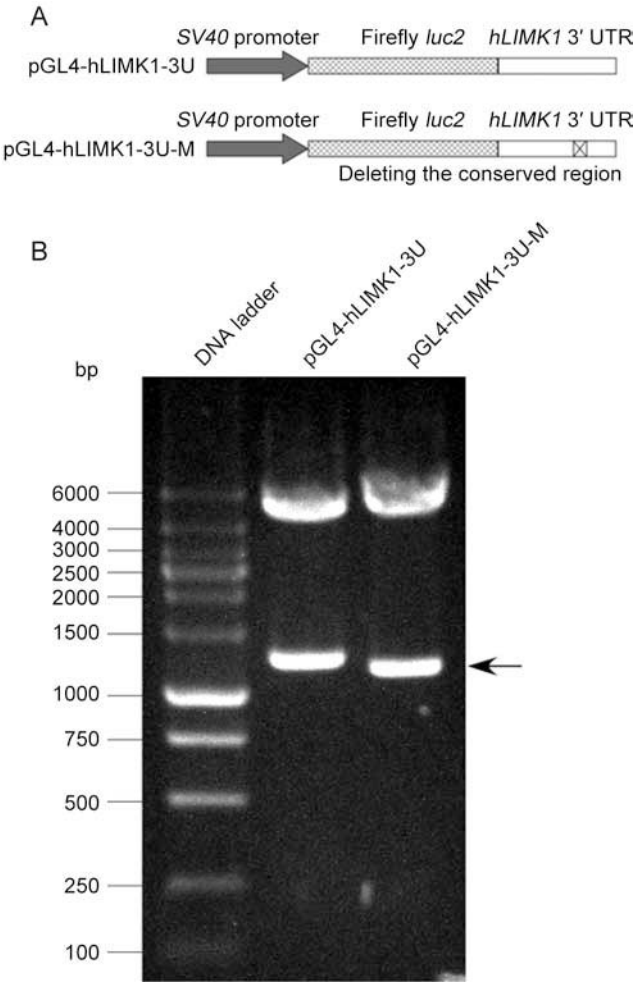

Fig. 3. A: Schematic of the reporter constructs used to determine the role of the *hLIMK1* 3′UTR in regulating the reporter gene expression. B: Identification of the reporter constructs by *Mlu* I/*Sal* I double digestion. The arrow indicates the bands for *hLIMK1*-3′ UTR and *hLIMK1*-3′ UTR-M.

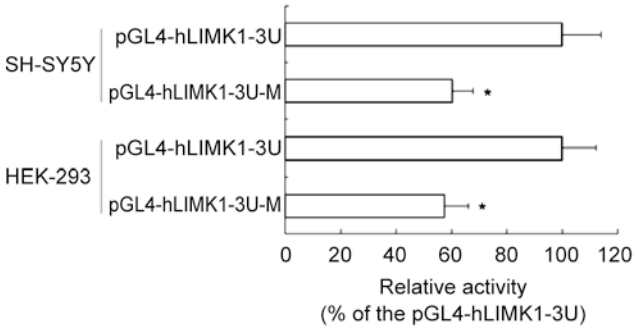

Fig. 4. Luciferase activity assays of human SH-SY5Y and HEK-293 cells transfected with pGL4-hLIMK1-3U or pGL4-hLIMK1-3U-M. The firefly luciferase activity of the reporter constructs in transfected cells was normalized to the *Renilla* luciferase activity of the control construct (pRL-TK). The relative activity in both cell lines transfected with pGL4-hLIMK1-3U was then normalized as 100%. Significant differences between the cells transfected with pGL4-hLIMK1-3U and pGL4-hLIMK1-3U-M were calculated using ANOVA and Tukey's *t* test ($^{*}P$ <0.001, n = 10).

internal control reporter. Human SH-SY5Y cells (a neuronal cell line) and human HEK-293 cells (a non-neuronal cell line) were used to test whether the role of the conserved region is neuron-specific. The relative activity (Luc2/*Renilla*) of the wild-type vector pGL4-hLIMK1-3U in each cell line was normalized to 100%. The luciferase assay showed that the relative luciferase activity (Luc2/*Renilla*) of the mutated construct pGL4-hLIMK1-3U-M in both SH-SY5Y and HEK-293 cells was only ~60% of that of the wild-type construct pGL4-hLIMK1-3U (Fig. 4), showing that the conserved region increased the reporter gene expression.

We then assessed the mRNA levels of the reporter gene in the transfected cells using real-time quantitative RT-PCR. The relative expression levels (*Luc2/β-actin*) of the two cell lines transfected with pGL4-hLIMK1-3U were normalized to 1.0. The result showed that, after 24-h transfection, the relative *Luc2* mRNA levels (*Luc2*/*β-actin*) in SH-SY5Y and HEK293 cells transfected with pGL4-hLIMK1-3U-M both decreased to ~50% of that of the cells transfected with pGL4-hLIMK1-3U (Fig. 5), indicating that the conserved region plays a role in maintaining the mRNA level of the reporter gene.

DISCUSSION

The 3′ UTR of eukaryotic transcripts has diverse functions[11,20]. A recent review has summarized that many regulatory elements in the 3′ UTR play critical roles in the post-transcriptional regulation of protein expression in mammalian cells[21]. An important way to identify regulatory elements in the 3′ UTRs of protein-coding mRNAs is to analyze the evolutionarily-conserved sequences using cross-species comparison[22,23]. In the present study, we found a highly-conserved region (~80 bp) in the 3′ UTR of *LIMK1* using cross-species sequence comparison, implying its important role in regulating gene expression. We also found >10 highly-conserved regions with lengths ranging

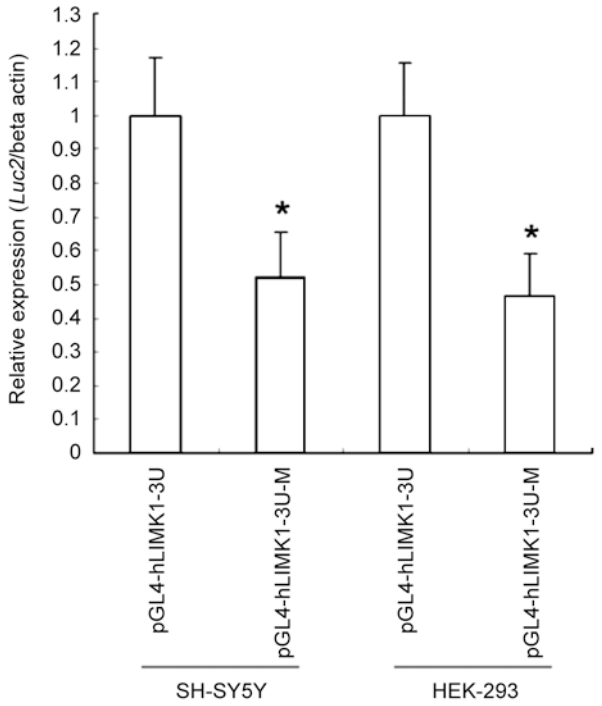

Fig. 5. Real-time PCR analysis of the reporter gene *Luc2* transcript levels in SH-SY5Y cells and HEK-293 cells transfected with pGL4-hLIMK1-3U or pGL4-hLIMK1-3U-M. The expression levels were normalized to the endogenous control housekeeping gene *β-actin* and the relative expression levels of pGL4-hLIMK1-3U in both cell lines were normalized to 1 (mean values with SD). Significant differences were determined with the Mann-Whitney U test between the cells transfected with pGL4-hLIMK1-3U and cells with pGL4-hLIMK1-3U-M (*P <0.001, n = 12).

from 10 to 20 bp (data not shown), which may be essential for the function of the 3′ UTR, since the microRNA miR-134 is located within one of these short conserved regions[16].

In this study, we demonstrated that the conserved region increased reporter gene expression in both neuronal SH-SY5Y cells and non-neuronal HEK-293 cells, suggesting that this region is essential for *LIMK1* expression at the post-transcriptional level. Using real-time quantitative RT-PCR, we also found that the mRNA levels of the reporter gene decreased significantly after the conserved region was deleted from the *LIMK1* 3′ UTR. This decrease may not occur at the transcriptional level because both the wild-type and the mutant constructs had an SV40 promoter (Fig. 2). By RNA secondary structure prediction, we found that the conserved region formed a loop-stem structure (Fig. 3). Since it has been shown that an extended stem-loop RNA structure in the 3′-UTR of the *BDNF* gene plays a role in mRNA stability[24], we speculate that the conserved region might affect mRNA maturation or mRNA degradation at the post-transcriptional level. Other studies have shown that conserved *cis*-acting elements in the 3′ UTR are sufficient for the regulation of RNA stability and are involved in diverse biological roles *in vivo*[25-27]. Therefore, we propose that the conserved region in *LIMK1* 3′ UTR also plays a critical role in regulating *LIMK1* expression in the nervous system. Future studies are needed to reveal the regulatory mechanisms of the conserved region and its biological functions.

The accurate expression of LIMK1 protein is essential for neuronal development and brain function[3,16]; its abnormal expression is associated with several neurological disorders[6,7,9]. However, little is known about the relationship between *LIMK1* 3′ UTR mutation and neurological diseases, although it has been demonstrated that mutations in the 3′ UTR elements influence the expression of specific genes at the level of translation, resulting in abnormal development and various diseases such as congenital heart disease, arrhythmogenic right ventricular cardiomyopathy type1, and IPEX (immune dysfunction, polyendocrinopathy and enteropathy, X-linked)[11,12,15]. The present study showed that the conserved region regulates reporter gene expression, suggesting that this region should be considered as a target for screening of mutations that may be associated with these diseases. Therefore, our findings will be helpful for further exploring the relationship between *LIMK1* 3′ UTR mutation and neurological diseases and the regulatory mechanisms of *LIMK1* expression in the normal brain.

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