



doi:10.3969/j.issn.1673-5374.2013.03.008 [http://www.nrronline.org; http://www.sjzsyj.org]

Zhang Y, Zhao S, Dong WR, He SF, Wang HH, Zhang LH, Tang YJ, Guo JS, Guo SQ. Cloning and bioinformatical analysis of the N-terminus of the sonic hedgehog gene. *Neural Regen Res.* 2013;8(3):258-263.

Cloning and bioinformatical analysis of the N-terminus of the sonic hedgehog gene[☆]

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Abstract

The sonic hedgehog protein not only plays a key role in early embryonic development, but also has essential effects on the adult nervous system, including neural stem cell proliferation, differentiation, migration and neuronal axon guidance. The N-terminal fragment of sonic hedgehog is the key functional element in this process. Therefore, this study aimed to clone and analyze the N-terminal fragment of the sonic hedgehog gene. Total RNA was extracted from the notochord of a Sprague-Dawley rat at embryonic day 9 and the N-terminal fragment of sonic hedgehog was amplified by nested reverse transcription-PCR. The N-terminal fragment of the sonic hedgehog gene was successfully cloned. The secondary and tertiary structures of the N-terminal fragment of the sonic hedgehog protein were predicted using Jpred and Phyre online.

Key Words

neural regeneration; basic research; sonic hedgehog protein; cloning; nested reverse transcription-PCR; secondary structure; tertiary structure; central nervous system; developmental neurobiology; rats; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) We screened out the amino acid coding region of the sonic hedgehog gene according to the sequences deposited in GenBank, and determined the sequences of the N- and C-terminal products of sonic hedgehog.

(2) The present study focused on the N-terminal product of sonic hedgehog, rather than the C-terminal product.

(3) The sequence of the N-terminal product of sonic hedgehog was cloned and its protein structure was predicted.

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Received: 2012-07-25

Accepted: 2012-10-26

(N20120315003/WJ)

INTRODUCTION

Sonic hedgehog is one of three proteins in the mammalian hedgehog signaling pathway family, the others being desert hedgehog and Indian hedgehog^[1]. It plays a

key role in regulating vertebrate organogenesis, such as the growth of digits^[2] and the organization of the brain^[3]. It exerts different effects on the cells of the developing embryo^[4], but also remains important in adults. It has been demonstrated that sonic hedgehog can be

identified in the subventricular zone *in vivo*. Blocking sonic hedgehog pathways in both adult and perinatal mice leads to reduced expression of Gli1 and reduced neurogenesis in subventricular zone^[5]. Sonic hedgehog is a chemoattractant for midbrain dopaminergic axons^[6]. It is involved in determining the structural diversity of dopaminergic projections, ensuring that midbrain dopaminergic axons project from the substantia nigra and the ventral tegmental area to rostral target tissues, including the striatum, pallidum, and hypothalamus. Additionally, preconditioning of cortical neurons with the N-terminal fragment of sonic hedgehog is sufficient to confer resistance^[7]. Similarly, sonic hedgehog may play an important role in injured motor neurons through the induction of brain-derived neurotrophic factor in crush-injured adult rat sciatic nerve^[8]. Sonic hedgehog, a large precursor protein, undergoes autoprolysis *via* an enzymatic activity contained at its C-terminal end, leading to an N-terminal product which is responsible for the biological activity of the whole protein^[9-10]. However, there is no functional evidence to indicate what role(s) the over-expressed N-terminal fragment of sonic hedgehog has in neurodegenerative disease.

We hypothesize that the over-expressed functional N-terminal fragment of sonic hedgehog may play a key role in neurodegenerative disease and stimulate neural regeneration. To further analyze the potential roles of the over-expressed N-terminal fragment of sonic hedgehog in the adult central nervous system and the mechanisms involved in the pathogenesis of neurodegenerative disease, we have successfully extracted total RNA from the notochord of a Sprague-Dawley rat embryo at embryonic day 9 and amplified the N-terminal product of sonic hedgehog by nested reverse transcription-PCR. It is important to analyze the amino acid sequence of the N-terminal fragment of sonic hedgehog and to define its functional domains by analyzing its secondary and tertiary structures to evaluate its biological activity and its possible therapeutic applications in neurodegenerative diseases, including Parkinson's disease.

RESULTS

Identification of the extracted total RNA

The purity of the extracted total RNA was examined spectrophotometrically, by monitoring the absorbance at 260 nm and 280 nm to estimate the amount of RNA (ng/ μ L) and the levels of protein and salt in the samples. The concentration of total RNA was 762.75 ng/ μ L; $A_{260\text{ nm}}=1.91$,

$A_{280\text{ nm}}=0.92$, $A_{260\text{ nm}}/A_{280\text{ nm}}=2.08$. We then examined the presence of ribosomal RNA subunits by gel electrophoresis (Figure 1), as an indicator of the intactness of the RNA product. Following the extraction procedure, we were able to detect the 28S and 18S ribosomal subunits as sharp bands on the gel (Figure 1), indicating that there was little or no degradation of the extracted RNA. The total RNA had high yield, purity, and integrity.

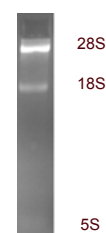


Figure 1 Electrophoresis of total RNA from the notochord of a Sprague-Dawley rat at embryonic day 9.

The gel shows one 28S band and one 18S band, indicating two ribosomal subunits.

The N-terminal fragment of sonic hedgehog was amplified by nested reverse transcription-PCR

According to the sequence of rat sonic hedgehog published in Genbank, we designed two pairs of nested PCR primers for the N-terminal fragment of sonic hedgehog and amplified the N-terminal fragment of sonic hedgehog by nested reverse transcription-PCR. The PCR product was 525 bp long (Figure 2), consistent with its theoretical size.

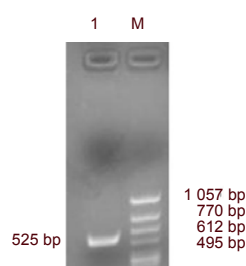


Figure 2 Amplification of the target gene, the N-terminal fragment of sonic hedgehog (525 bp), by nested reverse transcription-PCR.

The PCR product (1) was about 525 bp long, consistent with its theoretical size. M: Marker.

Sonic hedgehog gene sequence analysis

The overall length of sonic hedgehog is 1 715 bp, and the sequence coding for amino acids in the sonic hedgehog protein spans from 315–1 628 bp (1 314 bp), encoding 437aa amino acid residues. The amino acids from 25–198 constitute the N-terminal product of the sonic hedgehog protein (Figure 3), while the amino acids from 199–437 constitute the C-terminal product of the sonic hedgehog protein.

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ORIGIN
1  cspgrgfgkr qhpkkltpla ykqfipvae ktlgasrye skitrnsrf
keltprnypd
61  iifkdeentg adrlmtqrck dklنالaisv mraqpvgkrlr vtegwedgh
hseeslhveg
121  ravdittsdr drskygmlar laveagfdwv yyeskarihc svkaensvaa ksdg
    
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Figure 3 Amino acid sequence of the N-terminal fragment of sonic hedgehog (NCBI).

Prediction of the secondary structure of N-terminal fragment of sonic hedgehog

The secondary structure of the N-terminal fragment of sonic hedgehog was predicted by the online tool Jpred (UniProt accession number: Q62226; PDB ID: 2wg4; Figure 4).

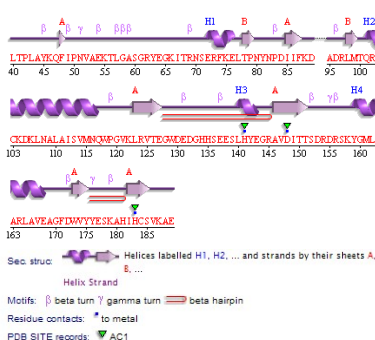


Figure 4 Secondary structure analysis of the N-terminal fragment of sonic hedgehog (Jpred).

Prediction of the tertiary structure of N-terminal fragment of sonic hedgehog

The secondary and tertiary structures of the N-terminal fragment of sonic hedgehog were predicted by the online tool Phyre (SCOP Code: 1vhha; Job Code: dd7001b6723f72e7; Figures 5, 6).

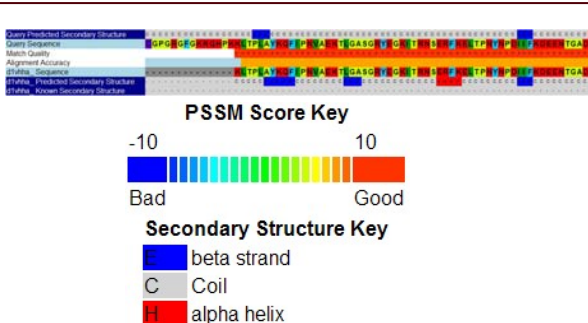


Figure 5 Secondary structure analysis of the N-terminal fragment of sonic hedgehog (Phyre).

variety of tissues and organs^[4], such as the limbs^[11], midline structures in the brain^[12], the spinal cord^[13], the thalamus^[14], and the teeth^[15]. Sonic hedgehog is produced by the notochord and the floor plate^[16] and is responsible for inducing ventral neural cell types and regulating neural stem cell proliferation and development in a concentration-dependent manner^[17-18]. The hedgehog signaling pathway plays a crucial role in several developmental processes^[19]. Sonic hedgehog displays inductive, proliferative, neurotrophic and neuroprotective activities on various neural cells and signals through a receptor complex comprising Patched and Smoothened^[20-22]. Although Wnt1, Otx2, Lmx1a, Msx1, Nkx2-2 and Nkx6-1 all participate in the development of midbrain dopaminergic neurons, sonic hedgehog is essential in this process^[23]. However, there is no evidence to show what the functions of sonic hedgehog are in the adult central nervous system.

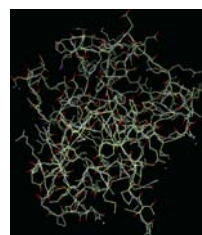


Figure 6 Tertiary structure analysis of the N-terminal fragment of sonic hedgehog (Phyre).

Moreover, many studies have shown the effects of the sonic hedgehog protein *in vitro*. It plays a crucial role in dopaminergic neuron specialization by promoting the differentiation of embryonic stem cells into dopaminergic neurons^[24-26]. Sonic hedgehog-knockout mice lack expression of dopaminergic neurons^[27]. In addition, sonic hedgehog also promoted maturation and survival of dopaminergic neurons, displaying a neuroprotective effect on dopaminergic neurons^[28]. Here, we have cloned the N-terminal product of the sonic hedgehog gene. This is the first key step to over-expressing a functional N-terminal fragment of sonic hedgehog protein in the adult central nervous system.

Synthesized as a large precursor protein, sonic hedgehog undergoes autoprocessing to generate an amino-terminal domain that appears to be responsible for both local and long-range signaling activities, and a carboxy-terminal domain that contains the autoprocessing activity. The N-terminal fragment of sonic hedgehog is further modified by fatty acids which appear crucial for its biological functions^[29], such as its morphogenetic activity and the patterning of the central nervous system. Detailed

DISCUSSION

Sonic hedgehog belongs to a family of secreted polypeptides involved in embryonic development. It plays an important role in a wide variety of regulatory functions during the development of vertebrate and invertebrate organisms, regulating morphogenesis of a

information concerning the higher order structure and functional domains of the N-terminal product is unavailable. Therefore, exact cloning of the N-terminal fragment of sonic hedgehog and its over-expression in a target area might be a new way to induce neurogenesis for the treatment of neurodegenerative diseases such as Parkinson's disease. Bioinformatical analysis of the N-terminal fragment of sonic hedgehog may provide a better understanding of its functions. So far, the current methods of clinical treatment for Parkinson's disease are satisfactory. Although cell replacement therapy is a potentially feasible strategy, it has met with such problems as uncertain survival, poor sources, poor functional efficacy and ethical issues^[30].

The N-terminal fragment of sonic hedgehog plays a key role in the development of the central nervous system. Thus, cell replacement therapy modified with the N-terminal fragment of sonic hedgehog may provide a new way to tackle neurodegenerative disease. We thus focused on the N-terminal fragment of sonic hedgehog in these experiments.

In this study, we successfully cloned the N-terminal product of sonic hedgehog, analyzed its nucleotide and amino acid sequences and its functional domains, and predicted its secondary and tertiary structural features with Jpred and Phyre. Primer design is the first key step in this procedure. The primers must have few hairpins and other secondary structures. We must also exactly analyze the N-terminal domain in the cDNA of sonic hedgehog and find restriction sites compatible with the multiple cloning site of the lentiviral vector into which we are cloning the fragment.

There were two appropriate restriction enzyme sites, *EcoRI* and *NotI*. On the Jpred website, the title of the N-terminal product of sonic hedgehog (2wg4) is "crystal structure of the complex between human hedgehog-interacting protein hip and sonic hedgehog without calcium." The fragment analyzed was the N-terminal signaling domain of sonic hedgehog, residues 40–191.

In summary, we successfully extracted total RNA from Sprague-Dawley embryonic rat and amplified the N-terminal fragment of sonic hedgehog by nested reverse transcription-PCR, which is useful for our further research concerning sonic hedgehog N-terminal fragment of over-expression, the analysis of its biological functions, and the exploration of specific mechanisms of the fragment in neurodegenerative diseases.

MATERIALS AND METHODS

Design

A molecular biology experiment.

Time and setting

Experiments were performed from September 2010 to December 2010 in the Department of Histology and Embryology, Southern Medical University, China.

Materials

A pathogen-free pregnant Sprague-Dawley rat was provided by the Animal Experimental Center of Southern Medical University (Guangzhou, China; license No. SCXK (Yue) 2006-0015). The animal was housed at 22 ± 3°C, with 55 ± 3% relative humidity and a 12-hour light/dark cycle, allowing free access to food and water. Animal procedures were in strict accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[31].

Methods

Design of nested reverse transcription-PCR primers

Two pairs of nested PCR primers for the N-terminal product of sonic hedgehog were designed using Primer Premier Software (Version 5.0, Canada), using the sequence of rat sonic hedgehog published in GenBank (NM_017221.1) as reference. Primer pairs were BLASTed (NCBI) to avoid annealing to non-specific sequences during amplification. PCR products using these primers were expected to be 525 bp long. Primers were diluted to a working concentration of 10 μM. Primer synthesis was conducted by Invitrogen Corporation (Shanghai, China). The primers used are as follows:

Gene	Sequence (5'–3')
First pair	Forward: TTC TGG TGG CCC TTG CTT Reverse: GAA GGT GAG GAA GTC GCT GT
Second pair	Forward: <u>CG GAATT</u> C ATG GCC TGT GGG CCC Reverse: <u>ATT GCGGCCGC</u> GCC GTC AGA TTT
β-actin	Forward: ACC ATG GAT GAT GAT ATC GCC Reverse: GTG CCA GAT TTT CTC CAT GTC

Underline: incision enzyme site (*EcoRI* and *NotI*).

Extraction and reverse transcription of total RNA

A pregnant Sprague-Dawley rat was anesthetized with 10% chloral hydrate (Shanghai Aladdin Reagent Co., Ltd., China); 0.64 g of notochord was excised from fetuses using Rnase-free materials and reagents. Total

RNA was isolated from the notochord using an RNAiso Plus kit (Takara, Guangzhou, Guangdong Province, China) according to the manufacturer's instructions. The RNA was run out on a 1% agarose gel to determine the quality of extraction. RNA yields were measured at a wavelength of 260 nm on a Bio-Rad Smartspect 3000 spectrophotometer (Bio-rad, Guangzhou, Guangdong Province, China). The concentration of total RNA in solution was then calculated using the formula: absorbance units \times 71/40 = μ g of RNA.

The cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Guangzhou, Guangdong Province, China), 5 μ L of RNA were heated at 65°C for 5 minutes with 1 μ L of OligodT primer (Shang Aladdin Reagent Co. Ltd., China) and this mixture was placed on ice for 3 minutes after heating. 2 μ L of dNTP mix (dATP, dCTP, dGTP, dTTP), 4 μ L of 5 \times reaction buffer, 1 μ L of RiboLock™ RNase Inhibitor and 1 μ L of RevertAid™ Reverse Transcriptase were added to the solution. This mix was heated at 42°C for 60 minutes. The reaction was terminated by heating at 70°C for 5 minutes, and the products were diluted 10-fold in RNase-free water and stored at -70°C.

cDNA amplification by nested PCR

PCR was performed using primers for β -actin or rat sonic hedgehog to amplify the cDNA with DreamTaq™ Green PCR Master Mix (Fermentas, Guangzhou, Guangdong Province, China). Reagents were added on ice into 200 μ L thin PCR tubes. The final reaction mix consisted of 2 μ L cDNA, 25 μ L Dream Taq Green PCR master mix, 1.5 μ L forward and reverse primers, and was made up to 50 μ L with PCR quality water. The reaction tubes were then placed into a thermal light-cycler (Biometra, Guangzhou, Guangdong Province, China) which was programmed for the first round as follows: 95°C for 3 minutes, then 25 cycles at 95°C for 30 seconds, 50.2°C for 1 minute, and 72°C for 1 minute. After the cycles were completed, the reaction was left at 72°C for 15 minutes before being cooled to 4°C. The PCR product from this first round was diluted 5-fold in ddH₂O. The second reaction mix consisted of 2 μ L diluted PCR product from the first round, 25 μ L Dream Taq Green PCR master mix, 1.5 μ L forward and reverse primers, and was made up to 50 μ L with PCR quality water. The second round was programmed as follows: 95°C for 3 minutes, then 35 cycles at 95°C for 30 seconds, 69.8°C for 1 minute, and 72°C for 1 minute. After the cycles were completed, the reaction was left at 72°C for 15 minutes before being cooled to 4°C. Products were run out on a 1% agarose gel to determine size and specificity.

Purification of the PCR product

The PCR product from the second round was purified using a GeneJET PCR purification kit (Fermentas, Guangzhou, Guangdong Province, China). First, the binding buffer (1:1) was added to the second round PCR mix, and mixed thoroughly. Then we transferred this mix solution to the GeneJET™ purification column and centrifuged the column at 13 000 \times g for 60 seconds before discarding the flow-through. We then added 700 μ L of wash buffer to the GeneJET™ purification column and centrifuged the column at 13 000 \times g for 60 seconds. After the flow-through was discarded, the purification column was placed back into the collection tube. The empty GeneJET™ purification column was centrifuged for an additional 1 minute to completely remove any residual wash buffer. Then we transferred the GeneJET™ purification column to a clean 1.5 mL microcentrifuge tube, after which 50 μ L of elution buffer was added to the center of the GeneJET™ purification column membrane and centrifuged for 1 minute. Finally, we discarded the GeneJET™ purification column and stored the purified DNA at -20°C.

Bioinformatical analysis of the N-terminal product of Sonic hedgehog with online tools

The nucleotide and amino acid sequences of the N-terminal product of sonic hedgehog were analyzed as previously described^[32]. The secondary structure of the N-terminal fragment of sonic hedgehog was predicted with the Jpred online tool (<http://www.compbio.dundee.ac.uk/www-jpred/>), and the tertiary structure of the N-terminal fragment of sonic hedgehog was predicted with Phyre (<http://www.sbg.bio.ic.ac.uk/~phyre/>).

Funding: This study was financially sponsored by the Guangdong Provincial Natural Science Foundation, No. S2012010009592. the Science and Technology Talent Foundation of Guangdong Provincial Natural Science Foundation, No. 30900725; the Joint Research Program by Southern Medical University-Shunde Guizhou Hospital, No. 09000608; the Science Foshan Municipal Key Project in Medical Sciences, No. 201008063; and the Shunde Medical Research Program, No. 2011050.

Author contributions: Yi Zhang was responsible for the whole experiment and wrote the final version of the manuscript. Weiren Dong and Suifen He were responsible for the design and funding. Shu Zhao was responsible for animal feeding. Lihua Zhang, Suifen He, Yinjuan Tang, Suiqun Guo, Haihong Wang and Jiasong Guo provided information and technical support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by Experimental Animal Ethics Committee of Southern Medical University, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Edited by Hao YL, Guo JS/Yang Y/Song LP)