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*Original Research*

# Molecular Characterization and Function of the Nogo-66 Receptor (*NgR1*) Gene in the Chinese Tree Shrew

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**Abstract**

**Background**: Nogo-66 receptor (NgR1) is a glycosylphosphatidylinositol-linked cell surface receptor with high affinity for Nogo-66. The binding of Nogo-66 to NgR1 plays a key role in inhibiting neurite growth, limiting synaptic plasticity and mediating Mammalian Reovirus (MRV) infection. The Chinese tree shrew (*Tupaia belangeri chinensis*) is, a new and valuable experimental animal that is widely used in biomedical research. Although susceptible to MRV, little is known about tree shrew NgR1 and its role in MRV infection. **Methods**: In this study, we cloned *NgR1* form the Chinese tree shrew by RACE technology and analyzed its characteristics, spatial structure and its tissue expression. We also examined the expression pattern of NgR1 in the response of tree shrew primary nerve cells (tNC) to MRV1/TS/2011 infection. **Results**: Tree shrew *NgR1* was found to have a closer relationship to human NgR1 (90.34%) than to mouse *NgR1*. Similar to the protein structure of human *NgR1*, the tree shrew NgR1 has the same leucine-rich repeat (LRR) domain structure that is capped by C-terminal and N-terminal cysteine-rich modules. The tree shrew *NgR1* mRNAs were predominantly detected in the central nervous system (CNS), and tree shrew *NgR1* can mediate infection by MRV1/TS/2011. **Conclusions**: Taken together, these results help to elucidate the function of *NgR1* and provide a basis for using the tree shrew as an animal model for studies of the nervous system and infectious diseases.

**Keywords:** Nogo-66 receptor; tree shrew; Mammalian Reovirus; phylogenetic analysis; molecular characterization

## 1. Introduction

Nogo is a myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein. It belongs to the reticulon (RTN) protein family and has three different subtypes: Nogo-A, Nogo-B and Nogo-C. These molecules are thought to inhibit neuronal regeneration in the central nervous system (CNS) through binding to Nogo-66 receptor (NgR1) [1–4]. NgR1 is a leucine-rich repetitive protein encoded by the *RTN4R* gene and is mainly expressed in the body of nerve cells and muscle *in vivo* [5–8]. It has a high affinity for Nogo-66, which is the main inhibitory region of Nogo-A [6,9,10]. Nogo-A is mostly expressed by myelinated oligodendrocytes in the CNS [11]. Nogo-66 binding to NgR1 plays a key role in inhibiting neurite growth and in limiting synaptic plasticity [6,12,13]. Other studies have suggested that Nogo and its receptor are associated not only with neurite regeneration but also with neurodegenerative disorders, such as Alzheimer's disease (AD) [10,14,15]. A study by Dermody [7] first identified NgR1 as a neural receptor for Mammalian Reovirus (MRV) that mediated infection by this virus.

So far, few animals that have proved suitable as models for CNS virus infection. The main MRV animal model currently in use is the mouse. Although this model provides a pathological basis for neurotropic virus nerve transmission, the molecular mechanism of nerve trafficking by the virus remain unclear. The Chinese tree shrew (*Tupaia belangeri chinensis*) is a new and valuable experimental animal model due to its low maintenance cost and short breeding cycle. It has a high brain-to-body mass ratio [16] and is used to study human conditions and diseases such as brain development and aging [17–19], depression [20], social stress [21], AD [16,22–24], Parkinson's disease (PD) [25], and MRV infection [26,27]. We previously isolated two new MRV strains from Chinese tree shrews and found these animals were susceptible to MRV infection [27]. The Chinese tree shrew is therefore a good animal model to study viral infection of CNS system. However, the mechanism of MRV infection is still unknown, while the molecular characterization and function of NgR1, one of the receptors for MRV infection, remains unclear.

To date, *NgR1* from the human, chick, mouse and fish have been cloned [8,28]. However, little is known about the structure and function of tree shrew *NgR1*. In the present study, we determined the sequence of tree shrew *NgR1* by RACE technology, described its biochemical characteriza-

tion, predicted its spatial structure, and examined its expression pattern in different tissues. We also studied the function of *NgR1* in tree shrew primary neuronal cell (tNC) in response to MRV1/TS/2011 infection. Our results indicate that *NgR1* has a closer relationship to human *NgR1* than to mouse *NgR1*, and that *NgR1* can mediate infection by MRV1/TS/2011.

## 2. Materials and Methods

### *2.1 Animals and Tissues from Tree Shrew*

Chinese tree shrews (1–3 days old newborns and 1–2 years old adults) were raised at the Center for Tree Shrew Germplasm Resources, Institute of Medical Biology, Chinese Academy of Medical Science and Peking Union Medical College, Kunming, China. After euthanasia with an overdose of pentobarbital sodium, 26 different tissues (brain, tongue, esophagus, heart, lung, liver, gallbladder, stomach, spleen, duodenum, jejunum, ileum, caecum, colon, rectum, kidney, bladder, testis, parietal lobe, cerebellum, temporal lobe, hippocampus, frontal lobe, optic chiasma, occipital lobe and blood) were dissected immediately, quickly frozen in liquid nitrogen and stored at –80 °C. This research project was approved by the institutional ethics committee and all procedures were conducted in accordance with ethical standards and practices.

### *2.2 Cloning of NgR1*

Primers were designed according to the predicted *Tupaia chinensis* reticulon 4-receptor (RTN4R) registered in GenBank (**Supplementary Table 1**). The detailed protocol can be found in our previous reports [16]. Briefly, RNA was extracted from brain tissue and the 5′UTR and 3′UTR were amplified using 5'/3'RACE specific primers (**Supplementary Table 1**) based on the manufacturer's instructions. The purified PCR product was cloned into pMD18-T vector and sequenced by Sangon Biotech (Shanghai) Co., Ltd. Sequences of *NgR1* were deposited in the NCBI (Accession Number: MW032435).

### *2.3 Sequence Alignments and Phylogenetic Analyses*

The MEGA 7.0 program (Mega Limited, Auckland, New Zealand) was used to analyze the homology of *NgR1* in tree shrew, human and several other common experimental animals (8 species). The *NgR1* phylogenetic tree was constructed using MEGA 7.0 (Mega Limited, Auckland, New Zealand), with Danio rerio used as the outgroup. The accuracies and statistical tests tree branch position were tested using the bootstrap method with 1000 replications.

### *2.4 Three Dimensional (3D) Structure of NgR1*

The predicted three-dimensional structure of NgR1 was constructed using SWISS-MODEL integrated with the visualization program PyMOL2.2.0 (DeLano Scientific, San Carlos, CA, USA). The full-length protein sequence was determined using MEGA7.0 (Mega Limited, Auckland, New Zealand). Tree shrew NgR1 protein sequence was imported into the SWISS-MODEL online analysis platform (https://swissmodel.expasy.org/) to generate a protein model PDB format file, with PyMOL used to visualize the 3D model. Search and download of the human NgR1 protein structure (Q9BZR6) template was performed using UniProt (https://www.uniprot.org/). The three-dimensional structures for tree shrew and human NgR1 were superimposed using PyMOL/align.

### *2.5 Quantitative Real-Time PCR*

Total RNA was prepared using MRC RNAzol (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription quantitative real-time PCR (RT-qPCR) was performed with gene specific primers (**Supplementary Table 2**) as reported previously [16] and using ts*GAPDH* as an internal control. Relative *NgR1* gene expression was calculated using the double-standard curve method.

### *2.6 Virus and Cells*

The preparation and culture of tree shrew primary neuronal cells (tNC) was described in our previous study [29]. Briefly, brain tissue was washed twice with pre-cooled PBS, cut into 1 mm blocks using micro-scissors and then digested with 0.25% trypsin (Gibco, Carlsbad, CA, USA) for 20 min. The digestion was stopped with DMEM /High Glucose (Hyclone, Logan, UT, USA) +5% FBS (Hyclone, Logan, UT, USA), and the cells centrifuged at 2000 g for 10 min. tNCs were resuspended and cultured at a density of 5 $\times$ $10^5$ cells/ well in Neurobasal medium (Gibco, Carlsbad, CA, USA) supplemented with B27 (1:50) (Gibco, Carlsbad, CA, USA), 2 mM glutamine (Gibco, Carlsbad, CA, USA) and, 1% penicillin /streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in 5% $CO_2$.

Tree shrew-derived Mammalian Reovirus (MRV1/TS/2011, $10^{6.5}$ TCID50/mL) was isolated as described previously [27] and stored in our department.

### *2.7 Virus Infection and Antibody Blocking*

The primary neuronal cells derived from tree shrew (tNC) were divided into 8 groups according to the treatment used: in group A, only PBS was added; group B, Neuraminidase (NA); group C, Phosphatidylinositol specific phospholipase C (PI-PLC, 50 mU/mL); group D, PI-PLC (100 mU/mL) and NA (50 mU/mL); group E, Anti-Nogo Receptor antibody (ab32890, 50 $\mu$g/mL); group F, Anti-Nogo Receptor antibody (50 $\mu$g/mL) and NA (NA, 50 mU/mL), group G, NEP1-40 (50 $\mu$g/mL), group H, NEP1-40 (50 $\mu$g/mL) and NA (50 $\mu$g/mL). All cells groups were cultured at 37 °C for 1 h and then washed to remove the reagents. The cells were then infected with MRV1/TS/2011 ($10^{6.5}$ TCID50/mL) for 1 h at 4 °C, with gentle shaking once every 15 min. The virus was then washed away with virus adsorption solution and cell culture continued at 37 °C

for 24 h. Image Pro Plus software 6.0 (Media Cybernetics, Bethesda, MD, USA) was used to evaluate the proportion of red fluorescence positive cells among the total number of cells and a histogram of these results was produced with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

### *2.8 Immunofluorescence Microscopy*

Twenty-four hours post infection, tNC cells were fixed with 1 mL of 4% paraformaldehyde for 20 minutes and the antiviral antigen $\sigma$1 (Reovirus Antibody clone YrreoV: GenWay, San Diego, CA, USA, 1:100) was added and incubated overnight at 4 °C. The second antibody Goat Anti Mouse IgG - H&L (Cy3) (KPL, Gaithersburg, MD, USA, 1:200) was added and incubated for 1 h at 37 °C. The virus antigen $\sigma$1 was then visualized using immunofluorescence.

### *2.9 Statistical Analysis*

One-way ANOVA was used to analyze for differences between groups. A significant difference was scored as **** $p < 0.0001$; *** $p < 0.001$; * $p < 0.05$.

## 3. Results

### *3.1 Identification and Molecular Evolution of NgR1 in the Chinese Tree Shrew*

The *NgR1* gene sequences were comprised of 3128 bp cDNA and coded for 731 amino acids (**Supplementary Fig. 1**). Multiple sequence alignment (MSA) revealed that *NgR1* was more homologous to human (90.34%) and Macaca fascicularis (89.91%) or Macaca mulatta (89.91%) than it was to mouse (89.06%) or rat (88.84%) *NgR1* (**Supplementary Fig. 2**). Evolutionary analysis revealed that *NgR1* was genetically closer to human (Homo sapien) and monkey (Macaca) than to mouse (Mus musculus) and rat (Rattus rattus), as illustrated in **Supplementary Fig. 3**.

The tree shrew and human protein sequences were 90.34% identical. This was higher than Macaca fascicularis (89.91%), Macaca mulatta (89.91%), mouse (89.06%), and rat (88.84%).

### *3.2 3D Structures of Tree Shrew NgR1*

The 3D structure of human and tree shrew NgR1 was created using the SWISS-MODEL integrated with visualization program PyMOL2.2.0 (DeLano Scientific, San Carlos, CA, USA). This was found to be similar between the two species. NgR1 has nine leucine-rich repeats (LRR), a C-terminal cap domain (LRRCT), and an N-terminal cap domain (LRRNT) (Fig. 1A,B). An alpha helix was present in the human LRRNT region and sixth LRR, but not in the tree shrew. The tree shrew NgR1 had an alpha helix at the LRRCT end, but not human NgR1 (Fig. 1C). These results show that tree shrew and human NgR1 have similar, but not identical structures (Fig. 1).

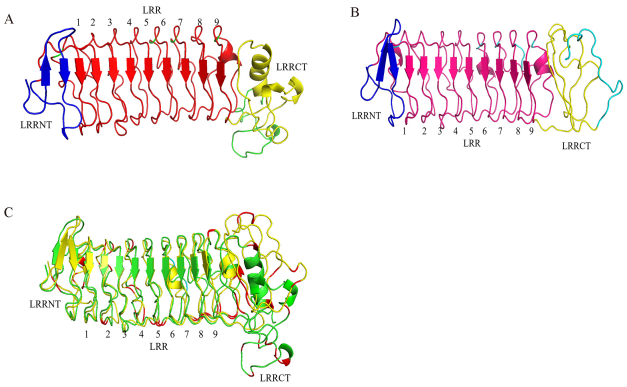


**Fig. 1. Predicted structure of NgR1 using the SWISS-MODEL integrated with visualization program PyMOL2.2.0.** (A) Tree shrew NgR1 protein. Specific domains include nine LRR (red), a LRRNT (blue) and a LRRCT (yellow). (B) Human NgR1 protein. Specific domains include nine LRR (purple), a LRRNT (blue) and a LRRCT (yellow). (C) The superimposed conformation of human (yellow) and tree shrew (green) NgR1, differential amino acids shown in red.

### *3.3 Expression Analysis of NgR1 mRNAs*

qPCR was used to measure the mRNAs expression in 26 tissues from the tree shrew in order to study the *NgR1* expression pattern. *NgR1* mRNAs were mainly distributed in the central nervous system, especially in the temporal lobe, frontal lobe and parietal lobe (Fig. 2). However, the *NgR1* mRNA level in the peripheral nervous system was relatively low. The tissue-specific expression pattern observed here for *NgR1* suggests that it may play a role in neurodegenerative diseases and in neurotropic virus infection.

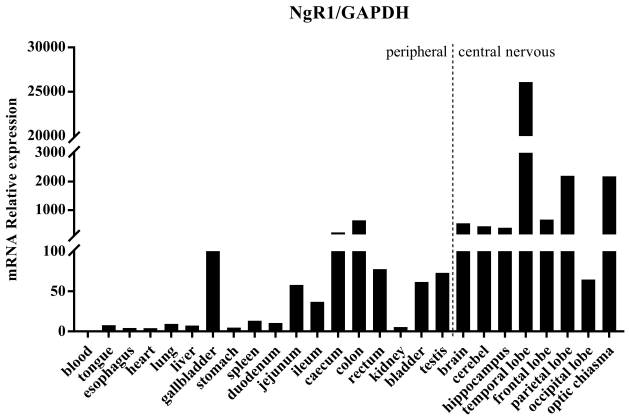


**Fig. 2. Expression pattern of *NgR1* in the tree shrew.** Gene-specific primers (**Supplementary Table 2**) were used to measure *NgR1* mRNA expression in 26 tissues from the tree shrews using RT-PCR, with the ts*GAPDH* gene used for normalization.

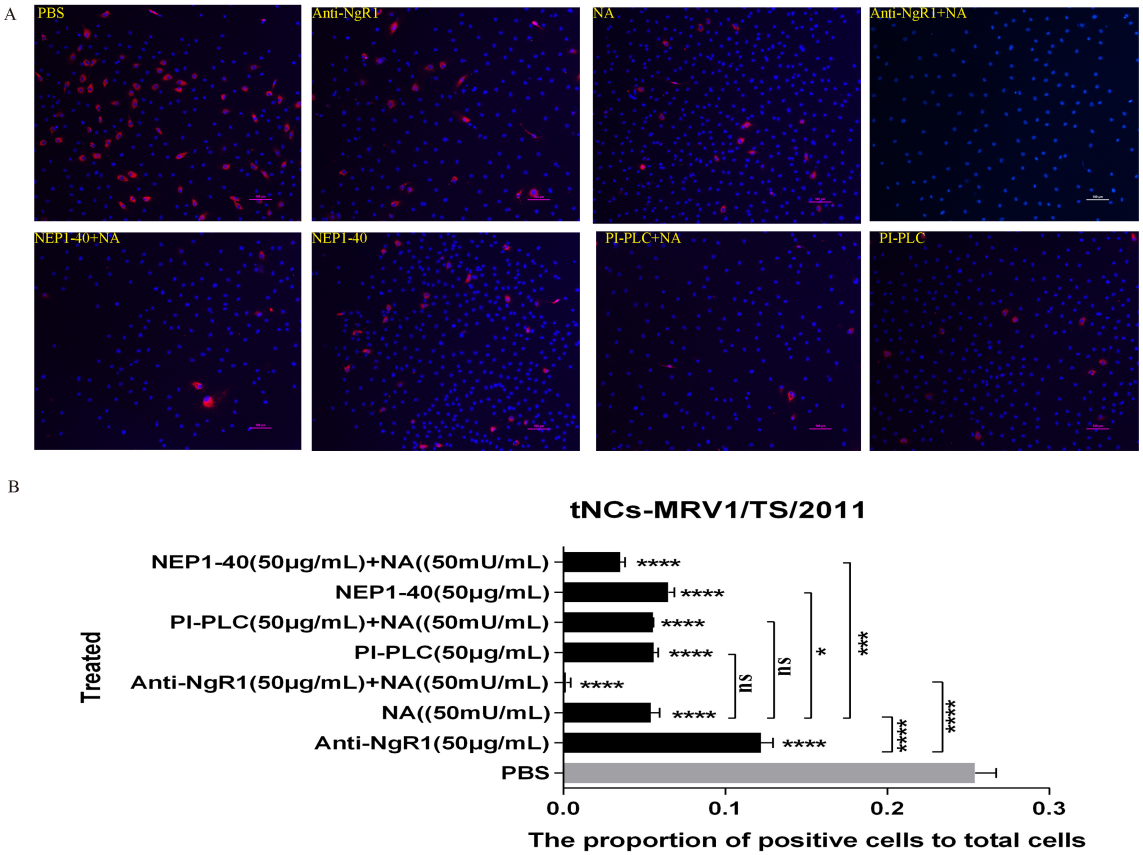


**Fig. 3. Infection blockade of MRV1/TS/2011 in tNC.** (A) The effect of different treatments on infection of tNC cells by MRV1/TS/2011. Infection was significantly reduced when anti-Nogo Receptor antibody (anti-NgR1) or Neuraminidase (NA) was added individually, and almost completely blocked, when anti-NgR1 and NA were added simultaneously. Infection was similarly reduced when Phosphatidylinositol specific phospholipase C (PI-PLC) or NEP1-40 was added. Simultaneous addition of PI-PLC and NA, or of NEP1-40 and NA, also reduced infection more than the single agent. (B) Image Pro Plus software 6.0 was used to evaluate the proportion of red fluorescence positive cells amongst the total number of cells shown in Fig. 3A. Significant differences were scored as: ****, $p < 0.0001$; ***, $p < 0.001$; *, $p < 0.05$.

### *3.4 The NgR1 Mediates MRV1/TS/2011 Infection in tNC*

To examine the function of NgR1 on infection by Mammalian Reovirus (MRV), anti-Nogo receptor antibody (anti-NgR1), Neuraminidase (NA), Phosphatidylinositol-specific phospholipase C (PI-PLC) and NEP1-40 were first used to block NgR1. NEP1-40 is an NgR1 antagonist peptide comprised of 40 amino acids from the N-terminal of Nogo-66. MRV1/TS/2011 was then incubated with tNC and the $\sigma$1 antigen of Reovirus was detected by immunofluorescence microscopy.

Simultaneous addition of anti-NgR1 and NA significantly decreased the MRV1/TS/2011 copy in tNC compared to the addition of anti-NgR1, NA, PI-PLC or NEP1-40 (Fig. 3). Furthermore, the addition of anti-NgR1, NA, NEP1-40 or PI-PLC significantly reduced the MRV1/TS/2011 copy in tNC compared to cells treated by PBS. Hence, these four NgR1 blockers used alone or in combination can reduce MRV1/TS/2011 copy in tNC compared to cells treated by PBS.

## 4. Discussion

Nogo is part of the reticulon protein family and consists of three subtypes: Nogo-A, Nogo-B and Nogo-C. Each subtype has a common 66 amino acid domain (Nogo-66) that can mediate an inhibitory effect on axon growth through the Nogo receptor (NgR) [1–3,30]. NgR is a leucine-rich repeat protein encoded by the *RTN4R* gene. *In vivo*, NgR is mainly expressed in muscle and neuronal cell bodies [5–8].

Here, we describe the Nogo-66 receptor (NgR1) in the Chinese tree shrew. The tree shrew *NgR1* is 3128 bp in length and the ORF sequence is 2193 bp, encoding 731 amino acids (**Supplementary Fig. 1**). In comparison, human *NgR1* consists of 473 amino acid residues [4] and zebrafish *NgR1* encodes 479 aa [28]. According to the multiple sequence alignment, tree shrew *NgR1* was more homologous to human (90.34%) than to mouse or rat (**Supplementary Fig. 2**). Previous reports showed that human homologous mouse *NgR1* gene cDNA had

89% amino acid homology [3]. Results from the phylogenetic tree showed that tree shrew *NgR1* was genetically closer to human and monkey *NgR1* than to rat and mouse (**Supplementary Fig. 3**). The NgR1 showed the same leucine-rich repeat (LRR) domain structure reported previously for human NgR1 [4,31]. It is also capped by C-terminal and N-terminal cysteine-rich modules termed LRRCT and LRRNT segments, respectively (Fig. 1). However, an alpha helix is present in the human NgR1 LRRNT region and in the sixth LRR, and whereas the alpha helix in tree shrew NgR1 is present in the LRRCT end (Fig. 1C). Furthermore, the amino acid length of *NgR1* is longer than human *NgR1*, while the 3D structure alignment starts from the 285th amino acid of tree shrew NgR1. Further studies are required to determine the possible impact of these differences on tree shrew *NgR1* founction.

Previous studies showed that NgR was predominantly expressed in the adult brain, eye and heart, with low expression level in the spinal cord and gill [28,32]. The expression pattern of NgR1 in the tree shrew is consistent with that reported in the human, mouse and zebrafish, with expression observed mainly in the CNS [3,32,33]. Therefore, the expression pattern of NgR1 in the tree shrew is similar to that of humans and other mammals, suggesting evolutionarily conserved functions.

In our previous studies we isolated two strains of MRV (MRV1/TS/2011 and MRV3/TS/2012) from the feces of tree shrews [27]. Other researchers have reported that cells expressing NgR1 can mediate Mammalian Reovirus binding and infection of non-susceptible cells. NgR1-specific antibody can effectively block the binding of Mammalian Reovirus to cells that expressed NgR1 [7]. In the present study, NgR1 blockers (anti-NgR1, NA, PI-PLC and NEP1-40), were used to inhibit the infection of reovirus in tree shrew primary neuronal cells (tNC). These four antagonists were observed to decrease MRV1/TS/2011 infectivity in tNC compared to cells treated with PBS. Furthermore, combined use of anti-NgR1 and NA significantly reduced MRV1/TS/2011 infectivity in tNC (Fig. 3). Our results indicate that NgR1 can mediate Mammalian Reovirus infection in tree shrew primary neuronal cells, but this can be blocked using NgR1 antagonists. These findings are consistent with those reported by Dermody [7].

## 5. Conclusions

In conclusion, we analyzed the structure and function of the *NgR1* gene in the Chinese tree shrew. Current knowledge regarding NgR1 is still limited and further studies are needed to investigate other functions of this gene. For example, nerve cells from the temporal and occipital lobes could be isolated and used for MRV1/TS/2011 infection and blocking experiments in order to investigate whether infectivity is related to the expression of NgR1 in different parts of the brain.

## Author Contributions

CXL, XYK and XFL designed the study, performed bioinformatics analysis, done the qRT-PCR experiments and cell culture and also drafted the manuscript; WGW, XMS, NL and PFT supplied the animals and collected the tissues; JJD designed the study and revised the manuscript. All authors have read and approved the manuscript.

## Ethics Approval and Consent to Participate

The research project was approved by the institutional Ethics Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences (DWSP201803034), and all the procedures were performed according to ethical standards and practices.


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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.jin2201022.

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