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Short Communication

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A novel protein tyrosine kinase Tec identified in lamprey, *Lampetra japonica*

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Abstract

Protein tyrosine kinase Tec, a kind of non-receptor tyrosine kinase, is primarily found to be expressed in T cells, B cells, hematopoietic cells, and liver cells as a cytoplasmic protein. Tec has been proved to be a critical modulator of T cell receptor signaling pathway. In the present study, a homolog of Tec was identified in the lamprey, *Lampetra japonica*. The full-length *Tec* cDNA of *L. japonica* (*Lja-Tec*) contains a 1923 bp open reading frame that encodes a 641-amino acid protein. The multi-alignment of the deduced amino acid sequence of Lja-Tec with typical vertebrate Tecs showed that it possesses all conserved domains of the Tec family proteins, indicating that an ortholog of Tec exists in the extant jawless vertebrate. In the phylogenetic tree that was reconstructed with 24 homologs of jawless and jawed vertebrates, the Tecs from lampreys and hagfish were clustered as a single clade. The genetic distance between the outgroup and agnathan Tecs' group is closer than that between outgroup and gnathostome Tecs' group, indicating that its origin was far earlier than any of the jawed vertebrates. The mRNA levels of *Lja-Tec* in lymphocyte-like cells and gills were detected by real-time quantitative polymerase chain reaction. Results showed that it was significantly upregulated under stimulation with mixed pathogens. This result was further confirmed by western blot analysis. All these results indicated that Lja-Tec plays an important role in immune response. Our data will provide a reference for the further study of lamprey Tec and its immunological function in jawless vertebrates.

Key words: tyrosine-protein kinase Tec, *Lampetra japonica*, expression pattern, molecular evolution

Introduction

The Tec (tyrosine kinase expressed in hepatocellular carcinoma) family kinases consist of five members: Tec, Btk, Bmx/Etk, Rlk/Txk, and Itk/Emt/Tsk. It is a subfamily of non-receptor protein tyrosine kinases (nrPTKs) [1,2]. The members of this subfamily are found to be abundantly expressed in hematopoietic tissues, where they are presumed to function in blood cell growth and differentiation [3]. The first member of the Tec family is Tec, which is cloned from human liver cancer cells [1]. The protein sequence of Tec is characterized by four domains including a pleckstrin homology domain (PH), an Src homology (SH)3 domain, an SH2 domain, and a tyrosine kinase domain (TKD) [4]. In contrast to receptor protein tyrosine kinases (rPTKs), the lack of an N-terminal hydrophobic transmembrane structure makes Tec to be a cytoplasmic PTK [5]. Tec was reported to be an important target molecule in signal transduction process of various cytokines, accessory molecules, and rPTKs [6]. It can also be regulated by a lot of nrPTKs, such as proto-oncogene protein tyrosine kinase Src (c-Src), Janus kinase (JAK), spleen tyrosine kinase (Syk), and focal adhesion kinase (FAK) family kinases [7]. Tec has also been proved to regulate different biological processes in various cells by adjusting several key signaling pathways such as phosphatidylinositol 3-kinase (PI3K) pathway, phospholipase C (PLCγ) pathway [8,9], and protein

kinase C pathway [10]. In these biological processes, Tec has been shown to be expressed in human T lymphocyte cells [11,12]. During lymphocyte development and activation, Tec plays an important role in the downstream pathways of T cell receptor (TCR) [13,14]. According to a previous study, Tec is activated after TCR/CD3 or CD28 ligation, and it interacts with CD28 receptor in an activation-dependent manner in T cells [15].

Agnathans, represented by lampreys and hagfish, are the oldest vertebrates currently proved to possess adaptive immune defenses [16,17]. They use variable lymphocyte receptors (VLRs) as counterparts of the immunoglobulin-based receptors that jawed vertebrates used for antigen recognition [18,19]. Three types of receptors, VLRA, VLRB, and VLRC, have been identified in VLRA$^+$, VLRB$^+$, and VLRC$^+$ lymphocyte-like cells of lampreys, which are presumed to be equivalent to gnathostome αβ T cells, B cells, and γδ T cells, respectively [20]. In contrast to the extensive studies of Tec in jawed vertebrates, little is known about the existence and phylogenetic relationship of Tec in jawless vertebrates. In the present study, for the first time, we reported on the molecular cloning and characterization of a Tec homolog (Lja-Tec) from *Lampetra japonica* based on the expressed sequence tag (EST) analysis of the cDNA library of lymphocyte-like cells. The transcription and distribution patterns of *Lja-Tec* were also investigated in order to confirm its importance in lymphocyte-like cell immune response.

Materials and Methods

Animals and stimulation by pathogens

The handling of lampreys and all experimental procedures were approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit No. SYXK2004—0029). Adult lampreys (*L. japonica*) were collected from Tongjiang Valley (Jiamusi, China) in December 2013. Adult lampreys (200–220 g in weight) were divided into two groups (20 per group), respectively. The lampreys in the experimental group were intraperitoneally injected with 100 μl mixed pathogens, whereas the control lampreys were injected with 100 μl saline only. The mixed pathogens were made by mixing saline with equal amount (1×10^7 cfu/ml each) of *Escherichia coli* DH5α, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* (TaKaRa, Dalian, China). The animals were killed on the third day after two injections at a 7-day interval, and the dissected tissues were immediately placed in an RNA protector (TaKaRa) and stored at −20°C.

Amplification of the cDNA fragment of *Lja-Tec* by RACE

Construction of a cDNA library from *L. japonica* lymphocyte-like cells and EST sequencing were carried out in our laboratory (data not shown). A Tec homolog was found through the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The outer primer, the inner primer of 3′-RACE, and the specific primers of the open reading frame (ORF) region listed in **Table 1** were designed on the basis of the EST of *Lja-Tec* and synthesized by TaKaRa Biotech (Dalian, China). The RNAiso (TaKaRa) was used for total RNA isolation, according to the manufacturer's instructions. The total RNA samples were dissolved in diethypyrocarbonate-treated water and stored at −80°C. The first strand of 3′ RACE-cDNA template was synthesized from 3 μg of total RNA by reverse transcriptase (TaKaRa), according to the manufacturer's instructions. *Taq* DNA polymerase (TaKaRa) was used to amplify the C-terminal region of *Lja-Tec* with the outer and inner primers (**Table 1**) under the following cycling conditions: 94°C for 5 min, followed by 30 amplification cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 150 s, and a final extension step at 72°C for 10 min. Polymerase chain reaction (PCR) products were analyzed by 2% agarose gel electrophoresis. The target PCR product was purified, cloned into a pMD19-T vector by using a DNA ligation kit (TaKaRa), transformed into *E. coli* DH5α, and then subject to DNA sequencing (TaKaRa).

Table 1. Primers used in this study

Name	Sequence
Primers designed for 3′-RACE	
Lja-Tec-outer	5′- CTGTGAATCATCAGAGTATCGGGGC-3′
Lja-Tec-inner	5′-CTCGGGCAGTGGAAAAGACAAAAGT-3′
Primers designed for ORF cloning	
5′-Lja-Tec	5′-ATGAGTAGGGAGGTGCTGCT-3′
3′-Lja-Tec	5′-CTACTCGCTGTAGTCCTG-3′
Primers designed for real-time PCR	
Lja-Tec (upstream)	5′-ATGAGTAGGGAGGTGCTGCT-3′
Lja-Tec (downstream)	5′-CGGTCCAGTGTTTTGCTC-3′
GAPDH (upstream)	5′-ACCAACTGCCTGGCTCCT-3′
GAPDH (downstream)	5′-TCTTCTGCGTTGCCGTGT-3′

Real-time quantitative PCR

The total RNA samples were extracted from supraneural myeloid bodies, gills, lymphocyte-like cells, kidneys, or hearts of lampreys using RNAiso reagent and then treated with DNase I (TaKaRa) to remove contaminated DNA. The reverse transcription was conducted with the PrimeScript™ RT reagent kit (TaKaRa). Real-time quantitative PCR was performed with the TaKaRa SYBR® PrimeScript RT-PCR kit. According to the manufacturer's protocol, 1× SYBR Premix Ex Taq, 0.6 μl of each specific primer (**Table 1**), and 2 μl of cDNA (100 ng/μl) in a final volume of 25 μl were used. The primer efficiency was analyzed in serial 10-fold dilutions of cDNA by calculating the regression line slope, which was defined as the cycle threshold (Ct) versus the cDNA relative concentration. The *GAPDH* was used as an internal control. The relative mRNA expression levels among groups were normalized against co-amplified *GAPDH* of each sample and then calibrated to the average expression levels of the negative control samples of lymphocyte-like cells. The amplification was performed under the following condition: initial denaturation at 95°C for 5 s to activate the DNA polymerase, followed by 40 cycles of 5 s at 95°C, 30 s at 60°C, 30 s at 72°C, and a final extension step at 65°C for 15 s. Each sample was analyzed in triplicate.

Sequence alignments and conserved motif analyses

The Lja-Tec amino acid sequence was used as query to search for the homologous sequences by BLAST in several databases such as NCBI and the Ensemble Genome Browser (http://www.ensemble.org/). The ORFs of some Tec homologs identified from their genomic sequences were deduced by BLASTN program with default settings [21]. The protein structural domains/regions were predicted with SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/). The amino acid sequences of Tecs were aligned with Clustal X 1.81 [22]. The phylogenetic tree was constructed with MEGA 4.0 [23]. The conserved motif analyses were performed using the online tool MEME (Multiple Em for Motif Elicitation, version 4.9.0, http://meme.nbcr.net/meme/) [24]. The motif widths were set to short lengths, ranging

from 3 to 20 amino acids, the number of different motifs was defined as 50, and the default settings were used for other parameters.

Western blot analysis

The immune defense-related tissues, including supraneural myeloid bodies, gills, lymphocyte-like cells, kidneys, and hearts, were isolated from the animals treated with mixed pathogens or control group. The tissues were homogenized by Dounce Tissue Grinders (Kimble Chase Life Science and Research Products LLC, New Orleans, USA). Total proteins extracted from tissues mentioned earlier were subject to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, USA) by a semi-dry transfer unit (Jingmai Biotech, Dalian, China). Membranes were blocked overnight with 5% fat-free milk at 4°C and incubated with rabbit anti-Lja-Tec antibody (1:500; Sangon Biotech, Shanghai, China) for 1 h at 37°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5000; Sangon Biotech) in 5% fat-free milk for 30 min at 37°C. After being washed with PBST (PBS containing 1% Tween-20), the signals were revealed by using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions. The optical density data were obtained from three independent experiments and were normalized to the signal intensity of β-actin, which was detected by an anti-β-actin antibody (1:5000; Abcam, Cambridge, UK) and then calibrated to levels in untreated samples.

Statistical analysis

Data were expressed as the mean ± SEM. The differences between the two groups were analyzed through Student's *t*-test in SPSS statistical software package. Differences were considered to be statistically significant at $P < 0.05$.

Results

Identification of Tec homolog in *L. japonica*

An EST sequence, which is homologous to human Tec in its N-terminal region, has been identified from a cDNA library of lymphocyte-like cells isolated from *L. japonica* previously. A cDNA fragment (accession no. KM255115) with 3029 nt that covered *Lja-Tec* ORF was amplified by 3′-RACE PCR. The cDNA fragment contains a 69 bp 5′-untranslated region (UTR), a 1037 bp 3′-UTR, and a 1923 bp ORF which encodes a polypeptide of 641 amino acids with an estimated molecular mass of 73.2 kDa. There is neither signal sequence nor the transmembrane domain in Lja-Tec, indicating that the Lja-Tec should be an intracellular or secretion protein.

The multiple sequence alignment of Lja-Tec with several typical Tecs from mammalian to teleost showed that it has ~53% identity with Tecs from *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus laevis*, and *Taeniopygia guttata* in their amino acid sequences (**Fig. 1**). Like the typical Tecs, the Lja-Tec also contains an N-terminal PH domain (Val_5-Asn_{115}), an SH3 domain (His_{198}-Thr_{254}), an SH2 domain (Ser_{261}-Gly_{347}), and a C-terminal TKD (Leu_{381}-Ile_{630}) that are characteristics of Tec. The PH, SH3, SH2, and TKDs, which cover 91% of the Lja-Tec sequence, possess ~43%, 50%, 54%, and 67% sequence identity with the corresponding domains of human Tec, respectively. Based on these protein sequences, we conclude that Tec exists in agnathans, which occupy the most ancient taxonomic position in vertebrates.

Phylogenetic analyses of Tec proteins in vertebrates

The amino acid sequences of the Tec homologs were compared by using the BLAST and the BLAST-like Alignment Tool (BLAT). To examine the evolutionary relationship of Lja-Tec in jawed vertebrates and invertebrates, phylogenetic analysis was conducted by using the neighbor-joining (NJ) methods. The phylogenetic tree was reconstructed with 24 homologs identified from agnathans to mammalian, and a protein of sea urchin (*Strongylocentrotus purpuratus*), which contains an SH2 domain, was used as the outgroup. The bootstrap values of each branch including Lja-Tec all exceeded 63% in the NJ phylogenetic tree (**Fig. 2**). The topology of the resulting NJ tree (**Fig. 2**) revealed that Tecs of jawless and jawed vertebrates including mammals, birds, reptiles, amphibians, teleosts, and agnathans (lampreys and hagfish) were unequivocally grouped into several clusters in accordance with their evolutionary position, and the primary Tec-like gene emerged early during the deuterostome radiation. Nevertheless, phylogenetic analysis based on the full sequences alignment clearly indicates that the agnathans' Tec-like sequences are the most primary orthologs to the vertebrates Tecs.

The molecular evolution profile of Tec-conserved domains

In order to explore the evolutionary dynamics of the domains that characterize Tecs, the analysis was conducted on the basis of the distribution profile of the conserved motifs identified by the online tool MEME with parameters described earlier. A total of 18 sequences of the Tec family were selected from NCBI databases. Among them, 17 Tecs were from jawless or jawed vertebrates including lampreys, fishes, amphibians, reptiles, birds, and mammals, the remaining one was a Tec-like identified in *S. purpuratus* which is the only ortholog of Tecs in invertebrate genomes available. The distribution profiles of the conserved motifs identified by MEME on TKDs are almost the same within these orthologs (data not shown). It means that the TKD is the most conserved domain of the Tec family. There are 17, 9, and 8 conserved motifs in PH, SH3, and SH2 domains, respectively (**Table 2** and **Fig. 3**). Tecs from birds and mammals contain all of the 34 conserved motifs. This means that the four characteristic domains of Tecs have developed into a complete form in warm-blood animals. There are 13 most highly conserved motifs in Lja-Tec, with motifs 2, 4, 6, 11, 13, and 15 in the PH domain; motifs 18, 20, and 22 in the SH3 domain; and motifs 28, 29, 31, and 33 in the SH2 domain. Compared with Tecs of fishes, although lack at least half of the conserved motifs, the Lja-Tec has all the four basic elements that characterize Tecs. It is easy to note that the conserved motifs of Tecs become more and more numerous from fishes to reptiles in the cold-blood animals world. Only one conserved motif (motif 12) in the PH domain is absent in the Tecs of reptiles. In Tecs of amphibians, two motifs (motifs 9 and 12) in the PH domain and one (motif 23) in the SH3 domain are absent. The Tecs of fishes are short of at least four motifs (motifs 7, 9, 12, and 17) in their PH domains and one (motif 26) in their SH3 domains. These data revealed that the SH2 domain is the second conserved element in this family and that the PH domain is the most diverse element among the domains that characterize Tecs from other non-receptor PTKs. From the distribution profile of the conserved motifs from lampreys to mammals, it can be concluded that the *Tec* gene evolved from primary to modern forms through a short insertion or deletion approach.

The transcription and distribution pattern of *Lja-Tec* in immune defense-related tissues

To determine the transcription pattern of *Lja-Tec* in immune defense-related tissues, the real-time quantitative PCR was performed (**Fig. 4**). In the negative control group, the relative expression level of *Lja-Tec* in gills is the highest, which is 1.81-, 1.74-, 1.27-, and

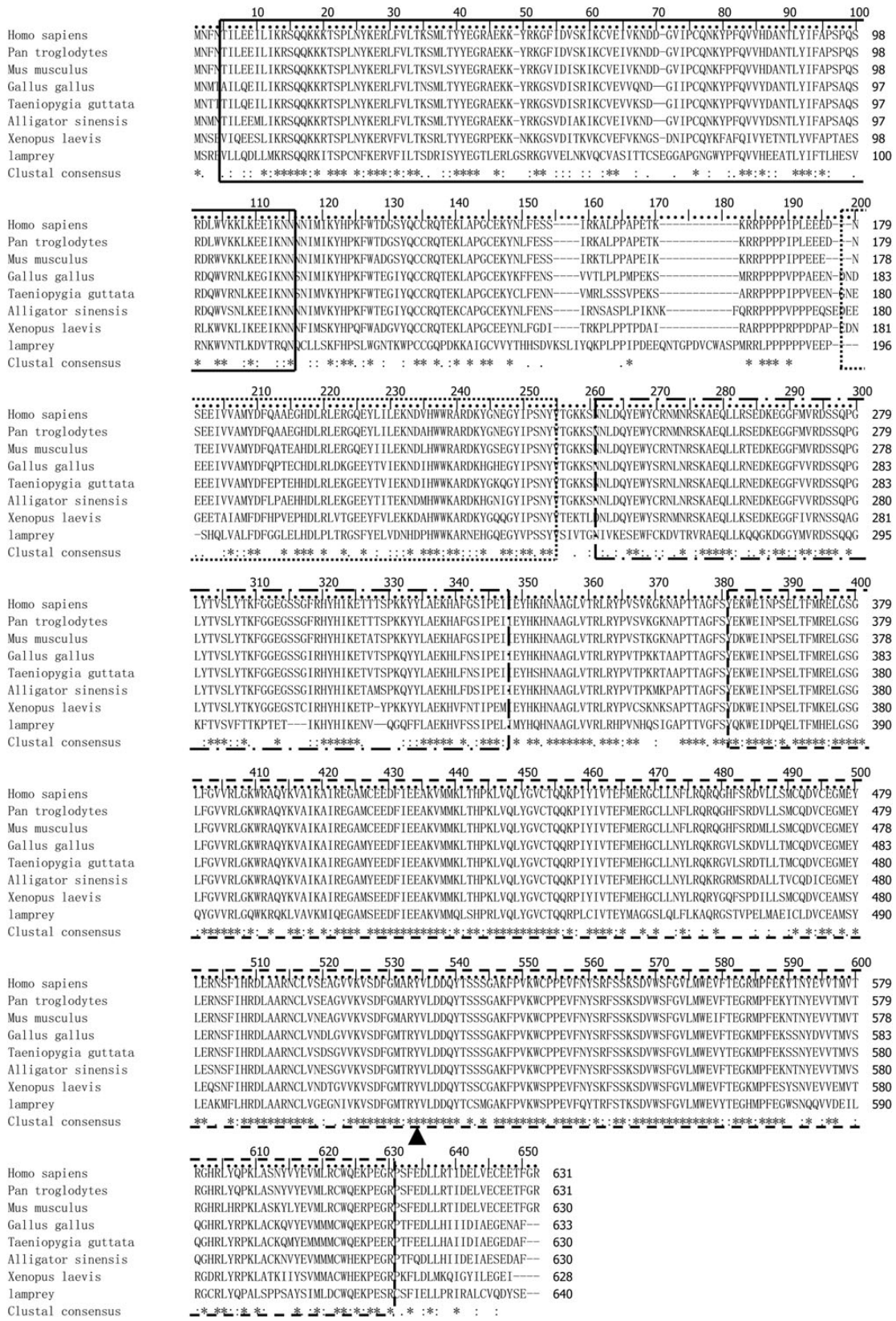

Figure 1. Sequence alignment of Lja-Tec with typical Tecs of other species by using Clustal X The accession numbers of the amino acid sequences are as follows: *H. sapiens*: NP_003206; *Pan troglodytes*: XP_517310; *M. musculus*: NP_001106931; *Alligator sinensis*: XP_006019065; *G. gallus*: NP_001025543; *T. guttata*: XP_002196927; *X. laevis*: NP_001089927; and *L. japonica*: KM255115. The triangle presents a conserved tyrosine residue. The solid straight line marks the N-terminal PH domain, the dotted line presents the SH3 domain, the broken and dotted line marks the SH2 domain, and the broken line marks the TKD. Identical (asterisk) and similar (colon) residues are indicated.

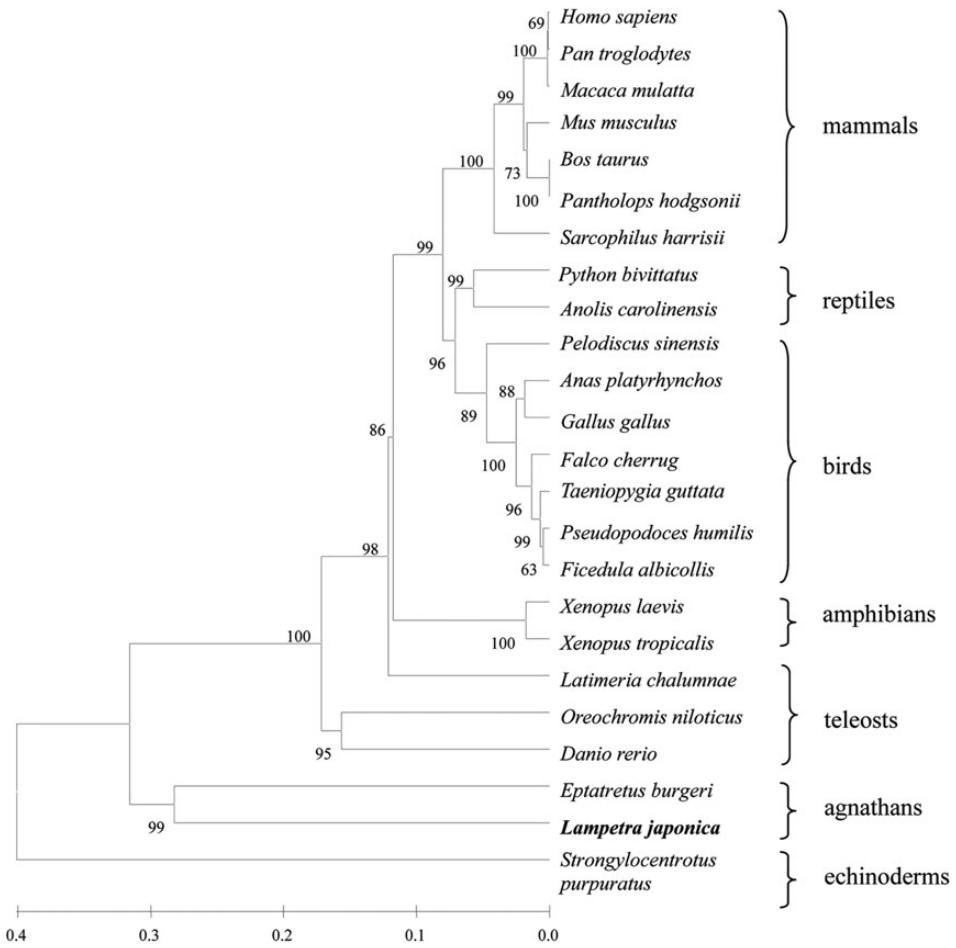

Figure 2. Phylogenetic relationship of Lja-Tec with 24 Tec homologs based on the NJ method The number at each node indicates the percentage of bootstrapping after 1000 replications. Mammals, birds, reptiles, amphibians, teleosts, and agnathans (lampreys and hagfish) and echinoderms are delineated by vertical brackets at the right. The bar indicates genetic distance. The GenBank accession numbers are as follows: *H. sapiens*: NP_003206; *P. troglodytes*: XP_517310; *Macaca mulatta*: XP_001103213; *M. musculus*: NP_001106931; *Bos taurus*: XP_002688295; *Pantholops hodgsonii*: XP_005958863; *Sarcophilus harrisii*: XP_003773307; *Python bivittatus*: XP_00743673; *Anolis carolinensis*: XP_008115817; *Pelodiscus sinensis*: XP_006113512; *Anas platyrhynchos*: XP_005028154; *G. gallus*: NP_001025543; *Falco cherrug*: XP_005433656; *T. guttata*: XP_002196927; *Pseudopodoces humilis*: XP_005517785; *Ficedula albicollis*: XP_005045321; *X. laevis*: NP_001089927; *Xenopus tropicalis*: XP_002933505; *Latimeria chalumnae*: XP_005998605; *Oreochromis niloticus*: XP_003456492; *Danio rerio*: NP_001108215; *Eptatretus burgeri*: BAD52302; *L. japonica*: KM255115; and *S. purpuratus*: XP_003730499.

1.15-folds as high as those in kidneys, supraneural myeloid bodies, lymphocyte-like cells, and hearts, respectively. After being challenged with mixed pathogens, the relative expression levels of *Lja-Tec* increased significantly in lymphocyte-like cells and gills (about 2- and 1.5-fold increase relative to their corresponding controls, respectively, $P < 0.05$). Only a slight increase was detected in the supraneural myeloid bodies and kidneys, whereas no difference was found in hearts.

Western blot analysis was performed with an anti-Lja-Tec antibody to determine the distribution pattern of Lja-Tec in the tissues mentioned earlier. The β-actin of *L. japonica* was detected as a band at 42 kDa and was used as the internal control (**Fig. 5A**). A 73 kDa band corresponding to the native Lja-Tec protein was recognized by an anti-Lja-Tec antibody in lymphocyte-like cells from saline-treated and mixed pathogens-stimulated *L. japonica* (**Fig. 5A**). By normalizing the optical density of the internal controls, the expression level of the Lja-Tec protein detected was about 6-fold higher in lymphocyte-like cells from mixed pathogens-treated animals than that from non-treated animals (**Fig. 5B**).

Discussion

Multiple nrPTKs are activated after TCR stimulation. These nrPTKs are responsible for the tyrosine phosphorylation of the TCR, the kinases themselves, and a number of cellular substrates [25,26]. Tec, similar to other nrPTKs, is an integral component of T cell signaling and plays an important role in T cell activation. One of the important Tec domains is the kinase catalytic domain, TKD. TKD has two lobes of different sizes in structure. Adenosine triphosphate binds to the small lobe, whereas the protein substrate binds to the large one. After the binding, catalysis of phosphate transfer occurs in a cleft between these two lobes [27]. Compared with the kinase activity center, the other three domains (PH, SH3, and SH2) of Tec also have specific binding substrates of their own. The SH3 domains have been proved to interact with proline-rich sequences of other proteins, whereas SH2 domains typically bind a phosphorylated tyrosine residue in the context of a longer peptide motif within a target protein [28,29]. The PH domain can interact with products of PI3K, as well as other phosphoinositides and probably other proteins [30].

Table 2. Conserved motifs discovered among Tecs from lampreys, fishes, amphibians, reptiles, birds, and mammals using the MEME software

Motifs	Width	Best possible match	Motifs	Width	Best possible match
1[a]	5	TILEE	18	5	HDLRL
2	15	KRSQQSPLNYR[LV]FVL	19	10	[RKT]G[EQ]EY[LI]EKND
3	5	TKSML	20	5	HWW[KR]A
4	5	TYY[ED]G	21	5	RDKYG
5	5	R[AP]EKK	22	10	GYIPSNYVT[GE]
6	5	KG[SF][VI]D	23	4	KKSN
7	3	[IV]SKIK	24	5	NLDQY
8	5	CVE[IV]V	25	5	WY[CS]RN
9	5	KND[DG][GI]	26	5	[LM]NRSK
10	5	IPCQN	27	5	AEQLL
11	5	FQVV[YH]	28	5	EDKEG
12	3	DAN	29	15	F[MV]VR[DN]SSQPGYTVSL
13	5	LY[IV]FA	30	10	KFGG[ED][SL]SG[IF]R
14	5	PSAQS	31	5	HYHIK
15	5	RD[QR]WV	32	5	PK[QK]YY
16	5	LKEEI	33	20	LAEKHIPE[IL]IYHKHNVTRLR
17	5	N[NS]NIM	34	5	YPV[ST][PT]

[a]Numbers correspond to the motifs described in **Fig. 4**.

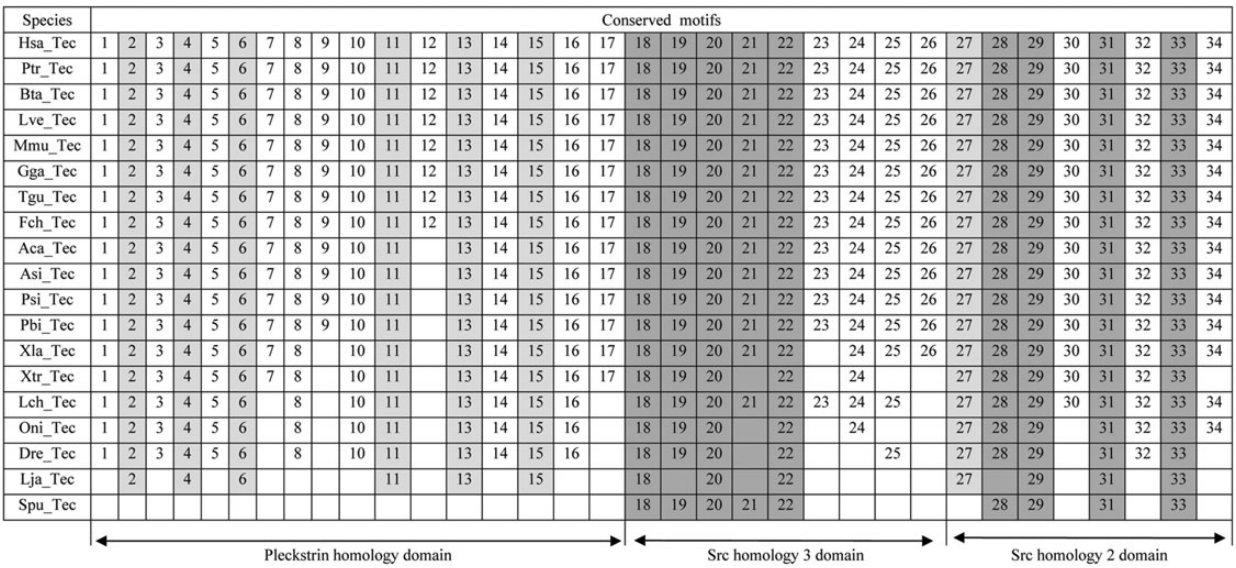

Figure 3. Type and distribution of conserved motifs discovered among Tec homologs from mammals, birds, reptiles, amphibians, teleosts, and lampreys using the MEME system Each number represents a special motif. The shaded part represents that the evolution of the motif is essentially completely conserved.

The agnathostome Lja-Tec also shares the same structural features as the gnathostome Tecs (**Fig. 1**). Especially, it is 43, 50, 54, and 67% identity to human Tecs in PH, SH3, SH2, and TKDs, respectively. The conserved property of PH, SH3, and SH2 domains of the Tec family was further revealed by the MEME system conserved motifs analysis. As shown in **Fig. 4**, Lja-Tec has 4 out of 8 motifs (50%) in the SH2 domain, 3 out of 9 motifs (30%) in the SH3 domain, and 6 out of 17 motifs (35%) in the PH domain. These facts indicated that an ortholog of Tec exists in the extant jawless vertebrate, and there is high divergence between jawless vertebrate Tecs and jawed vertebrate Tecs. In jawed vertebrates, lymphocyte receptors, immunoglobulins, and TCR are the main components of their adaptive immune system. In jawless vertebrates, three subsets of lymphocytes use VLRA, VLRB, and VLRC for antigen recognition, instead of TCR and B cell receptor (BCR) of jawed vertebrates [31]. Thus, there should be differences between the VLRA$^+$, VLRB$^+$, and VLRC$^+$ cell signaling pathways and the B and T cell signaling pathways. The relatively low identities of PH, SH3, and SH2 domains between Tecs from jawless and jawed vertebrates may have two reasons. One possibility is that because of the far genetic distance, the specific binding substrates of these domains in jawless or jawed vertebrates Tecs are genetically different from each other, even if they are homologs. The other possibility may be that their binding substrates are not the same at all.

Gene families, especially those with a large gene repertoire, usually provide invaluable information for the trace of gene and chromosome evolution history [32]. A phylogenetic tree constructed to investigate

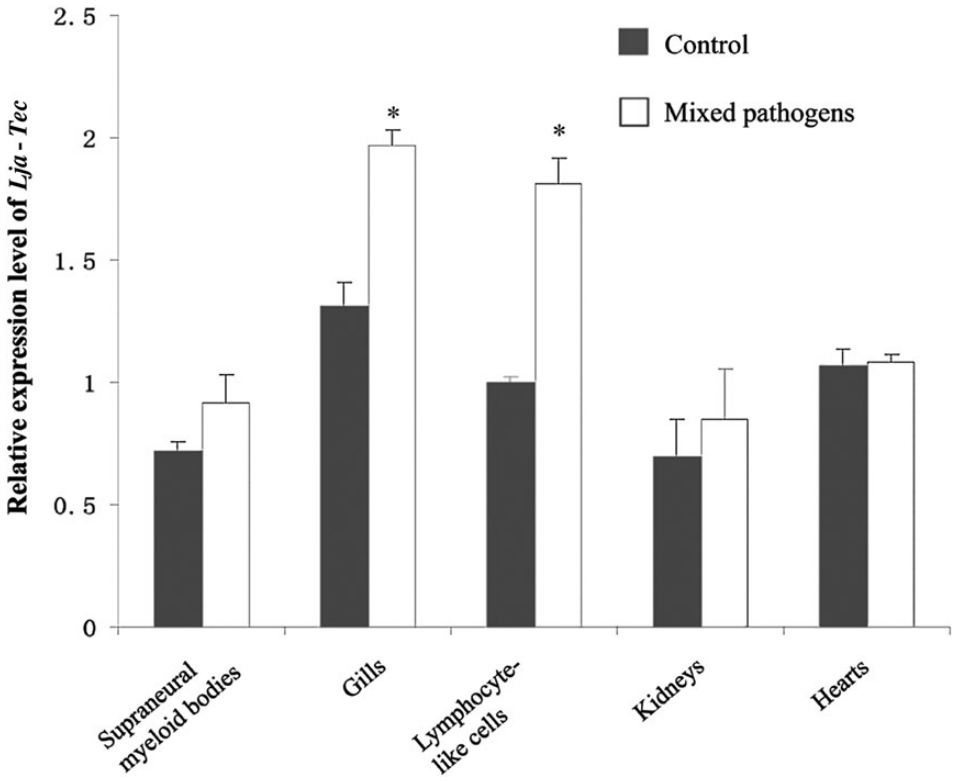

Figure 4. *Lja-Tec* mRNA expression is significantly upregulated in the gills and lymphocyte-like cells after treatment with mixed pathogens The lamprey *Tec* mRNA levels were determined using real-time quantitative PCR in various immune tissues. Total RNA was extracted from the supraneural myeloid bodies, gills, lymphocyte-like cells, kidneys, and hearts of lampreys after stimulation with mixed pathogens. Lamprey *GAPDH* was used as an internal control, and the saline-treated groups served as the negative control groups. The relative mRNA expression levels among groups were normalized against *GAPDH* of each sample and calibrated to the average expression levels of the negative control samples of lymphocyte-like cells. The significant difference ($P < 0.05$) in *Lja-Tec* mRNA expression between the challenged and the control groups is indicated with asterisks.

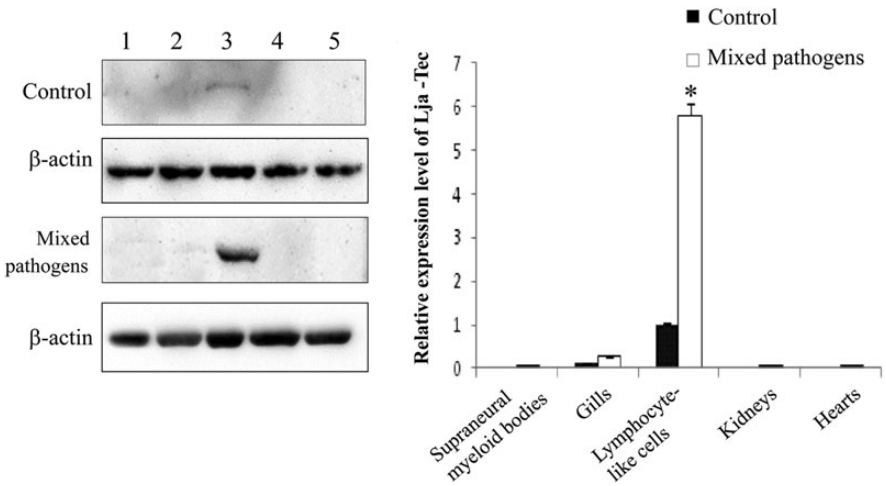

Figure 5. Western blot analysis of Lja-Tec by rabbit anti-Lja-Tec polyclonal antibodies The distribution of Lja-Tec and actin in lamprey tissues from non-treated and mixed pathogen-stimulated groups was determined by the western blotting method. (A) Line 1, supraneural myeloid bodies; line 2, gills; line 3, lymphocyte-like cells; line 4, kidneys; line 5, hearts. (B) Statistical results of three independent experiments are shown. Data were presented as mean ± SEM of three independent experiments. The asterisks indicate statistically significant difference ($P < 0.05$).

the molecular evolution of the Tecs in vertebrates showed that Tecs were clustered into one big clade including those from jawed vertebrates and one distinct jawless vertebrate branch (Lja-Tec and a Tec-like of hagfish) (**Fig. 2**). Although the evolution pattern of the Tec family is in accordance with the classical interpretations of the origin of species, there is a far genetic distance (above 0.4) between Tecs from jawless and jawed vertebrates. From our phylogenetic analysis using full-length sequences, we proposed that Lja-Tec can be regarded as a

primary ortholog of vertebrate Tecs. This novel Tec shares the structural feature and amino acid motif common to other known gnathostome Tecs.

In the present study, *Lja-Tec* was shown to be widely distributed and transcribed in a number of immune-associated tissues and yielded significant upregulation in the gills and lymphocyte-like cells after challenged with mixed pathogens (**Fig. 4**). Western blot analysis further confirmed that the Lja-Tec protein was expressed in the lymphocyte-like cells from both control and mixed pathogens-treated *L. japonica*, and the level of Lja-Tec is upregulated in the lymphocyte-like cells from the mixed pathogens-stimulated group (**Fig. 5**). These results indicated that Lja-Tec participated in the immune response of lampreys. Tec can be activated after BCR or TCR excitation. Besides BCR or TCR, Tec can also be activated by a combination of anti-CD9 and anti-CD38 antibodies which were immature B cell surface molecule [13]. Although *L. japonica* does not have any genuine B/T lymphocytes, it uses VLRs instead of BCR/TCR to trigger signal transduction in lymphocyte-like cells. So, Lja-Tec is most likely to participate in the VLR-mediated immune response of *L. japonica* B ($VLRB^+$) and T ($VLRA^+$ and $VLRC^+$) lymphocyte-like cells.

In summary, we reported the primary characterization of a Tec homolog, Lja-Tec, from lamprey *L. japonica*, a representative of jawless vertebrates. The newly discovered Lja-Tec offers a new paradigm of the vertebrate Tec family origin. Phylogenetic analysis indicated that Lja-Tec could be regarded as a primary type of Tec in jawless vertebrates and an ortholog of Tecs in jawed vertebrates. The vertebrate Tec members shared a common ancestor gene and evolved via short insertion or deletion strategy. As the accurate function of Lja-Tec remains unknown, further studies are needed to clarity its functions.

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