Lab Note

A novel member of B cell linker protein identified in lamprey, *Lampetra japonica*

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The B cell linker protein (BLNK) is an adaptor molecule and plays an important role in signal transduction of B-cell receptor (BCR) and pre-B-cell antigen receptor [1,2]. The BLNK contains a conserved C-terminal Src homology 2 (SH2) domain, a proline-rich region and an N-terminal acidic region [2]. As an adaptor protein, BLNK can bind with some signaling molecules such as Bruton’s tyrosine kinase (BTK), growth factor receptor-bound protein 2 (Grb2), spleen tyrosine kinases (Syk) etc through its SH2 domain [3]. When BCR signaling pathway was activated, phosphorylation of BLNK could recruit phospholipase C\(\gamma\) (PLC\(\gamma\)), BTK, Grb2, guanine nucleotide exchange factor Vav (Vav) and non-catalytic region of tyrosine kinase adaptor protein (Nck), and regulate downstream signaling pathways [4].

Agnathans, represented by lamprey and hagfish, are the oldest vertebrates currently proved to possess the adaptive immune defenses [5]. Though T-cell receptor (TCR) and B-cell receptor (BCR) do not exist in jawless vertebrates, recent findings in lamprey have revealed that it possesses an alternative immune system that could specifically recognize and respond to external pathogens [6].

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
(Permit Number: SYXK2004—0029). Adult lampreys were collected from Tongjiang section of the Heilongjiang River (Tongjiang City, China) in December, 2012. Adult lampreys (200–220g in weight) were divided into two groups (20 animals per group); one group animals were immunized with 0.1 mg of LPS (Escherichia coli 0111:B4) (Sigma-Aldrich, St Louis, USA) in 0.1 ml 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 analysis expressed sequence tags (EST) of the cDNA library constructed with lamprey lymphocyte-like cells by our lab previously, a BLNK ortholog was found using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Total RNA was isolated from lamprey lymphocyte-like cells [7] using RNAiso (TaKaRa, Dalian, China) reagent following the manufacturer instructions, and dissolved in DEPC-treated water and stored at −80°C. First strand 3’ and 5’ RACE cDNAs were synthesized from 3 µg of total RNA by Reverse transcriptase M-MLV at 30°C for 10 min, 42°C for 60 min, 70°C for 15 min with the 3’-CDS primer and 5’-CDS primer and Random 9 mers primer following the manufacturer instructions (TaKaRa). The 3’- and 5’-end sequences of Lj-BLNK were obtained by PCR with outer primer, inner primer (TaKaRa) and specific primers (Supplementary Table 1). Taq DNA polymerase (TaKaRa) was used for amplification with the following cycling conditions: 94°C for 5 min, followed by 40 amplification cycles at 94°C for 30 s, 65°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 a pMD19-T vector using a DNA Ligation kit (TaKaRa) and then subject to DNA sequencing (TaKaRa).

Total RNAs were separately extracted from different lamprey tissues including lymphocyte-like cells, gill, heart, liver, intestine and kidney using RNAiso reagent (TaKaRa), and the total RNAs were treated with DNase I (TaKaRa), and then subject to reverse transcription using PrimeScript™ RT reagent kit (Perfect Real Time) (TaKaRa). Real-time quantitative PCR experiments were performed with a TaKaRa TP800 Real Time PCR System (TaKaRa) using 2 µl cDNA with 16.8 µl SYBR green PCR mastermix (TaKaRa) and 0.6 µl of each specific primer (Supplementary Table 1). The efficiency of the primers was analyzed in 10-fold serial dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Cts) versus the relative concentration of cDNA. The GAPDH of
**Lampetra japonica** was used as an internal control to normalize the starting quantity of RNA because it showed a constant expression level and did not fluctuate under the different experimental conditions. Melting curve analysis was also performed to verify that primer dimmers were not amplified. The cycling is performed as follows: 95°C for 30 s, followed by 40 amplification cycles at 95°C for 5 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 65°C for 10 min. Results were expressed as the mean±SD of three parallel experiments for each sample. The differences of gene expression between two groups were analyzed using Student’s t-test by SPSS statistical software package. Differences were considered statistically significant at P<0.05.

All BLNK sequences were obtained from the National Center for Biotechnology Information (NCBI) ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) and the Ensemble genome browser ([http://www.ensemble.org/](http://www.ensemble.org/)). Firstly, a search for B cell linker amino acid sequences was performed using BLNK and B cell linker as keywords to maximize the number of hit sequences. Secondly, a number of BLNK 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.org/ Web site according to previously verified sequences.

All BLNK amino acid sequences were aligned with ClustalX 1.81 using default settings but identity matrix set for protein weight matrix. Result of multiple sequence alignments was converted into mega format and directly imported to MEGA 4.0 for constructing phylogenetic tree. A Neighbor-Joining (NJ) tree was constructed based on pair-wise deletion of gaps/missing data and the distance matrix of amino acids model with 1000 bootstrap replicates. Conserved motif analyses were performed online using the MEME system version 4.9.0 ([http://meme.nbcr.net/meme/](http://meme.nbcr.net/meme/)), except that the distribution of motif occurrences, the minimal and maximal motif widths, and the number of different motifs were defined as any number of repetitions, 6, 30, and 15, respectively.

The molecular cloning and characterization of a BLNK ortholog molecule from the lamprey (**Lampetra japonica**) (Lj-BLNK) were reported for the first time. The Lj-BLNK could well be the counterpart of jawed vertebrates, which is known to play important roles in lymphocytes signal transduction.

A sequence fragment was found in a lymphocyte-like cDNA library which has been constructed and performed EST sequencing previously in our lab. Through PCR followed by 5’-RACE and 3’-RACE, the
full-length cDNA of Lj-BLNK with 4684 bp nucleotides was obtained (NCBI accession number is KF692036), which contained a 2268 bp open reading frame (ORF) encoding a polypeptide of 755 amino acids with an estimated molecular mass of 83,200 Da, a 152-bp 5’-untranslated region (UTR) and a 2308-bp 3’-UTR. The theoretical isoelectric point of Lj-BLNK is 6.8. The signal sequence and the transmembrane domain do not exist in Lj-BLNK, which indicate it may be an intracellular protein.

Multiple sequence alignments of Lj-BLNK with other BLNKs of jawed vertebrates revealed that Lj-BLNK had a low sequence identity with the BLNKs from jawed vertebrates (24.32% with Homo sapiens, 24.90% with Mus musculus, 25.62% with Gallus gallus, 23.85% with Xenopus laevis and 23.12% with Danio rerio) (Fig. 1). Though the conservation is low in amino acid sequence, Lj-BLNK protein also had a conserved SH2 domain at its carboxyl terminal and a proline-rich region that are characteristics of BLNK family. It is worth pointing out that the SH2 domain of Lj-BLNK possesses over 50% sequence identity with the SH2 domains of BLNK from Gallus gallus.

The expression pattern of Lj-BLNK was examined using real-time quantitative PCR with total RNA extracted from lymphocyte-like cells, gill, heart, liver, intestine and kidney of lampreys before and after being challenged with LPS (Supplementary Fig. S1). The expression level of Lj-BLNK was obviously higher in heart than that in any other tissues when treated with PBS (negative control). For the LPS-stimulated group, the expression level of Lj-BLNK transcript was the highest in the lymphocyte-like cells, about 10-fold increase relative to controls (P<0.05). Substantial increases of Lj-BLNK transcripts were also found in kidney and heart; 7 and 2.5 folds increases relative to controls (P<0.05), respectively. The significant up-regulation in various immune associated tissues such as the lymphocyte-like cells, kidney and heart after challenged with LPS indicated that Lj-BLNK may play an important role in immune reaction of lampreys.

In order to investigate the phylogenetic relationship of vertebrate BLNKs, the metazoan genome databases were analyzed to mine the homologues of BLNK with online programs of BLAST and BLAT. It showed that BLNK genes were only existed in vertebrates and Lj-BLNK was the original one found in agnathans. The phylogenetic tree was reconstructed by NJ method with 25 homologues identified from agnathans to mammalian, and an Uncharacterized LOC575645 protein of sea urchin (Strongylocentrotus purpuratus) which contains a SH2 domain (30% identity with Gallus gallus) was used as the out group. The topology of the resulted NJ tree (Supplementary Fig. S2) showed that, BLNKs of jawed vertebrates were unequivocally grouped into several clusters in accordance with their
evolutionary position. The first one (named cluster I) includes BLNKs from mammals, reptiles, birds and amphibians; the second one (named cluster II) includes teleosts and the third one (named cluster III) includes Lj-BLNK only. Cluster I can be further classified into three subgroups, one includes BLNKs from mammals, and the other two include those from reptiles and birds, amphibians, respectively. Phylogenetic analysis indicated that the Lj-BLNK was clustered as the out group of BLNKs from jawed vertebrates and its origin is far earlier than the one from the common ancestor of jawed vertebrates.

In order to explore the evolutionary dynamics of conserved motifs of the BLNK family further, amino acid sequences of BLNKs from jawless and jawed vertebrates were analyzed by the MEME system with parameters described above. There are totally 15 conserved motifs elicited from vertebrate BLNKs (Supplementary Table 2), and they comprise 432 amino acid residues. Most BLNKs from higher Mammals contain all of the 15 motifs except those from Carnivora that lack motif 10, while in lower mammals such as Ornithorhynchus anatinus and Sarcophilus harrisii, the motif 11 and 13 are both absent. The BLNKs in reptiles and birds comprise 11 conserved motifs (motif 3, 4, 14, 5, 6, 9, 8, 2, 12, 1 and 7) and the motif 11 and 13 are both absent too. The BLNKs in Amphibians and fishes share 8 conserved motifs (motif 15, 3, 6, 14, 2, 12, 1 and 7), while that in lamprey there are only 5 conserved motifs (motif 14, 2, 12, 1 and 7) exist. According to the type and distribution of the motifs, motifs 2, 12, 1 and 7 which comprise the SH2 domain exist in all BLNK sequences (Fig. 2). The results revealed that the SH2 domains of BLNKs were conserved throughout the BLNK gene family in vertebrates during gene evolution.

In conclusion, a novel member of BLNK was identified in lamprey. Phylogenetic analysis of the BLNKs indicated that the Lj-BLNK could be regarded as a primary type of BLNK in vertebrates and an ortholog of BLNKs in jawed vertebrate. The BLNK gene came into existence after multiple gene duplications that took place in the emergence of jawless vertebrates. The Lj-BLNK might play an important role in signal transduction, and involve in the immune response of lymphocyte-like cells in lamprey. Since the accurate function of Lj-BLNK remains unknown till now, more thorough studies are needed to elucidate this speculation.

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References


Figure legends

Figure 1. Sequence alignment of Lj-BLANK with other typical BLNKs using Clustal X The GenBank accession numbers are as follows: Homo sapiens: AAH18906; Gorilla gorilla gorilla: XP_004049898; Mus musculus: NP_032554; Gallus gallus: NP_990239; Xenopus laevis:
NP_001082264; *Danio rerio*: NP_998003; *Lampetra japonica*: KF692036. The broken line marks the N-terminal leucine zipper motif, the double dotted line shows an "acidic" region, the trilateral presents 4 conserved tyrosine residues, the solid straight line marks the proline-rich region and the dotted line presents the SH2 domain. Identical (asterisk) and similar (colon) residues are indicated. The conserved sequences are indicated by the box: black, ≥90% identity; gray, 60%–89% identity.

**Figure 2.** Type and distribution of conserved motifs among BLNK homologues from mammals, reptiles, bird, amphibians, teleosts and agnathans using the MEME system. Each number represents a special motif. Repeated numbers in one sequence indicate that the motif represented by the number is present in multiple copies.

**Supplementary Figure S1.** Lj-BLNK mRNA expression is significantly upregulated in the lymphocyte-like cells, heart and kidney after treatment with LPS. The Lj-BLNK mRNA levels were determined using real-time quantitative PCR in various immune associated tissues. Total RNA was extracted from the lymphocyte-like cells, gill, heart, liver intestine, and kidney of lampreys after stimulation with LPS. Lamprey GAPDH served as an internal control to calibrate the cDNA template for all of the samples, and PBS served as a negative treatment control. The significant differences (*P*<0.05) in Lj-BLNK mRNA expression between the challenged group and the control group are indicated with asterisks.

**Supplementary Figure S2.** Phylogenetic tree reconstructed with 25 BLNK homologues based on the NJ method. Bootstrap values are indicated as percentages at the nodes. Mammals, reptiles, bird, amphibians, teleosts, agnathans and echinoderms clades are delineated by vertical brackets at the right. The bar indicates genetic distance. The GenBank accession numbers are as follows: *Gorilla gorilla* gorilla: XP_004049898; *Homo sapiens*: AAH18906; *Macaca mulatta*: XP_001100700; *Ictidomys tridecemlineatus*: XP_005335339; *Bos Taurus*: NP_001039519; *Sus scrofa*: XP_001928268; *Canis familiaris*: XP_543943; *Mustela putorius furo*: XP_004794740; *Felis catus*: XP_003994350; *Rattus norvegicus*: NP_001020938; *Mus musculus*: NP_032554; *Equus caballus*: XP_001500570; *Sarcophilus harrisii*: XP_003755296; *Ornithorhynchus anatinus*: XP_001505857; *Gallus gallus*: NP_990239; *Taeniopygia guttata*: XP_002190670; *Anolis carolinensis*: XP_003224778; *Xenopus*
Xenopus laevis: NP_001082264; Xenopus tropicalis: NP_001072164; Danio rerio: NP_998003; Salmo salar: ACN10516; Takifugu rubripes: XP_003963710; Oryzias latipes: XP_004076950; Lampetra japonica: KF692036; Strongylocentrotus purpuratus: XP_003730298.