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SHP1 tyrosine phosphatase gets involved in host defense against *Streptococcus agalactiae* infection and BCR signaling pathway in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP1), a kind of protein tyrosine phosphatases (PTPs), is a critical regulator of antigen receptor signal transduction. Signal transduction of BCR is regulated by phosphatases in teleost as in mammals. In this study, SHP1 from Nile tilapia (*Oreochromis niloticus*) (OnSHP1) was identified and characterized, including the expression pattern against bacterial infection and regulation function in BCR signaling pathway. The open reading frame of OnSHP1 contains 1749 bp of nucleotide sequence, encoding a protein of 582 amino acids. The OnSHP1 protein was highly conversed compared to that of other species, including two amino-terminal SH2 domains at the N terminus and a PTP catalytic domain. Transcriptional expression analysis revealed that OnSHP1 was detected in all examined tissues and highly expressed in spleen. The up-regulated OnSHP1 expression was observed in peripheral blood, spleen and anterior kidney following challenge with *Streptococcus agalactiae* or lipopolysaccharide (LPS) *in vivo*, as well as that displayed in leukocytes stimulated with *S. agalactiae* or LPS *in vitro*. Further, after induction with mouse anti-tilapia IgM monoclonal antibody *in vitro*, *OnSHP1* was significantly up-regulated in leukocytes. When spleen leukocytes treated with PTP Inhibitor II *in vitro*, the phosphorylation level of OnSHP1 at the phosphorylation sites (Y^{535} and Y^{557}) and the cytoplasmic free Ca^{2+} concentration were up-regulated significantly. Overall, the findings of this study indicate that SHP1 gets involved in host defense against bacterial infection and BCR signaling pathway in Nile tilapia.

1. Introduction

The balance between the opposing activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulates the signal transduction pathways, which guarantee the normal activity of proliferation, differentiation and death [1,2]. Until now, more than one hundred putative mammalian PTPs are identified, which are the positive or negative specific regulators in signaling pathways [3,4]. It is classified as four groups based on these PTPs substrate specificity and the largest one is Cys-based PTPs, named 'classical' PTPs with true tyrosine-specific. Based on the cellular location, the 'classical' PTPs can be further sorted as transmembrane (TM) and non-TM families [5,6]. Among them, CD45 and CD148 are the representative TM PTPs and as the receptors for some undefined ligands [7]. The non-TM PTPs contain a single PTP catalytic (PTPc) domain for localization or regulation [8] and two PTPs containing Src homology 2 (SH2) domains are identified in mammals, SHP1 and SHP2 [9–11]. Both of them share many structures, such as the two SH2 domains at the N terminus and an inhibitory C-terminal tail. However, the regulatory features of these two enzymes are somewhat different *in vivo*, where SHP1 is accepted as a negative regulator but SHP2 is thought as a positive promotor [4,12–14].

SHP1, encoded by tyrosine-protein phosphatase non-receptor type 6 gene (PTPN6), is a member of the PTP family with two SH2 domains (NH_2-terminal) and a PTPc domain (C-terminal), expressed predominantly in hematopoietic cells of all lineages but low levels in epithelial cells [9,11,15]. The NH_2-terminal SH2 domains act as the PTP binding domains and regulate the interaction of SHP1 with its substrates [4,16]. It is recruited to membrane-bound inhibitory

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receptors via the binding of its SH2 domains to tyrosine-based inhibitory motif (ITIM) within the cytoplasmic domain of a receptor. There is a conserved PTP signature motif, (I/V)HCXAGXXR(S/T)G, in PTPc domain. Two tyrosines in the C-termini of SHP1 (Y^{536} and Y^{564}) can be phosphorylated upon stimuli and might further influence the function and activities of these PTPs [17]. In mice, a splicing mutation in the SHP1 locus results in the *motheaten* (*me/me*) phenotype (have no detectable SHP1 protein), characterized as severe haemopoietic disruption and death at about 2–3 weeks after birth [18]. Another different mutation (by insertion or deletion of a few amino acids within the phosphatase domain) in the SHP1 locus causes *motheaten viable* (me^v/me^v) phenotype, showed dead at 8–12 weeks after birth [19]. SHP1 functions mainly as a negative regulator of signaling pathways in lymphocytes when responding the activation signals by direct binding to either the regulated receptor itself or an associated co-receptor [20]. The available data about the action of SHP1 in B cells indicate that SHP1 down-regulates activation signaling cascades in BCR signaling pathway, and BCR-induced proliferative responses enhanced in SHP1-deficient B cells [21]. Moreover, a phosphatase-inactive SHP-1 increased the calcium (Ca^{2+}) mobilization after B cell antigen receptor engagement [22].

Teleost fish are the most advanced of all fish with true bones and are dominant in both marine and freshwater habitats with an amazing amount of diversity [23]. They contain an adaptive immune system similar in many respects to that of mammals [24]. Until now, the study of SHP1 in teleosts is limited, with a study of the gene clone and the expression in zebrafish (*Danio rerio*) [25]. SHP1 was recruited by phosphorylated ITIM motif of immunoglobulin superfamily protein in jawless vertebrate (lamprey) as well as in teleost fish (carp and channel catfish) [25–27]. No more study about the functional characterization of SHP1 in teleosts is reported to date. In this study, we identified and characterized the functional characterization of SHP1 (OnSHP1) in Nile tilapia (*Oreochromis niloticus*). The open reading frame of OnSHP1 was cloned and analyzed with bioinformatics methods, including multiple sequence alignment and phylogenetic analyses. The expression profiles of OnSHP1 in healthy adult tissues and leukocytes from different immune tissues, as well as when infected by the challenge, *Streptococcus agalactiae* (*S. agalactiae*) or lipopolysaccharide (LPS) *in vivo* and *in vitro*, were investigated. Moreover, the OnSHP1 expression in B cells after stimulation with mouse anti-tilapia IgM (OnIgM) monoclonal antibody (mAb) was explored as well. The role of OnSHP1 tyrosine phosphorylation sites (Y^{535} and Y^{557}) and cell Ca^{2+} mobilization upon induction of the PTP Inhibitor II were determined. Our results suggest that OnSHP1 is likely playing important roles in pathogen infection and BCR signaling pathway in Nile tilapia.

2. Materials and methods

2.1. Identification of OnSHP1

The predicted SHP1 in Nile tilapia (*Oreochromis niloticus*) (accession number XM_005455447.2) was found in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) by basic local alignment search tool (BLAST). The open reading frame (ORF) sequence was amplified from the anterior kidney cDNA sample [28] with specific primers (Table 1). Polymerase chain reaction (PCR) was performed using DNA polymerase (TaKaRa, Japan) under the following conditions: 95 °C for 3 min, then 35 cycles of 95 °C for 30 s, 60 °C for 3 s, 72 °C for 2 min, followed by a final extension at 72 °C for 10 min and 4 °C forever by BioRad T100 (BioRad, USA). The PCR product was inserted into pMD18-T vector (TaKaRa, Japan) and transformed into the competent *Escherichia coli* (*E. coli*) DH5α (Tiangen, China). Two positive clones were selected and sequenced at TSINGKE Biological Technology Corporation (Guangzhou, China).

The website of ExPASy Molecular Biology Server (http://us. expasy.org), NCBI and Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/) were used to analyze protein structure. The three-dimensional structure was predicted using SWISS-MODEL (http://swissmodel.expasy.org/). The multiple sequence alignment and similarity analysis were performed by DNAMAN software analysis (Lynnon Biosoft, USA) and NCBI. The phylogenetic analysis was performed with Molecular Evolution Genetics Analysis (MEGA) software (version 7.0) and the neighbor-joining method with 1000 bootstraps were used.

Table 1
The primers used in this study.

Primers	Sequence (5′-3′)	Application
SHP1-F	ATGGTTCGATGGTTCCACAGAGATA	gene sequence
SHP1-R	TCTCTTTTTCACGGAGCCACTCTTC	gene sequence
qSHP1-F	CTACAAACGCAAAGGCATCG	qRT-PCR
qSHP1-R	GCCATCCTGCTGCTTCTGT	qRT-PCR
qCD79a-F	CATCATAACAAAACTCAGGAGG	qRT-PCR
qCD79a-R	GTAGACACGCAGGTAGGTTCCAT	qRT-PCR
qLYN-F	GATGCCTCAGCCCGACAACT	qRT-PCR
qLYN-R	TGTCCCTCTGTGGCGGTGTA	qRT-PCR
qBLNK-F	CCTCCCCAAAGCCTCCTGAA	qRT-PCR
qBLNK-R	GCGAAACAAGGCATCGTCAG	qRT-PCR
β actin-F	AACAACCACACACCACACATTTC	qRT-PCR
β actin-R	TGTCTCCTTCATCGTTCCAGTTT	qRT-PCR

2.2. Fish maintenance

Healthy Nile tilapia (*Oreochromis niloticus*) of about 100 ± 10 g were obtained from the Guangdong Tilapia Breeding Farm and maintained at the South China Normal University (Guangzhou, China) by an automatic filtering aquaculture system at 28 ± 2 °C with a recirculating water system. Fish were fed one time a day with a commercial diet (Guangdong, China). All the experiments animal protocols were complied with the guidelines of the University Animal Care and Use Committee of the South China Normal University.

2.3. Tissue collection

Healthy fish were anesthetized in water with 0.04% 3-aminobenzoic acid ethyl ester (MS-222; Aladdin, China), then the peripheral blood from the caudal vein was extracted with a heparinized needle/syringe. Other tissues, including spleen (SPL), peripheral blood (PBL), intestine, anterior kidney (AK), thymus, gills, terminal kidney, liver, brain, heart, muscle and skin were collected. They were frozen by liquid nitrogen immediately and stored at −80 °C before use [29–31].

The infection experiment was performed by peritoneal injection with live *S. agalactiae* (ZQ1901) 100 μL (1×10^7 CFU/mL) and LPS (1 mg/mL) (*E. coli* 055:B5, Sigma, USA) in sterile PBS, respectively (n = 3) [28,29,32]. The control stimulation was injected with sterile PBS. Fish were anesthetized first, then PBL, SPL and AK tissues were collected at 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h post-infection (p.i.). Collected and placed them in liquid nitrogen and kept at −80 °C before usage [29].

2.4. OnSHP1 expression in tissues

Total RNA was prepared from tissue samples using the Trizol (Vazyme, China) and quantified with Nanodrop 2000 spectrophotometer (Thermo, USA). The RNA was eluted in RNase-free water, and cDNA was synthesized by 1 μg total RNA using Hifair™II 1st StrandcDNA Synthesis SuperMix Kit (gDNA digester plus) (YEASEN, China) following manufacturer's directions. The obtained cDNAs were diluted ten folds in nuclease-free water and stored at −20 °C.

To explore the expression levels of *OnSHP1* in healthy fish, quantity real-time PCR (qRT-PCR) was performed in ABI 7500 Sequence Detection System instrument (Applied Biosystems, USA) using Hieff®

qPCR SYBR Green Master Mix (Low Rox Plus) (YEASEN, China) and specific primers (Table 1). All the samples were measured in duplicate under the following conditions: 3 min at 95 °C, followed by 40 amplification cycles (15 s at 95 °C and 2 min at 60 °C) [29,31]. A dissociation curve was used to ensure only a single product was amplified. The reaction without templates were performed as negative controls in all experiments. The expression of *OnSHP1* in healthy fish was normalized to that of tilapia *β* actin and transcription levels calculated using the $2^{-\Delta Ct}$ [33]. The relative expression in each tissue was compared to the expression in skin and the results were shown as the ratio. For the *S. agalactiae* or LPS infected groups, the relative expression of *OnSHP1* was presented as the ratio of infection group to control group [29].

2.5. Leukocyte isolation and simulation from Nile tilapia

Whole blood from healthy tilapia were extracted from the caudal vein with a heparinized needle/syringe when the fish were anesthetized with 0.04% 3-aminobenzoic acid ethyl ester (MS-222; Aladdin, China). Then, the leukocytes from PBL, SPL and AK were obtained as we previous did [29–31] with some modification. The leukocytes were obtained after purified with Histopaque 1077 (Sigma, USA). Trypan blue (0.4%; Sigma, USA) was used to determine the cell quantity and viability.

To investigate the expression of *OnSHP1* in leukocytes separated from PBL, SPL and AK, cells (1×10^6) were suspended in 1 mL Trizol (Vazyme, China) for RNA extraction or freezing at −80 °C for Western blotting. For infection *in vitro* study, a concentration of 4×10^6 cells/mL in RPMI-1640 with 10% fetal bovine serum (FBS; Gibco, USA) was re-suspended and added to the 96-well cell culture plates with 100 μL per well (4×10^5 cells) (Thermo, USA). The formalin-inactivated *S. agalactiae* [28] and LPS were added to the well with the final concentration 4×10^7 CFU/mL and 100 μg/mL, respectively. After incubated the stimulus, the cells were cultured at 25 °C. At 0 h, 3 h, 6 h, 12 h, 24 h and 48 h p.i., cells were collected in 1 mL Trizol (Vazyme, China) for RNA extraction and cDNA preparation. To explore the role of OnSHP1 in B cell singling pathway, leukocytes were incubated with mouse anti-OnIgM mAb [28,29,32], 2 μg/mL final concentration. The cells were collected at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h and 48 h p.i. in 1 mL Trizol (Vazyme, China) for RNA extraction or freezing at −80 °C for Western blotting directly. The control stimulation was incubated with sterile PBS.

The qRT-PCR was performed with specific primers (Table 1) as described in section *2.4*, and the relative expressions of *OnSHP1* were calculated using the $2^{-\Delta Ct}$ and performed as the ratio of stimulated group to control group.

2.6. Effects of PTP inhibitor II on OnSHP1

PTP Inhibitor II (Selleck Chemicals, China) is a covalent inhibitor of PTP by binding with SHP1 (SH2 domain) [34]. According to the product instruction, PTP Inhibitor II was dissolved in DMSO (Sigma, USA) with the concentration of 250 mM. The leukocytes from SPL isolated as described in *2.5* were incubated with the inhibitor at the final concentration of 100 μM for half an hour at 25 °C. The inhibitor dose chosen was based on mammalian studies and has been tested in our preliminary experiments where it was deemed effective. Then, the groups were challenged with mouse anti-OnIgM mAb (2 μg/mL) or LPS (10 μg/mL, was taken as a positive control) for 1 h, 3 h and 6 h. The cells were collected and lysed with Trizol for RNA extraction or freezing directly for Western blotting. The same dose of DMSO was taken as the control group here and only incubated inhibitor was performed as well.

2.7. Western blotting

The collected leukocytes for Western blotting were performed as our previous study described [30,31]. Briefly, 20 μL lysed cell in loading buffer (with *β*-mercaptoethanol; Sigma, USA), equaled to about 5×10^5 cells, were electrophoresed on 12% SDS-PAGE gels, then transferred to 0.22 μm polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were washed three times with tris-buffered saline supplemented with 0.1% Tween (TTBS), followed blocking with 0.5% bovine albumin (BSA) in TTBS for 1 h at 37 °C. After washed three times, the membranes were covered with rabbit anti-human/mouse/rat phospho-SHP1 (Tyr536) polyclonal Ab (1:1000) (Affinity Biosciences, China; in order to detect the phosphorylation level of phospho-OnSHP1 Y^{535}), rabbit anti-human/mouse/rat phospho-SHP1 (Tyr564) polyclonal Ab (1:1000) (Affinity Biosciences, China; in order to detect the phosphorylation level of phospho-OnSHP1 Y^{557}), and goat anti-human/mouse/rat SHP1 IgG polyclonal Ab (1:1000) (R&D Systems, USA). The internal control was *β* actin, detected by mouse anti-*β* actin mAb (YEASEN, China). After washed three times, the corresponding secondary antibody labeled with horseradish peroxidase (HRP) were added to cover the membrane, including goat anti-rabbit IgG polyclonal Ab (1:2000; Southern Biotech, USA), goat anti-mouse IgG mAb (1:2000; Southern Biotech, USA) and donkey anti-goat IgG polyclonal Ab (1:5000; YEASEN, China). After washed three times, the protein was visualized by Tanon 5200 Multi (EWELL Biotechnology, China) with the BeyoECL Plus (Beyotime, China). Image J software (National Institutes of Health, USA) was used to analyses the data.

2.8. The cytoplasmic free Ca^{2+} concentration change was analyzed by flow cytometric

For exploring the Ca^{2+} mobilization of the leukocytes incubated with PTP Inhibitor II, the Ca^{2+} indicator Fluo-3 AM (Beyotime, China) was used as the manufacturer's instructions. Fluo-3 AM was dissolved in DMSO with 5 mM concentration. Splenocytes were incubated with PTP Inhibitor II or the same concentration of DMSO (as the control group) for half an hour, and then in the presence or absence of LPS (10 μg/mL) and anti-OnIgM mAb (2 μg/mL) [28,29,32] during 1 h, 3 h and 6 h. The cells were diluted in PBS after each time point, and incubated with Fluo-3 AM at a final concentration of 5 μM for 1 h. The cells were washed three times, and suspended with PBS for 30 min, then analyzed by BD FACS Aria III flow cytometer (BD, USA) under the emission of fluorescence (525 nm).

2.9. Statistical analysis

The significant differences among the groups were defined as different degree significance, where * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$. Figures were made by GraphPad Prism 5 software.

3. Results

3.1. Analysis of the tilapia SHP1amino acid sequence and structure

Based on the predicted nucleic acid sequence of Nile tilapia SHP1 on NCBI, *OnSHP1* sequence was identified with the designed specific primers in Table 1. The ORF of the OnSHP1 gene was 1749 bp encoding a predicted protein of 582 amino acid (Fig. S1A). Protein structure analysis indicated that OnSHP1 was without signal peptide region and transmembrane region. By analyzing the conserved domains of OnSHP1 on NCBI and SMART, there discovered that OnSHP1 contains three conserved domains, two SH2-domains and a PTPc domain (Fig. S1B). Three-dimension structural analysis revealed OnSHP1 contains two SH2 domains, the N-SH2 and C-SH2, and a typical PTPc domain (Fig. S1C) as in humans [35].

Multiple alignment of OnSHP1 with other known mammals (human and mouse), amphibian (frog) and fish (catfish, trout and zebrafish) sequences indicated that the two SH2-domains (N- and C-terminal) and

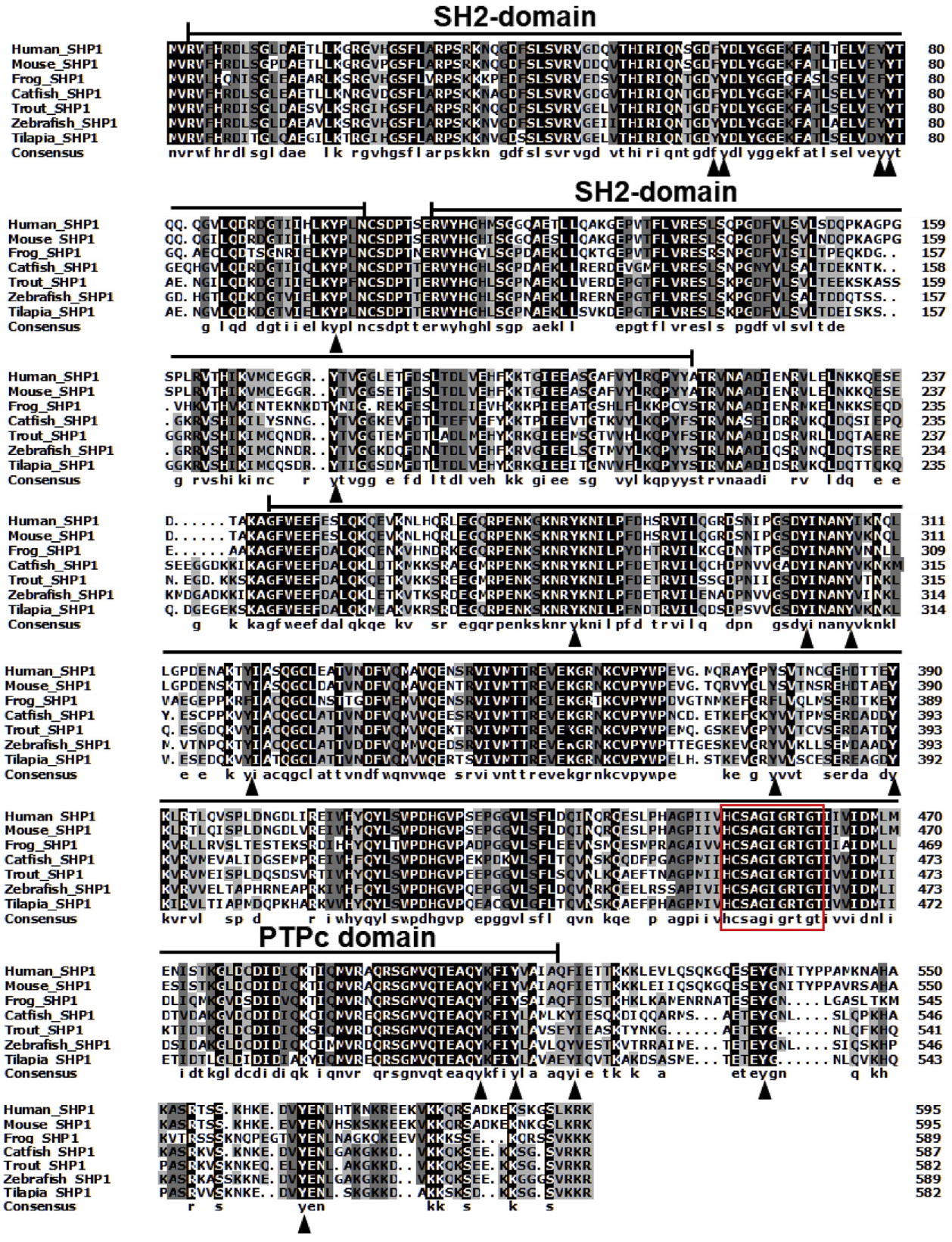

Fig. 1. Alignment of the amino acid sequences of *Oreochromis niloticus* SHP1 (OnSHP1) with that of other species. The accession numbers of these proteins from GenBank are as follows: Human (*Homo sapiens*, P29350.1), mouse (*Mus musculus*, P29351.2), frog (*Xenopus tropicalis*, NP_001116928.1), catfish (*Ictalurus punctatus*, AHH39690.1), trout (*Salmo salar*, NP_001133922.1) and zebrafish (*Danio rerio*, NP_956254.1). The identical and similar amino acids are highlighted in black and gray, respectively. The N- and C-terminal Src homology 2 (SH2) domains are indicated with a line above. The signature motif (HCSAGIGRTGT) of protein tyrosine phosphatase (PTP) is indicated on the alignments with a box as well. The conversed tyrosine phosphorylation sites are indicated with triangles.

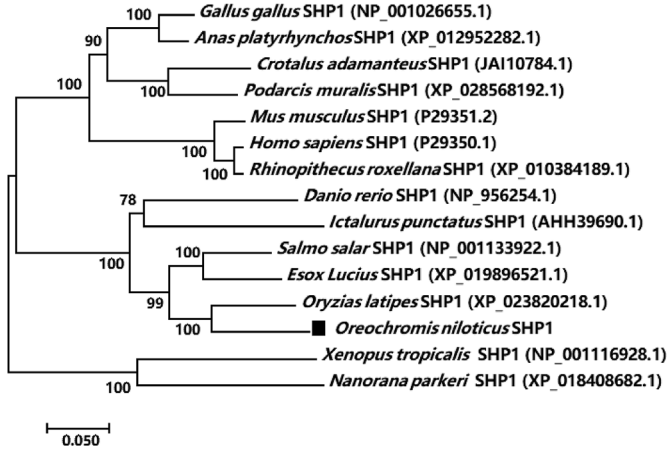

Fig. 2. Phylogenetic tree analysis of OnSHP1 constructed by neighbor-joining method with those of other vertebrates. Numbers at each branch indicate the percentage bootstrap values on 1000 replicates in MEGA7.0 software. The sequences are aligned by CLUSTAL W program. OnSHP1 is marked with a solid black square.

PTPc domain are highly conserved among vertebrates. The PTP signature motif (HCSAGIGRTG) is identical in OnSHP1 in mammals and fish (Fig. 1). There are 17 predicted conserved tyrosine phosphorylation sites, including Y^{60}, Y^{61}, Y^{78}, Y^{79}, Y^{98}, Y^{174}, Y^{279}, Y^{304}, Y^{309}, Y^{323}, Y^{379}, Y^{392}, Y^{507}, Y^{511}, Y^{517}, Y^{535} and Y^{557} were shown in Fig. 1 with triangles. The percent identity of OnSHP1 compared to human (*Homo sapiens*), mouse (*Mus musculus*), frog (*Xenopus tropicalis*), catfish (*Ictalurus punctatus*), trout (*Salmo salar*) and zebrafish (*Danio rerio*) are 63.5%, 62.83%, 59.73%, 71.09%, 78.16% and 72.20%, respectively. Phylogenetic analysis of the OnSHP1 with other predicted or identified fish SHP1 sequences indicated a close relationship among fish, mammals and reptiles (Fig. 2).

3.2. OnSHP1 transcription pattern in tilapia tissues and leukocytes

The constitutive transcription level of *OnSHP1* was studied in different tissues from healthy Nile tilapia by qRT-PCR. *OnSHP1* expressed widely in the examined tissues but highest in the spleen and lowest in the skin (Fig. 3A). The detection of *OnSHP1* transcription in leukocytes isolated from PBL, SPL and AK, showed highest *OnSHP1* mRNA level in leukocytes from SPL and lowest in AK (Fig. 3B). Before to further study the expression level of OnSHP1 at protein level, by BLAST there found that goat anti-human/mouse/rat SHP1 IgG polyclonal Ab might recognized the OnSHP1. Western blotting detection indicated that a single protein band about 65 kDa was detected, same as the predicted size of OnSHP1 protein, in spleen leukocytes (Fig. S2). It was similar to the study in zebrafish [25]. Moreover, β actin was used as the internal control for Western blotting. The result indicated a band about 42 kDa was detected (Fig. S2). Ensured the effectiveness of the antibody, the protein level of OnSHP1 in leukocytes from PBL, SPL and AK were measured by Western blotting, which indicated highest OnSHP1 protein level in SPL (1.19) and lowest expression in AK (0.69) (Fig. 3C).

3.3. The expression profiles of OnSHP1 after S. agalactiae and LPS challenges in vivo and in vitro

To establish the role of *OnSHP1* in pathogen infection, healthy Nile tilapia were challenged with *S. agalactiae* and LPS *in vivo* and OnSHP1 expression profiles were detected by qRT-PCR (Figs. 4 and 5). After infected upon *S. agalactiae* and LPS *in vivo*, *OnSHP1* was up-regulated differently in PBL, SPL and AK (Fig. 4). When infected by *S. agalactiae*, *OnSHP1* was up-regulated significantly at 3 h p.i. in PBL, SPL and AK, and the peak were at 3 h (3.84-fold), 24 h (10.98-fold) and 3 h (6.42-fold), respectively (Fig. 4A, B and C). Challenged with LPS, *OnSHP1* was up-regulated significantly in PBL, SPL and AK, and all of them reached the peak at 3 h p.i. with 1.35-fold, 7.58-fold and 3.21-fold, respectively (Fig. 4D, E and F).

To further explore the role of *OnSHP1* in immune reaction, leukocytes from the immune tissues were isolated from PBL, SPL and AK, then stimulated with formalin-inactivated *S. agalactiae* and LPS *in vitro*. Incubated with inactivated *S. agalactiae*, *OnSHP1* was up-regulated 4.11-fold, 1.59-fold and 1.35-fold in PBL, SPL and AK, respectively (Fig. 5A, B and C). Stimulation with LPS, *OnSHP1* increased the mRNA expression level in leukocytes (Fig. 5D, E and F). In SPL leukocytes, *OnSHP1* was up-regulated and reached the peak first at 1 h p.i. (1.39-fold) significantly (Fig. 5E.) While in PBL and AK, *OnSHP1* was up-regulated at 6 h p.i. and in PBL, and reached the peak at 6 h p.i. (1.45-fold) and at 12 h p.i. (2.38-fold) in AK (Fig. 5D and F).

3.4. OnSHP1 expression in leukocytes induced by mouse anti-OnIgM mAb

To study the role of OnSHP1 in B cell singling pathway, leukocytes from PBL, spleen and AK were separated and incubated with anti-OnIgM mAb (Fig. 6). Stimulated with anti-OnIgM mAb, *OnSHP1* was significantly up-regulated at 1 h p.i. and reached the peak at 6 h p.i. (1.67-fold) in PBL leukocytes (Fig. 6A). In SPL, *OnSHP1* was up-regulated at 12 h p.i. (2.37-fold) significantly (Fig. 6B); but in AK, there was no significant up-regulation after anti-OnIgM mAb incubation (Fig. 6C). The expressions of *CD79a* and *BLNK* were detected for ensuring anti-OnIgM mAb worked in AK leukocytes (Fig. S3).

3.5. Effect of PTP inhibitor II on the Y^{535} and Y^{557} phosphorylation and cytoplasmic free Ca^{2+} concentration of spleen leukocytes induced by mouse anti-OnIgM mAb or LPS

In order to explore the effect of inhibitor on phosphorylation level of OnSHP1, the spleen leukocytes were incubated with PTP Inhibitor II. The PTP Inhibitor II is a covalent inhibitor of PTP by binding with SH2 domain of SHP1 [34], which provides a practical method to investigate the effect of SH2 in OnSHP1. The results revealed that the two phosphorylation sites of OnSHP1, Y^{535} and Y^{557}, were significantly up-regulated after adding the inhibitor into leukocytes, but the protein level of OnSHP1 remained at the same level (Fig. 7). Moreover, after adding the inhibitor into leukocytes, the cells were stimulated with LPS or the mouse anti-OnIgM mAb. We found that the phosphorylation level of Y^{535} and Y^{557} were significantly up-regulated after both stimulations with LPS and the mouse anti-OnIgM mAb. But compared to LPS, the measurable higher level at both Y^{535} and Y^{557} sites were presented when stimulated with mouse anti-OnIgM mAb. However, the level of OnSHP1 protein stayed at a steady level after stimulation.

Before detecting the cytoplasmic free Ca^{2+} concentration after treated with PTP Inhibitor II, the downstream signaling molecule of BCR, LYN and BLNK were detected at the mRNA expression level. As shown in Fig. S4, the expression of LYN and BLNK were significantly up-regulated when the cells incubated with mouse anti-OnIgM mAb. However, the expression ns were down-regulated when the cells treated with the inhibitor before incubated with mouse anti-OnIgM mAb. These results indicated the inhibitor worked in these leukocytes. It was discovered that significantly up-regulated level of free Ca^{2+} concentration occurred when treated with the inhibitor (Fig. 8), by only treated the inhibitor or then incubated with LPS/mouse anti-OnIgM mAb.

4. Discussion

The phosphorylation and dephosphorylation of tyrosine in proteins regulate the signal transduction pathways and guiding cells to proliferation or differentiation, and even death [1,36]. SHP1, one of the non-transmembrane PTP, containing two SH2 domains and a classic PTP domain, plays important roles in inflammation and cell signaling

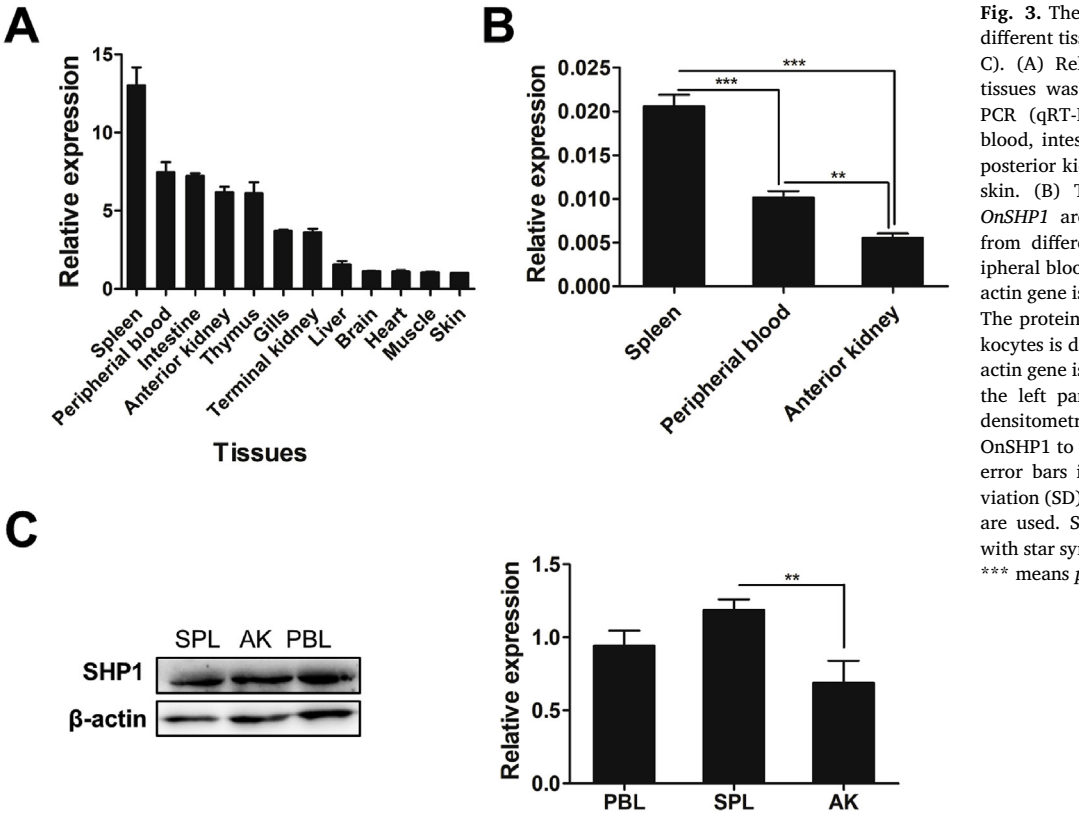

Fig. 3. The expression profiles of OnSHP1 in different tissues (A) and tissue leukocytes (B and C). (A) Relative expression level in different tissues was detected by quantitative real-time PCR (qRT-PCR), including spleen, peripheral blood, intestine, anterior kidney, thymus, gills, posterior kidney, liver, brain, heart, muscle and skin. (B) The mRNA transcription levels of *OnSHP1* are detected in leukocytes separated from different immune tissues, including peripheral blood, spleen and anterior kidney. The β actin gene is used as the endogenous control. (C) The protein expression level of OnSHP1 in leukocytes is detected by Western blotting, which β actin gene is taken as the endogenous control (in the left panel). The histogram represents the densitometric quantification of the ratio of total OnSHP1 to total β actin (in the right panel). The error bars indicate the mean ± standard deviation (SD) and three biological repeats (n = 3) are used. Significant differences are presented with star symbol, where ** means $p < 0.01$ and *** means $p < 0.001$.

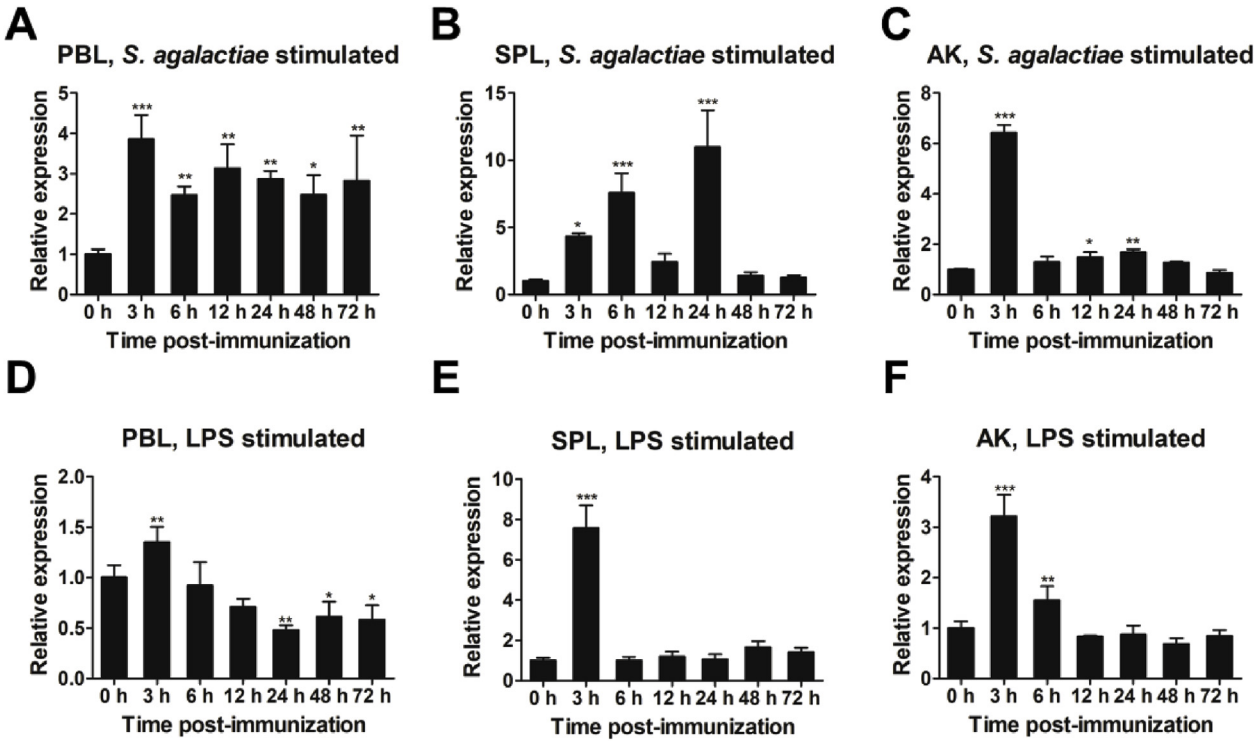

Fig. 4. The transcription levels of *OnSHP1* in peripheral blood (PBL, A and D), spleen (SPL, B and E) and anterior kidney (AK, C and F) against *Streptococcus agalactiae* (*S. agalactiae*) and lipopolysaccharide (LPS) infection in Nile tilapia, respectively. The infected fish versus control fish are measured post-infection 0 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h. Statistical differences are evaluated by one-way ANOVA followed by Tukey's multiple comparison test. Bars represent the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

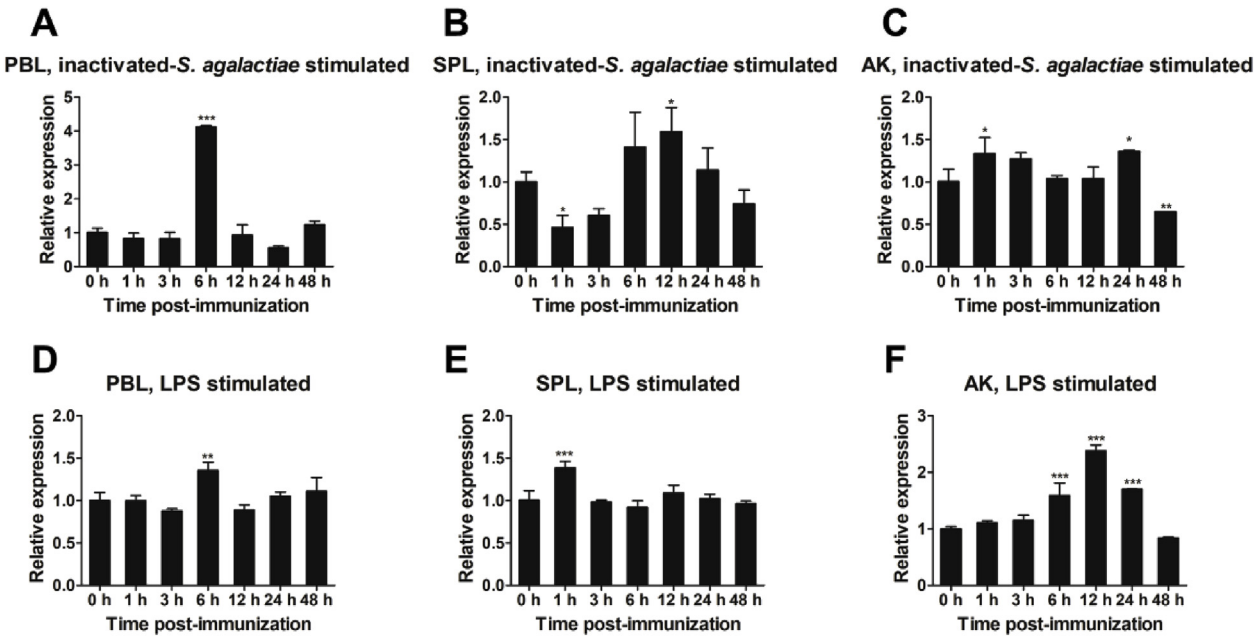

Fig. 5. *OnSHP1* mRNA relative expression levels in leukocytes separated from PBL (A, C), SPL (B and E) and AK (C and F) after stimulation with inactivated *S. agalactiae* and LPS, respectively. The data represent means ± SD (n = 5). Statistical differences are evaluated by one-way ANOVA followed by Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

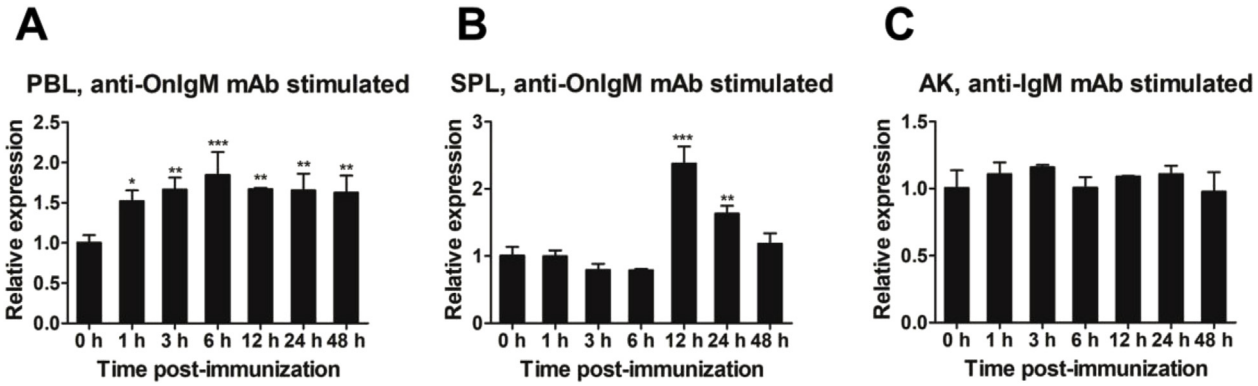

Fig. 6. Relative expression of OnSHP1 in leukocytes separated from PBL (A), SPL (B) and AK (C) after stimulation with mouse anti-OnIgM monoclonal antibody (mAb). Statistical differences are evaluated by one-way ANOVA followed by Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

[22]. Studying the role of SHP1 in Nile tilapia during the pathogen invasion or the B cell activation would enrich the research of SHP1 in teleosts and fill the blank of the downstream molecular pathway of BCR signaling.

It is known that SHP1 contains two SH2 domains and a cytoplasmic PTP domain in mammals [9,22], and a similar structure is discovered in zebrafish [25]. In Nile tilapia, two SH2-domains and a PTPc domain were also existed in OnSHP1 (Fig. S1) with a PTP signature motif (HCSAGIGRTG), which was not only highly conserved among the vertebrates but had a close relationship with the mammalian and reptile (Figs. 1 and 2). Although OnSHP1 contains 17 predicted conserved tyrosine phosphorylation sites, there are two sites (Y^{535} and Y^{557}) corresponding to Try536 and Try564 in human and mouse, which have been proved to be phosphorylated upon various stimuli and might affect its activity [37]. Similar tyrosine phosphorylation sites exist in the fish as shown in the multiple sequence alignment (Fig. 1), which might imply similar performance existing in teleost fish [25–27].

It is reported that SHP1 mainly expresses in hematopoietic cells but with a low level in epithelial cells in mammals [9]. In Nile tilapia, *OnSHP1* expressed highest in spleen, followed by similar levels in PBL, intestine, AK or thymus, and expressed in other detected tissues as well (Fig. 3A). The expression pattern was consisted with the finding in zebrafish [25], which might indicate that SHP1 functions widely in the individual but plays important roles in immune response. The expression in leukocytes separated from the main immune tissues might support this hypothesis (Fig. 3B and C), which showed high expression level (both molecular and protein levels) in SPL. When the pathogen (*S. agalactiae*) or endotoxin (LPS) infected the fish, the expression of *OnSHP1* could be up-regulated in PBL, SPL and AK for 72 h (Fig. 4). *S. agalactiae* is the predominant pathogen in Nile tilapia, while LPS is the main component of gram-negative bacterial cell wall and widely used the study of inflammatory response [28,38]. In mice, the *me/me* phenotype (without detectable SHP1 protein) presents severe inflammation and systemic autoimmunity [18]. The performance in *me/me* phenotype mice implies that SHP1 might play essential role in inflammation; however, until now, there is no study about the function of teleost fish SHP1 during the bacterial infection. Moreover, the leukocytes isolated from the PBL, SPL and AK were treated with inactivated *S. agalactiae* or LPS *in vitro* performed the similar expression pattern as did *in vivo* (Fig. 5). The data above implied that OnSHP1 might play defense response against pathogenic infection in Nile tilapia.

The motheaten phenotype mice exhibits B cell defects and the

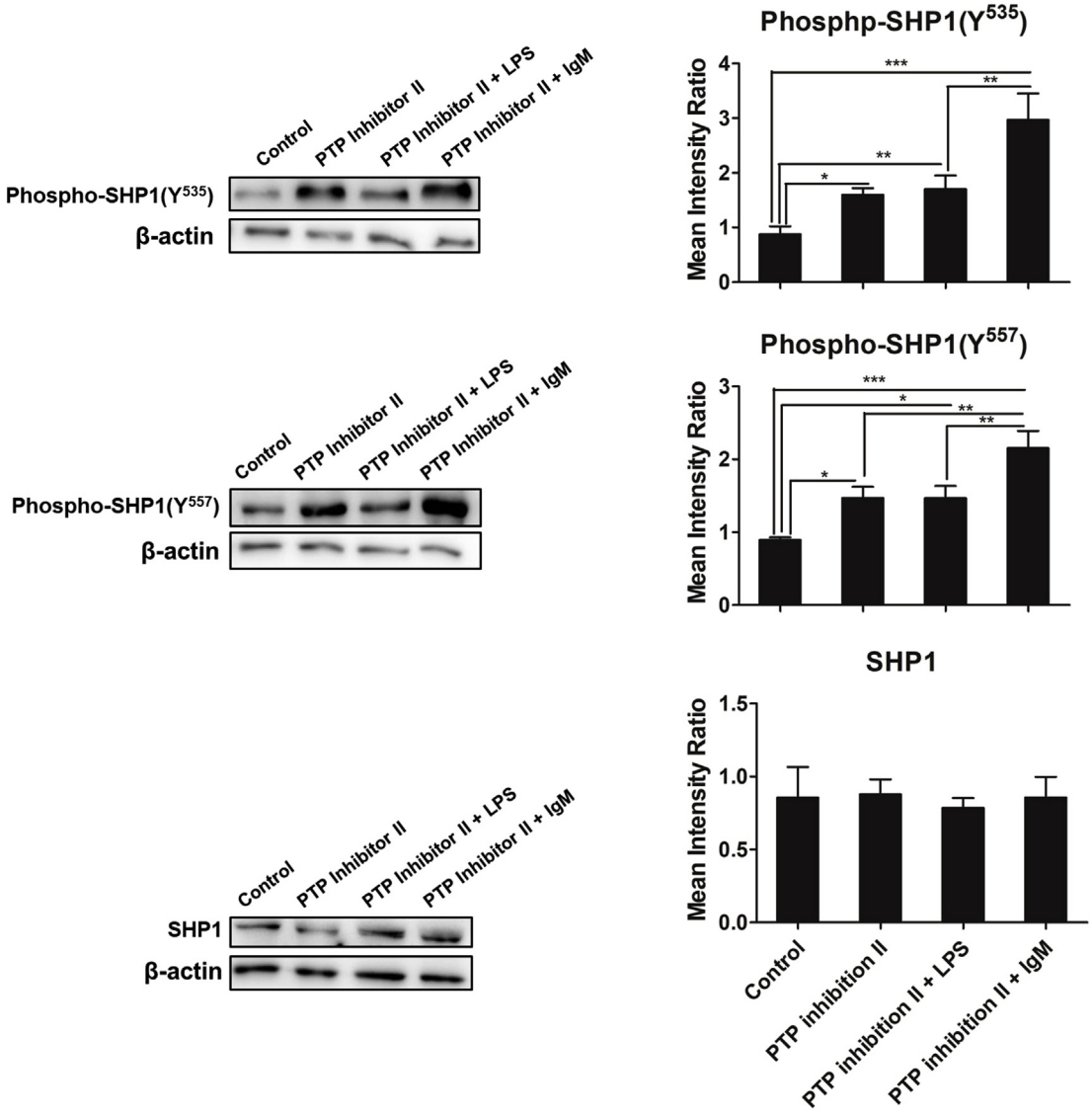

Fig. 7. The detection of tyrosine phosphorylation level (A and B) and protein expression (C) of OnSHP1 by Western blotting after the splenic leukocytes were incubated with PTP inhibitor II *in vitro* for 1 h. Phospho-OnSHP1 (Y^{535}) (A) and phospho-SHP1 (Y^{557}) (B) were detected by rabbit anti-phospho-SHP1 (Tyr536) and phospho-SHP1 (Tyr564) antibody, respectively. The protein expression level of OnSHP1 was detected with anti-mouse SHP1 Ab (C). The internal control was β actin, which was detected by mouse anti-β actin mAb. The histogram represented the densitometric quantification of the ratio of total OnSHP1 to total β actin. Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test. The error bars represented SD (n = 3) and significant difference was indicated by asterisks (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

published data indicate the capacity of SHP1 in suppressing BCR signaling pathway [21]. In mammals, SHP1 associates constitutively with BCR complex in resting B cells and to dephosphorylate CD79a and CD79b *in vitro* [22]. Further, previous studies indicated that IgM mAb combined with mIgM specifically, resulted in B-cell activation and immune reactions in teleosts [28,29,32]. Here, by stimulation with mouse anti-OnIgM mAb, the expressions of OnSHP1 in leukocytes from different immune tissues (PBL, SPL and AK) were detected (Fig. 6). PBL and SPL performed similar expression pattern, but there was no significant change in AK although the leukocytes were activated (Fig. S3). It might owe to that the different B-cell subpopulations existing in different immune tissues as the report in rainbow trout [39], indicating that OnSHP1 might play similar role in Nile tilapia as in mammals [17].

In mammals, deletion of the SHP1 N-terminal SH2 domain (not the C-terminal SH2 domain) would result in SHP1 activation by released the PTP domain [40]. Since the SH2 domains and PTP domain with tyrosine sites in OnSHP1 were conserved as that of mammals, it might be happened in tilapia as same as in mammals. PTP Inhibitor II is a covalent inhibitor of PTP by binding with SH2 domain of SHP1 [34]. As hypothesized above, incubation with the inhibitor would result in the down-regulation of the downstream molecules of BCR signaling pathway (Fig. S4), and up-regulation the tyrosine phosphorylation of Y^{535} and Y^{557} (Fig. 7). The phenomenon implied that, as in mammals, SH2 domain (but no sure the N- or C-terminal SH2) of OnSHP1 might insert into PTP domain and inactivated the PTP activity; however, the inhibitor bound with SH2 domain activate the enzyme and the increase of phosphorylation level at OnSHP1 Y^{535} and Y^{557} [14–16]. Moreover, the cytoplasmic free Ca^{2+} concentration increased (Fig. 8) as in *me/me* phenotype mice in response to BCR stimulation [18]. However, the mechanism of this regulation remains to be determined as in mammals [13]. It indicated that OnSHP1 might negatively regulate BCR signaling pathway.

Together, these studies indicated that OnSHP1 is a highly conserved non-transmembrane PTP with two SH2 domains and PTP domain. The expression profiles during challenges *in vivo* or *in vitro* demonstrated that OnSHP1 gets involved in host defense against *S. agalactiae*. The

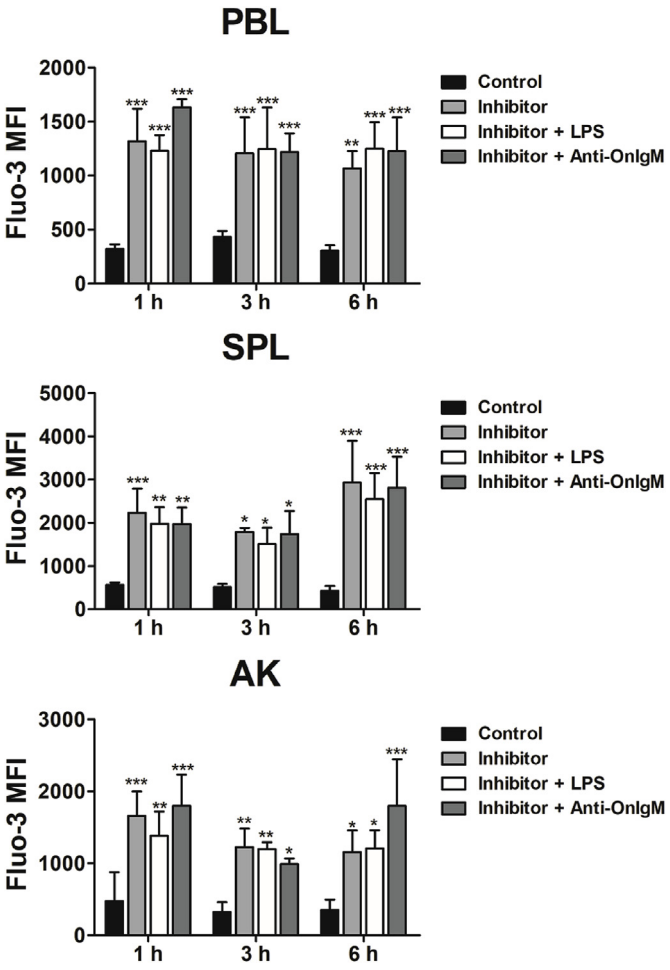

Fig. 8. The cytoplasmic free Ca^{2+} concentration in lymphocytes from PBL (A), SPL (B) and AK (C) upon incubation by PTP inhibitor II and stimulation with LPS or anti-OnIgM mAb for 1 h, 3 h and 6 h. At the different incubation periods, cells were loaded with Fluo-3 AM (5 μM final concentration). Mean fluorescence intensity (MFI) ± SD of intracellular Ca^{2+} levels (Fluo-3) in leukocytes was shown (n = 5). Statistical differences were evaluated by one-way ANOVA followed by Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

analysis of stimulus of specific mouse anti-OnIgM mAb and the effect of PTP Inhibitor II on lymphocytes indicated that OnSHP1 might play roles in BCR signaling pathway.

CRediT authorship contribution statement

Liting Wu: Funding acquisition, Writing - original draft. **Along Gao:** Writing - review & editing. **Yang Lei:** Formal analysis. **Jun Li:** Writing - review & editing. **Kangsen Mai:** Writing - review & editing. **Jianmin Ye:** Funding acquisition, Writing - original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.02.026.

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