Lab Note

A novel member of lymphocyte-specific protein tyrosine kinase protein identified in lamprey, *Lampetra japonica*

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Lymphocyte-specific protein tyrosine kinase p56 (Lck) is a member of the Src family of non-receptor protein tyrosine kinase. Lck plays an important role in mediating T-cell receptor (TCR) signal transduction and the development, differentiation, proliferation, and activation of T-cells [1]. Lck contains N-terminus of myristylation sequence, a unique amino-terminus region, followed by Src homology 3 (SH3) and SH2 domains and a C-terminus of tyrosine kinase catalytic domain [2]. Lck can associate with the inner face of the plasma membrane through its myristoyl glycine and palmitoyl cysteines in the amino-terminus [3]. Following the myristylation sequence, there is the unique region, a short region of ~80 amino acids. This unique region is involved in the interaction of Lck with specific cellular proteins [4,5]. Downstream of this unique region are SH3 and SH2 domains which are involved in protein–protein interactions [6]. The tyrosine kinase domain is the catalytic domain of Lck catalyzing the transfer of the gamma-phosphate from ATP to tyrosine residues in proteins. The catalytic domain of human Lck contains a site of autophosphorylation (Tyr-394), which plays an important role in regulating the protein kinase activity [7]. Agnathans, represented by lamprey and hagfish, are the oldest vertebrates currently identified possessing the adaptive immune defenses [8]. A recent study of jawless vertebrate has provided a clue for the origin of adaptive immune defense. Though TCR and B-cell receptor system do not exist in jawless vertebrates, lamprey has been confirmed to possess an alternative immune system that could specifically recognize and respond to external pathogens [9].

The handling of lamprey (*Lampetra japonica*) and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University. Adult lampreys were purchased from Tongjiang section of the Heilongjiang River (Tongjiang, China) in December. Adult lampreys (200–220 g in weight) were divided into two groups (20 animals per group); one group of animals was immunized with 0.1 mg of lipopolysaccharide (LPS) (Sigma-Aldrich, St Louis, USA) in 0.1 ml phosphate-buffered saline (PBS), and the control animals were injected with 0.1 ml PBS only. The animals were immunized at 8-day intervals by four intraperitoneal injections.

Based on the expressed sequence tag analysis of the cDNA library which was constructed with lamprey lymphocyte-like cells in our laboratory, a Lck homolog was found using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Total RNA was isolated from lamprey lymphocyte-like cells [10] using RNAiso (TaKaRa Biotechnology, Dalian, China) reagent following the manufacturer instructions. The 3′- and 5′-RACE cDNAs were synthesized from 5 μg of total RNA by Reverse Transcriptase M-MLV at 30°C for 10 min, 42°C for 30 min, 70°C for 15 min, 95°C for 5 min, 4°C for 60 min with the 3′-coding sequence primer and 5′-coding sequence primer and Random 9-mers primer following the manufacturer instructions (TaKaRa Biotechnology). The 3′- and 5′-end sequences of *Lj-Lck* were obtained by polymerase chain reaction (PCR) with outer primer, inner primer (TaKaRa Biotechnology), and specific primers (Supplementary Table S1). LA Taq DNA polymerase (TaKaRa Biotechnology) was used for amplification with the following cycling conditions: 94°C for 3 min, followed by 40 amplification cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and a final extension step at 72°C for 10 min. Products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. The target band of PCR product was isolated and purified, subcloned into
pMD19-T vector using DNA Ligation kit (TaKaRa Biotechnology), and then subject to DNA sequencing (TaKaRa Biotechnology). Through PCR followed by 5′-RACE and 3′-RACE, the full-length cDNA of Lj-Lck with 2651 bp nucleotides was obtained (NCBI accession number: KJ17 4499), which contained a 1485 bp open reading frame, a 460 bp 5′-untranslated region (UTR), and a 706 bp 3′-UTR, encoding a polypeptide of 494 amino acids with an estimated molecular mass of 56.4 kDa. The signal sequence and the transmembrane domain do not exist in Lj-Lck, which indicated that it may be an intracellular protein.

All Lck sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and the Ensemble genome browser (http://www.ensemble.org/). First, a search for lymphocyte-specific protein tyrosine kinase sequences was performed using Lck and lymphocyte-specific protein tyrosine kinase as keywords to maximize the number of hit sequences. Secondly, a number of Lck genomic sequence segments were obtained from the genomic databases by online programs of BLAST and the BLAST-like Alignment Tool (BLAT) in the NCBI and Ensemble genome browser databases. Partial sequences and potential pseudogenes were excluded and some predicted sequences were used to search coding exons at the Ensemble genome browser according to previously verified sequences. All Lck amino acid sequences were aligned with ClustalX 1.81 using default settings except for identity matrix set for protein weight matrix. Multiple sequence alignments of Lj-Lck with other Lcks of jawed vertebrates (62% with jawed vertebrates revealed that Lj-Lck had a high sequence conservation, the Lcks from mammals, birds, and amphibians; the second one (named cluster II) includes Lcks from teleosts, and the third one (named cluster III) includes Lj-Lck only. Cluster I can be further classified into four subgroups, one includes Lcks from mammals except for ornithorhynchus, and the other three include those from ornithorhynchus, birds, and amphibians, respectively. Phylogenetic analysis indicated that the Lj-Lck was clustered as the out group of Lck from jawed vertebrates and its origin was far earlier than the one from the common ancestor of jawed vertebrates. Considering that Agnathans, represented by lamprey and hagfish, are agreed to be the oldest vertebrates possessing the primary lymphocyte-like cells which take the function of adaptive immune defenses [9], the origination of Lck seems highly related to the emergence of lymphocytes. Though the evolution pattern of the Lck family is in accordance with the classical interpretations of the origin of species, there is a far genetic distance (>0.2) between Lcks from jawless and Lcks from jawed vertebrates. From Fig. 2, the common ancestor gene of vertebrate Lcks was found to possess short genetic distance with the tyrosine-protein kinase Src42A from sea squirt, which illustrated that the origin of the Lck gene can be traced back to invertebrates.

In order to explore the evolutionary dynamics of conserved motifs of the Lck family, amino acid sequences of Lcks from jawless and jawed vertebrates were analyzed by the MEME Suite. Conserved motif analyses were performed online using the MEME system version 4.9.0 [11](http://meme.nbcr.net/meme/). The default settings of minimal and maximal motif widths and the number of different motifs were defined as 6, 30, and 25, respectively. There are totally 25 conserved motifs elicited from vertebrate Lcks (Supplementary Table S2). According to the type and distribution of the motifs, motif 17 represents N-terminus of myristylation sequence, motif 9 indicates the SH3 domain, motifs 11, 22, 8, 16, and 6 comprise the SH2 domain. The C-terminus of tyrosine kinase catalytic domain is made up of motifs 11, 3, 18, 2, 13, 4, 12, 1, 21, 5, 19, and 7 (Table 1). These 19 motifs exist ubiquitously in nearly all sequences. Almost all Lcks from mammals and birds contain all of the 24 motifs, but motif 24 is absent in Lck from amphibians. As to the Lcks from fishes, motif 15 is absent, and motifs 14 and 24 are replaced by motifs 23, 25, respectively, reflecting that motifs 23, 25 are primitive. In lamprey Lck sequence, motifs 23, 15, and 25 are absent.
These results revealed that myristylation sequence, the SH3 and SH2 and C-terminus of tyrosine kinase catalytic domains of Lcks were conserved throughout the Lck gene family in vertebrates, and the ‘unique region’ was the most divergent region during gene evolution. The existence of these novel motifs in jawed vertebrates suggested that the
evolution of unique amino-terminus regions may arise through ‘short insertion or deletion’ neomorphic mutation events. Thus, we concluded that Lck gene may originate from ancient jawless vertebrates and diversified to versatile members in jawed vertebrates through multiple gene mutations mechanism.

The expression pattern of Lj-Lck was examined using real-time quantitative PCR with total RNA extracted from different lamprey tissues of LPS-treated lampreys (Fig. 3). Total RNAs were separately extracted from different lamprey tissues including lymphocyte-like cells, supraneural myeloid body, gill, heart, liver, intestine, and kidney using RNAiso reagent (TaKaRa Biotechnology). The total RNAs were treated with DNase I (TaKaRa Biotechnology), and then subject to reverse transcription using PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa Biotechnology). Real-time quantitative PCR experiments were performed with TaKaRa TP800 Real-Time PCR System (TaKaRa Biotechnology) using 2 μl cDNA with 16.8 μl SYBR green PCR mastermix (TaKaRa Biotechnology) and 0.6 μl of each specific primer (Supplementary Table S1). The efficiency of the primers was analyzed in serial 50-fold dilutions of

Figure 2. Phylogenetic tree reconstructed with 29 Lck homologs based on the NJ method Bootstrap values are indicated as percentages at the nodes. Mammals, reptiles, bird, amphibians, teleosts, agnathans, and sea squirt clades are delineated by vertical brackets at the right. The bar indicates genetic distance. The GenBank accession numbers are as follows: Felis catus: XP_003989862; Canis lupus familiaris: XP_851972; Allorhopoda melanoleuca: XP_002921912; Loxodonta Africana: XP_003415510; Mustela putorius furo: XP_004783242; Aotus nancymaee: AAV70114; Pan troglodytes: XP_003307997; H. sapiens: AAA59502; Gorilla gorilla gorilla: XP_004025416; Bos taurus: AA102047; Ovis aries: ACJ53945; Sus scrofa: ACK36990; Tursiops truncatus: XP_004318259; Orcinus orca: XP_004266569; Odobenus rosmarus divergens: XP_004406474; Sarcophius harrisii: XP_003765473; M. musculus: AAB59674; Rattus norvegicus: AA160881. Ornithorhynchus anatinus: XP_001509275; Ficedula albicollis: XP_005058509; G. gallus: XP_004947872; Anas platyrhynchos: XP_005020507; X. (Silurana) tropicalis: XP_002939341; Anguilla japonica: BAL48858; S. salar: NP_001133379; Carassius auratus langsdorffii: BAF56886; Larimichthys crocea: AEL33718; Oryzias latipes: XP_004082690; C. intestinalis: XP_002121011.
cDNA by calculating the slope of the regression line of the cycle thresholds (Cts) versus the relative concentration of cDNA. The GAPDH of *L. japonica* was used as the internal control to normalize the starting quantity of RNA. The cycling was performed as follows: 95°C for 30 s, followed by 40 amplification cycles at 95°C for 5 s, 55°C for 30 s, 72°C for 30 s, and a final extension step at 65°C for 15 min. The results were expressed as the mean ± standard deviation of three independent experiments for each specimen. The differences of gene expression between two groups were analyzed using Student’s *t*-test by SPSS statistical software package. The differences were considered statistically significant at *P* < 0.05. It was found that the expression level of *Lj-Lck* was higher in liver than in other tissues in the control (treated with PBS). For the LPS-stimulated group, the expression level of *Lj-Lck* transcript reached the highest in the lymphocyte-like cells, which was ∼7-fold increase compared with the control (*P* < 0.05). The increase of *Lj-Lck* expression were also found in gill and supraneural myeloid body (4- and 3-fold increase relative to the controls (*P* < 0.05), respectively). The significant up-regulation in various immune associated tissues such as the lymphocyte-like cells, gill, and supraneural myeloid body when treated with LPS indicated that Lj-LCK may play an important role in immune reaction of lampreys. LPS is a major component of the outer membrane of Gram-negative bacteria and has been used in mammals, amphioxus, fish, and lamprey for immunological studies [10]. LPS is a potent activator of T-cells by signaling through CD27 molecule [12]. Based on the fact that lampreys have thymus-like lympho-epithelial structures in the tips of the gill filaments [13], and the supraneural myeloid body serves as hemocytology in sea lamprey [14], it is possible that the injection of LPS could activate lymphocyte-like cells in the gills, supraneural myeloid body, and blood to recruit signaling molecule such as Lj-Lck for participating in the immune response.

In conclusion, a novel member of Lck was identified in lamprey. Phylogenetic analysis of the Lcks indicated that the Lj-Lck could be regarded as a primary type of Lck in vertebrates and an ortholog of Lcks in jawed vertebrate. The Lck gene came into existence after multiple gene mutation processes that took place in the emergence of jawless vertebrates. The Lj-Lck might play an important role in signal transduction and involve in the immune response of

<p>| Table 1. Type and distribution of conserved motifs discovered among typical Lck homologs from mammals, reptiles, bird, amphibians, teleosts, and agnathans using the MEME system |
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*Each number represents a specific motif.
Detailed information can be found in **Supplementary Table S2.**

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**Figure 3.** *Lj-Lck* mRNA expression was significantly up-regulated in the lymphocyte-like cells, heart, and kidney after treatment with LPS *P* < 0.05 compared with the control group.
lymphocyte-like cells in lamprey. Since the accurate function of Lj-Lck still remains unknown, further studies are needed to elucidate this speculation.

**Supplementary Data**

Supplementary Data are available at *ABBS* online.

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**References**