

Research Article

Role of Positive Selection in Functional Divergence of Mammalian Neuronal Apoptosis Inhibitor Proteins during Evolution

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Neuronal apoptosis inhibitor proteins (NAIPs) are members of Nod-like receptor (NLR) protein family. Recent research demonstrated that some *NAIP* genes were strongly associated with both innate immunity and many inflammatory diseases in humans. However, no similar phenomena have been reported in other mammals. Furthermore, some *NAIP* genes have undergone pseudogenization or have been lost during the evolution of some higher mammals. We therefore aimed to determine if functional divergence had occurred, and if natural selection had played an important role in the evolution of these genes. The results showed that *NAIP* genes have undergone pseudogenization and functional divergence, driven by positive selection. Positive selection has also influenced *NAIP* protein structure, resulting in further functional divergence.

1. Introduction

Increasing interest has recently focused on the importance of nucleotide binding and oligomerization domain (Nod-like receptors (NLRs)), as important pattern recognition receptors (PRRs) [1, 2]. In humans, NLR family is composed of 22 intracellular PRRs and carboxy-terminal leucine-rich repeats (LRRs) regions [3–5]. NLR families were divided into three different subfamilies based on their phylogenetic distribution, each characterized by a specific molecular structure [6, 7]. NALPs represent the largest NLR subfamily, for which 14 genes have been identified in humans. NALP proteins harbor a NACHT domain, some LRRs, and an amino-terminal pyrin domain (PYD) domain. The second subfamily of NLRs includes the caspase recruitment domain (CARD), such as NOD1, NOD2, NOD4, and CIITA. This subfamily also contains a slightly separated group, containing NOD3 and NOD5/NLRX1 [1, 5]. NOD5/NLRX1 has not a defined *N* terminal domain, whereas at least one splice variant of CIITA was reported to harbor a CARD.

The NAIP belongs to the third subfamily, which is characterized as the baculovirus inhibitor of apoptosis repeat-containing protein 1 (BIRC1). This group is characterized by one or three baculovirus inhibitors of apoptosis repeat-containing domains (BIRs). In humans, NAIP is a member of the inhibitor of apoptosis protein (IAP) family and has been cloned as a candidate gene for the neurodegenerative disorder spinal muscular atrophy (SMA) [8–10]. Some NAIPs, either alone or in combination with ICE-protease-activating factor (IPAF), have been shown to be involved in the formation of inflammasomes [11, 12]. NAIPs also appear to be involved in the pathogenesis of Alzheimer's disease, Down's syndrome, multiple sclerosis, and Parkinson's disease [13–16]. NAIPs, but not IAPs in general, have been identified as cancer targets [17–21]. A potential role of some NAIPs in innate immunity has also been reported in mouse where NAIP polymorphisms determined whether macrophages restrict or support intracellular replication of *Legionella pneumophila* (*L. pneumophila*) and whether

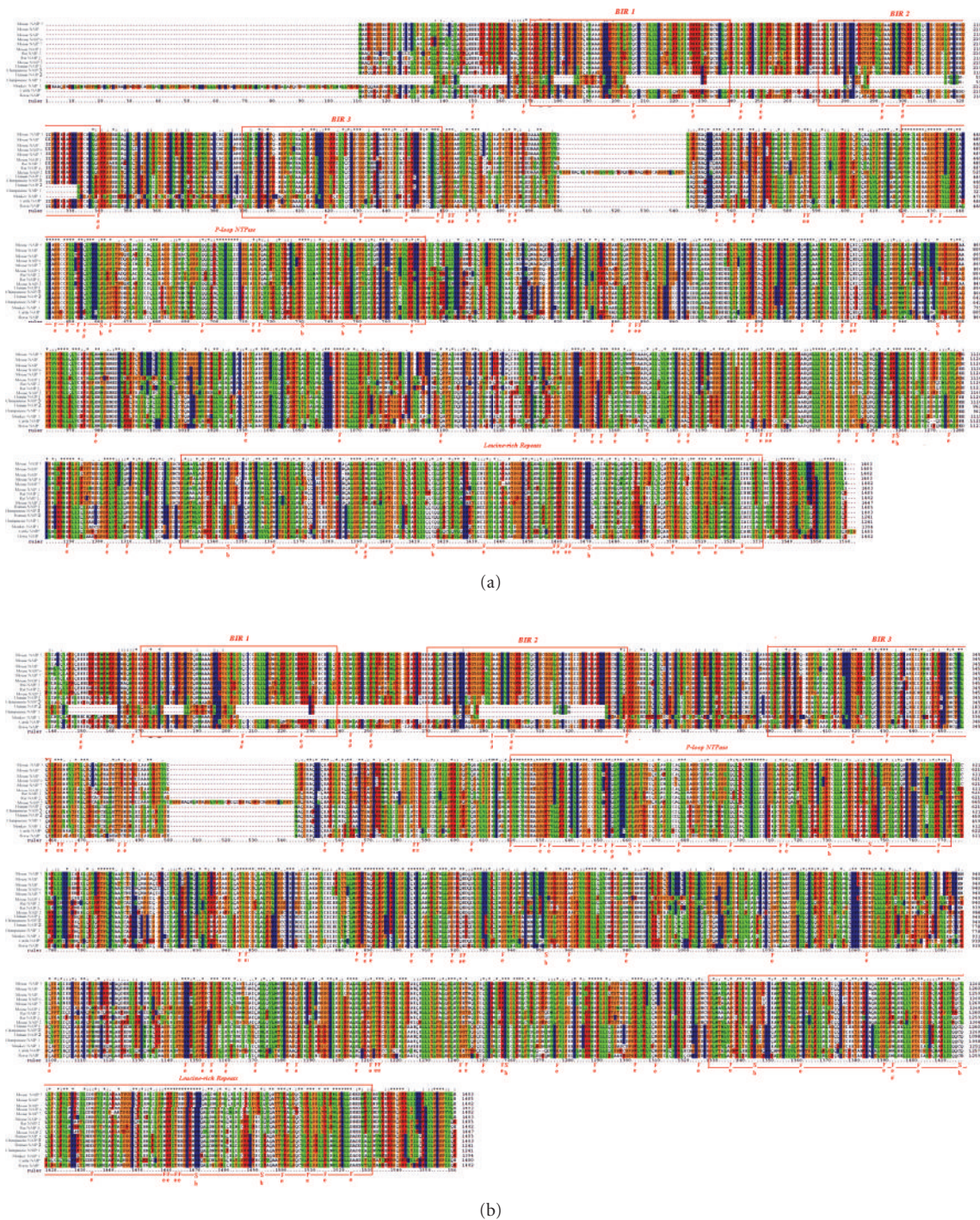


FIGURE 1: Manually corrected alignments. (a) Alignments before manual correction; (b) Alignments after manual correction. Alignment were constructed using Clustal X and modified with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Functional domains are designed as BIR, P-loop NTPase, and LRP. “*” indicated highly conserved residues. Functional (f) or structural (s) residues (f indicates highly conserved and exposed, s indicates highly conserves and buried); “#” indicates amino-acids had changed during the evolution.

mice are resistant or (moderately) susceptible to *Legionella* infection [22].

Overall, human NAIPs genes play important role not only in innate immunity, but also many inflammatory diseases. However, although NAIP genes are strongly associated

with many inflammatory diseases in humans, no similar situation had been found in other mammals [23–25]. Furthermore, some NAIP genes have undergone pseudogenization or been lost during their evolution of higher mammals. In this context, the present study aimed to determine whether

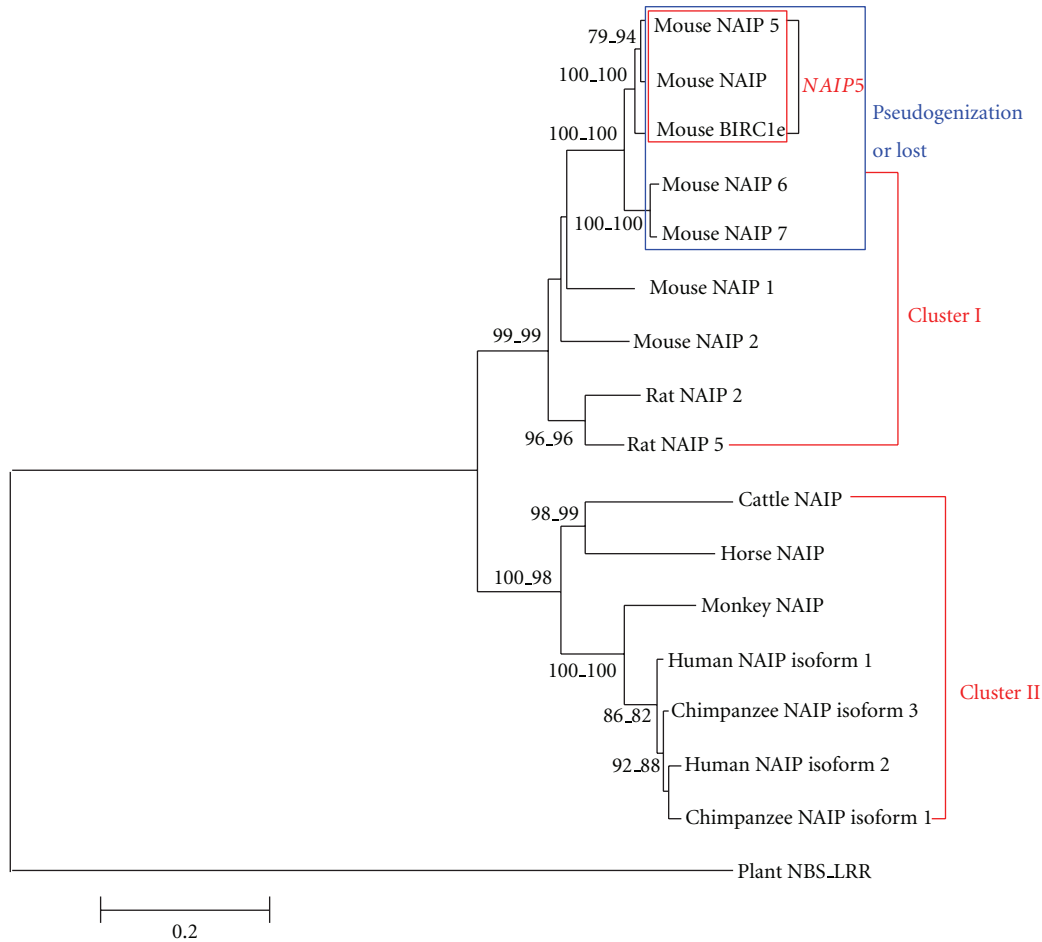


FIGURE 2: Phylogenetic tree of NAIPs reconstructed using two different methods (NJ and ME). A total of 16 NAIP protein sequences from seven mammalian species (cow, horse, human, chimpanzee, monkey, mouse, and rat) were included in the analysis. Bootstrap values are shown on the branches for the two different methods (following the order of the NJ and ME methods).

functional divergence of *NAIP* genes had occurred and if natural selection had played an important role during their evolution.

2. Materials and Methods

2.1. Sequences. The protein sequences of all known NAIPs were retrieved from GenBank (Reference Proteins, Refseq protein) by *PSI-BLAST*, using human NAIP isoform 1 protein sequences as queries [26]. Coding gene sequences for NAIP proteins were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>).

2.2. Phylogenetic Analysis. Redundant sequences were removed by *DAMBE* software. Multiple sequence alignments were performed using *Clustal X* [27] then were manually corrected, and the alignments were shaded using the *Boxshade* program (http://www.ch.embnet.org/software/BOX_form.html). All alignment gaps were removed before phylogenetic analysis. Phylogenetic trees were reconstructed using neighbor-joining (NJ) and minimum-evolution (ME)

methods with *MEGA4.0* [28]. Bootstrap values were estimated from 1000 replicates.

2.3. Analysis of Functional Divergence of NAIP Genes. *DIVERGE 2.0* program [29] was used to estimate type I functional divergence [30] between different clusters of *NAIP* genes. Type I sites represented amino-acid residues conserved in one cluster, but highly variable in another, which suggests that these residues had been subjected to different functional constraints. Statistically, the functional divergence between two clusters was measured as the coefficient of functional divergence, θ (ranging from 0-1). A null hypothesis of $\theta = 0$ indicated that the evolutionary rates of two duplicate genes at each site were virtually identical [31, 32]. If the null hypothesis was rejected, a site-specific profile was used to predict the critical amino-acid residues most likely to be responsible for the functional divergence detected.

The phylogenetic tree used for *DIVERGE 2.0* was reconstructed by *MEGA 4.0* with the ME method [28]. The coefficients of functional divergence (θ) between gene

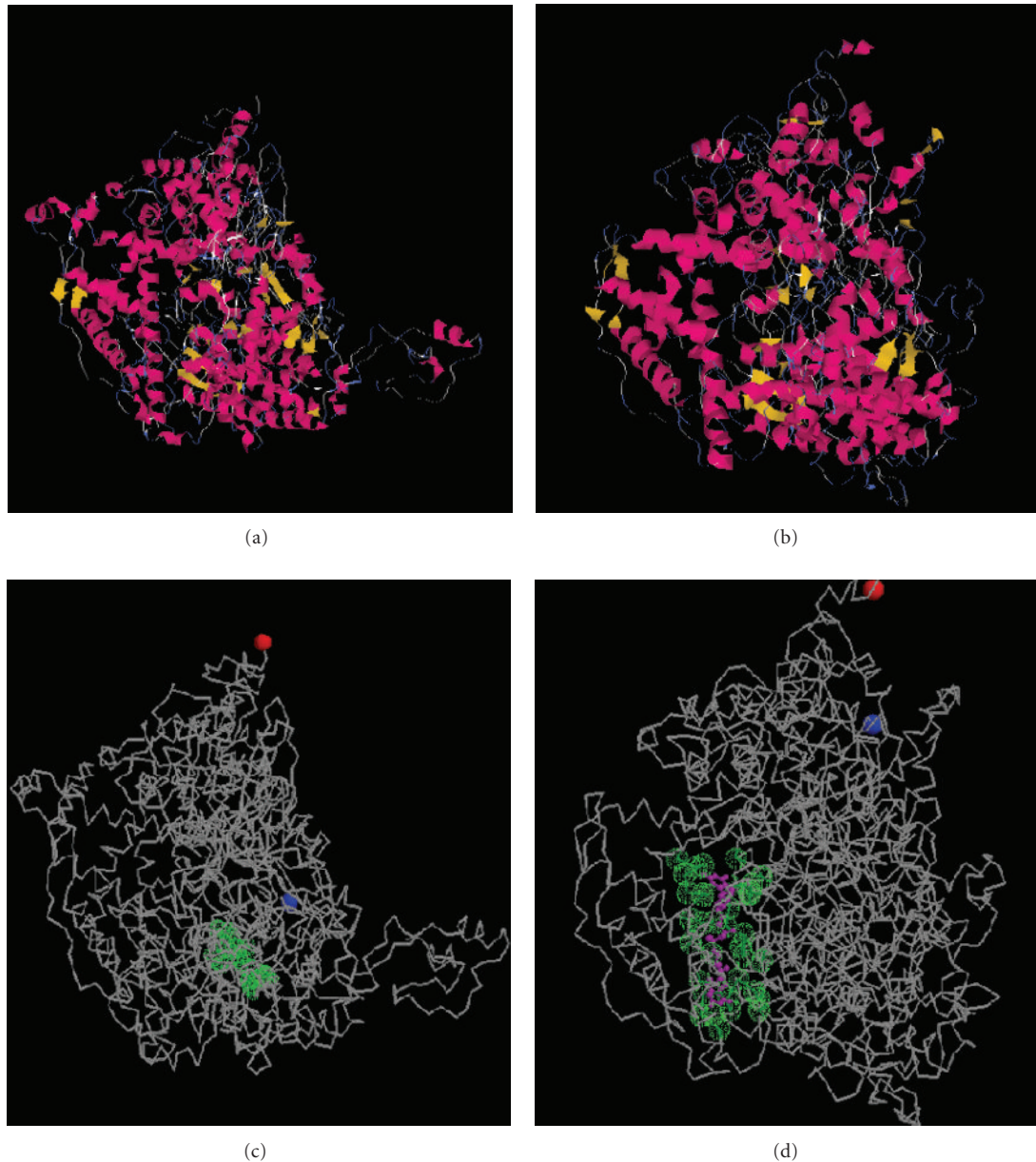


FIGURE 3: Predicted models of human (a) and mouse (b) NAIPs. (c) indicates predicted binding sites on human NAIP; (d) indicates predicted binding sites on mouse NAIP.

clusters were calculated by model-free estimation (MFE) and maximum-likelihood estimation (MLE) under a two-state model (MLE) to detect amino-acid residues reflecting functional divergence.

2.4. Test for Selective Force. Patterns of molecular evolution were assessed for NAIP gene subfamily using *MEGA 4.0* [33]. *ClustalX* alignments of nucleotide sequences were used as inputs in the analysis. The dN and dS values were calculated within every cluster, using modified Nei and Gojobori's [34, 35]. A dN/dS > 1 suggested that the gene had undergone positive selection.

2.5. Model Prediction for NAIPs. Models prediction for NAIPs were conducted using *I-TASSER server*. (<http://zhang-lab.ccmb.med.umich.edu/I-TASSER/>).

3. Results

3.1. NAIP Genes Pseudogenized during Evolution. A total of 52 NAIP amino-acid sequences were retrieved from the GenBank. The 52 sequences included 13 humans (*Homo sapiens*: NP_004527, NP_075043, NP_001157, NP_892007, NP_0011561, AAI36274, A55478, AAC62261, BAB87181, BAG58144, BAD96320, AAC52045, and AAI43762), three chimpanzees (*Pan troglodytes*: XP_001156951, XP_001156604,

TABLE 1: The sequences used in this study.

NAIP name	The other name	Species	Chromosome	Structure	Gene accession numbers	Protein accession numbers
NAIP isoform 1	BIRC1	Human	5q13.1	BIR3x-NACHT-LRR	NM_004536	NP_004527
NAIP isoform 2	BIRC1	Human	5q13.1	BIRx-NACHT-LRR	NM_022892	NP_075043
	BIRC1 isoform 1	Chimpanzee	5	BIRx-NACHT-LRR	XM_001156604	XP_001156604
	BIRC1 isoform 3	Chimpanzee	5	BIR3x-NACHT-LRR	XM_001156951	XP_001156951
	BIRC1	Monkey	6	BIRx-NACHT-LRR	XM_001093532	XP_001093532
NAIP		Cow	20	BIR3x-NACHT-LRR	XM_589415	XP_589415
NAIP		Horse	14	BIR3x-NACHT-LRR	XM_001504041	XP_001504091
NAIP1	Birc1a, NAIPa,	Mouse	13 D1-D3	BIR3x-NACHT-LRR	NM_008670	NP_032696
NAIP2	Birc1b, NAIPb	Mouse	13 D1	BIR3x-NACHT-LRR	NM_001126182	NP_001119654
NAIP5	Birc1e, Naip-rs3,	Mouse	13 D1	BIR3x-NACHT-LRR	NM_010870	NP_035000
NAIP6	Birc1f, NAIPf	Mouse	13 D1	BIR3x-NACHT-LRR	NM_010871	NP_035001
NAIP7	Birc1 g, NAIPg,	Mouse	13 D1	BIR3x-NACHT-LRR	NM_021545	NP_067520
NAIP	BIRC1e	Mouse		BIR3x-NACHT-LRR	AF381771	AAN77912
NAIP		Mouse		BIR3x-NACHT-LRR	AF135492	AAD56764
NAIP2	Birc1b	Rat	2q14-q16	BIR3x-NACHT-LRR	XM_226742	XP_226742
NAIP5		Rat	2q14-q16	BIR3x-NACHT-LRR	XM_226743	XP_226743

TABLE 2: Test for selection by modified Nei and Gojobori's.

	N: no. of nonsynonymous sites		S: no. of synonymous sites		dN/dS
	dN	Std. Err.	dS	Std. Err.	
Cluster 1	2954.186	24.204	1202.397	14.174	2.46
Cluster 2	2780.340	26.770	1123.943	14.187	2.47

Note: Cluster 1 includes mouse NAIP 5, mouse NAIP, mouse BIRC1E, mouse NAIP6, mouse NAIP 7, rat NAIP5, rat NAIP2, mouse NAIP 1, mouse NAIP 2; Cluster 2 includes human NAIP isoform 2, chimpanzee NAIP isoform 1, human NAIP isoform 1, chimpanzee NAIP isoform 3, monkey NAIP, cow NAIP and horse NAIP, respectively. dN/dS > 1 means positive selection.

and XP_001156888), one cow (*Bos taurus*: XP_589415), one monkey (*Macaca mulatta*: XP_001093532), one horse (*Equus caballus*: XP_001504091), 29 mice (*Mus musculus*: NP_032696, NP_001119654, NP_035000, NP_035001, NP_035002, NP_067520, AAB69223, AAB69223, AAC73002, AAD56759, AAD56761, AAD56762, AAD56763, AAD56764, AAD56765, AAI16627, AAI16348, AAN60208, AAN77585, AAN77586, AAN77587, AAN77588, AAN77612, AAN77615, AAN77911, AAN77912, AAN77913, EDL00813, and Q9QWK5), and five rats (*Rattus norvegicus*: XP_226742, XP_226743, XP_001070799, XP_001070842, and AAI99667). After removal of redundant sequences using DAMBE (data not shown), the 16 remaining sequences were aligned using *Clustal X*. The alignments were manually corrected (Figure 1). The 16 sequences were included in the final dataset and included two humans, two rats, one horse, one cow, seven mice, two chimpanzees, and one monkey (Table 1).

Alignments of the 16 NAIP sequences showed that all NAIP sequences included three BIRs, except for human NAIP isoform 2, chimpanzee BIRC1 isoform 1 and monkey BIRC1, NACHT domains, and several LRRs (Figure 1 and Table 1). NACHT belongs to the AAA⁺NTPase superfamily, a sister group of another family of ATPases. NACHT domain

included seven conserved motifs, the ATP/GTPase-specific *p*-loop, the Mg²⁺-binding site (Walker A and B motifs, resp.), and five specific motifs (motifs I–V).

Phylogenetic trees for NAIPs were shown in Figure 2. Phylogenetic trees were reconstructed by two different methods (ME and NJ) with high bootstrap values. Two major clusters (I and II) of NAIPs were statistically supported. The plant NBS_LRR was used as the root. Cluster I mainly included rodent NAIPs, namely, mice NAIPs 1, 2, 5, 6, and 7, rats NAIPs 2 and 5. Cluster II contained cow, horse, monkey, chimpanzee and human NAIPs. NAIPs 1, 6 and 7 were mice-specific and could be related to the reproduction-related NAIP 2 or NAIP 5, which indicated the possible origin of the ancestral NAIP 2 and 5 genes. Mouse NAIP 5 included three copies, NAIP 5, NAIP 6, and NAIP 7, each with high bootstrap values. This indicated that these gene copies were produced by gene duplication and shared similar functions. Mouse NAIP genes had been extensively duplicated, and duplicates were located on mouse chromosome 13. No other instances of gene duplication were detected in higher mammals [36, 37]. The present study aimed to determine whether the gene duplications were lost or pseudogenized in higher mammals. Human NAIP genes were used as queries against the human genome

TABLE 3: The prediction model evaluation.

Type	C-score	TM-score	RMSD	No. of decoys	Cluster density
Human NAIP	-1.24	0.56 ± 0.15	12.8 ± 4.2 Å	93	0.1275
Mouse NAIP2	-1.69	0.50 ± 0.15	14.2 ± 3.8 Å	99	0.0787

Note: C-score is a confidence score for estimating the quality of predicted models by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of $[-5, 2]$, where a C-score of higher value signifies a model with a high confidence and vice versa.

TM-score and RMSD are known standards for measuring structural similarity between two structures which are usually used to measure the accuracy of structure modeling. TM-score >0.5 indicates a model of correct topology.

using tBLASTn. Trace of *NAIP* pseudogenes was found on chromosome 5. Nine pseudogenes were found beside the *NAIP* genes (NW 922908, NW 926600, NW 001841555, NW 924990, NW 925558, NW 001841789, NW 927458, NW 001838943, and NW 001838948).

3.2. Functional Divergence of *NAIP* Genes during Evolution.

Functional divergence was estimated using *DIVERGE 2.0* to determine whether functional divergence had occurred between different species during *NAIP* evolution, functional divergence was estimated by *DIVERGE 2.0* program. After alignments and removal of the gaps, a total of 1324 amino-acid sites were included in the analysis. Pairwise comparisons of the two *NAIP* clusters were conducted, and the rate of amino-acid evolution at each sequence position was estimated. The MFE θ was 0.69 ± 0.08 , and MLE θ was 0.67 ± 0.09 . Functional divergence was significant between the comparison of the two clusters ($\theta > 0$ with $P < 0.001$) indicating that site-specific selective constraints on change might contribute to the functional evolution of *NAIP* genes.

Furthermore, the important amino-acid residues responsible for functional divergence were predicted by calculating the site-specific profiles based on posterior analysis of all pairs of clusters with functional divergence. *Cutoff* values were established to extensively reduce false positives, by progressively removing the highest scoring residues from the alignments until θ dropped to zero. RFD NO was 319, covering 24.1% of the total of 1324 aligned sites with a *cutoff* of 0.93. RFD was generally detected in all functional domains of *NAIPs*, implying that shifts in functional constraints might have acted on every protein domain.

3.3. Functional Divergence of Mammals *NAIP* Genes Driven by Positive Selection.

To determine whether positive selection drove the functional divergence, *MEGA 4.0* was used to investigate a model of the selective force acting on certain codons within every cluster during their evolution using the modified Nei and Gojobori's method. The dN/dS value in cluster I was 2.46, while it was 2.47 in cluster II. This showed that the *NAIP* genes had undergone positive selection (Table 2).

3.4. Functional Divergence Led to Structural Changes in *NAIPs*.

To further investigate whether the functional divergences of *NAIPs* resulted from selective forces acting on structures, models of human and mouse *NAIPs* were predicted using

the *I-TASSER* server. The C-scores, TM-scores, and RMSDs of the two models were as follows: human *NAIP* -1.24 , 0.56 ± 0.15 , 12.8 ± 4.2 Å, respectively; mouse *NAIP* -1.69 , 0.50 ± 0.15 , and 14.2 ± 3.8 Å, respectively (Table 3, Figures 3(a) and 3(b)), indicating that the models were highly reliable. The predicted binding sites of the model had been changed as follows: human *NAIP* predicted binding sites were 281, 283, 284, 285, 286, 287, 291, 292, 294, 316, 317, 319, 320, 321, 324, 325, 326, 359, 368, 388, 389, 390, 391, 392, 393, 394, 401, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 553, 559, and 560. Mouse *NAIP* predicted binding sites were 314, 315, 316, 317, 318, 319, 320, 321, 324, 395, 396, 529, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 868, 880, 882, 893, 894, 895, 896, 897, 898, 899, 901, 902, 905, 908, and 912 (Figures 3(c) and 3(d)).

4. Discussion

NAIPs belong to the *IAP* family, which are responsible for sequestering activated caspases [38]. Published data showed that *NAIP* genes were strongly associated with innate immunity. In addition, human *NAIPs* contribute to inflammatory diseases and cancer development. However, no similar results have been reported in mice. Furthermore, *NAIP* has also an expanded gene number in the mouse, with five tandem copies reported in C57BL/6J and at least seven in the 129 strain [39]. Reported data showed no incomplete functional overlap among mouse *NAIP* loci [40]. It was suggested that *NAIP* was encoded within a region undergoing rapid evolution. However, it was not clear whether *NAIP* gene was derived by evolutionary force, whether *NAIP* gene underwent functional divergence during evolution, or if functional divergence was caused by selective forces. Clarification of the evolutionary process driving changes in the *NAIP* genes is thus important for understanding its genetic trend and exploring its new functions.. The present study analyzed 16 *NAIPs* genes from different mammals using phylogenetic analysis and showed that *NAIP* genes experienced lineage-specific duplications in rodents. Mice *NAIP* genes, especially, have been extensively duplicated, and the duplicates were located on mouse chromosome 13. The duplication of *NAIP* genes might have occurred before and after separation. Gene duplication is thought to be a major driving force facilitating the evolution of tissue specialization. However, lineage-specific duplications in *NAIPs* genes appear not to be universal in mammals, other than rodents. It was speculated that these duplications might

have been lost or undergone pseudogenization during the evolution of higher mammals (Figure 1) [41]. To test this hypothesis, human *NAIP* genes were used as queries against human genome, which identified nine pseudogenes on chromosome 5. However, the biological significance of the loss of *NAIP* genes remains unknown. It was hypothesized that *NAIP* gene duplications generated functional redundancy in higher mammal. In other words, mutations disrupted the structure and function of one of the two genes. However, the duplications were not deleterious, and they were not removed by selection. Gradually, the genes containing mutations became pseudogenes, which were either unexpressed or functionless.

To determine whether the evolution of *NAIP* genes was driven by positive selection. *MEGA 4.0* was implemented to investigate a model of selective force acting on *NAIP* genes during their evolution. The results suggested that *NAIP* genes were driven by positive selection, in accordance with other reports indicating that genes in duplication blocks could be maintained by positive selection (Table 2) [42, 43].

Gene duplication driven by positive selection has also been thought to be an essential source of novel genes with new or altered functions, as evidenced by the widespread existence of gene families. The present work shows that functional divergence between clusters of *NAIP* genes could have occurred, and the role of selective forces acting on the tertiary structure of *NAIP* proteins was also investigated. Predicted models of human and mouse *NAIP* were constructed using *I-TASSER* server, and structural differences in binding sites suggest that the expressional divergence of *NAIP* during evolution may lead to functional specialization.

5. Conclusions

NAIP genes have undergone pseudogenization and functional divergence during evolution. Functional divergence has been driven by positive selection forces, which have also influenced *NAIP* protein structure, leading to further functional divergence.

Conflict of Interests

The authors declares that they have no conflict of interests.

Acknowledgments

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