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Involvement of H-Ras in the adaptive immunity of Nile tilapia by regulating lymphocyte activation

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ABSTRACT

H-Ras is a guanosine triphosphatase (GTPase), which acts as a molecular switch and controls multiple important cellular processes including lymphocyte activation and function. However, regulatory mechanism of adaptive immune response by H-Ras remains unclear in non-mammalian animals. In the present study, we investigated the involvement of H-Ras in lymphocyte activation with a teleost model *Oreochromis niloticus*. H-Ras from *O. niloticus* (On-H-Ras) is highly conserved with those from other vertebrates. The mRNA of On-H-Ras showed a wide expression pattern in the lymphoid-tissues and with the highest level in liver. After *Aeromonas hydrophila* infection, transcription of On-H-Ras was significantly induced on day 8 but came back to basal level on day 16, suggesting that On-H-Ras potentially participated in primary response during the adaptive immunity. Furthermore, On-H-Ras mRNA was obviously up-regulated when leukocytes were activated by T lymphocyte mitogen PHA in vitro. Meanwhile, protein level of H-Ras was also augmented once leukocytes were stimulated with lymphocyte receptor signaling agonist PMA and ionomycin. More importantly, once Ras activity was inhibited by specific inhibitor, the up-regulation of lymphocyte activation marker CD122 was obviously impaired during lymphocyte activation process. Therefore, On-H-Ras regulated lymphocyte activation through both mRNA and protein level. Altogether, our results illustrated the involvement of H-Ras in teleost adaptive immunity via controlling lymphocyte activation, and thus provided a novel perspective to understand evolution of the lymphocyte-mediated adaptive immunity.

1. Introduction

The Ras is a superfamily of guanosine triphosphate (GTP)-binging proteins containing over 150 monomeric members with the molecular weight of approximately 21 kDa [1]. This superfamily can be further divided into several subfamilies including Ras, Rho, Rheb, Rab, Arf and Ran [2]. Among them, members in Ras subfamily, such as N-Ras, H-Ras and K-Ras function as binary molecular switches to control the signal transduction from cell surface to cytoplasm. The activity of Ras protein depends on alternative bond of GTP or guanosine diphosphate (GDP). The GTP-bound is an active form of Ras, whereas once GTP is hydrolyzed by GTPase-activating proteins (GAPs) the Ras turns to deactivate [3,4]. Generally, the GDP-binding Ras is a predominant existing form, and the percentage of GTP-binding Ras mushrooms upon upstream stimulation [5,6]. The tight signaling regulated by Ras switch drives a series of cellular effects such as cell proliferation, differentiation and growth [2,7]. Lack of or dysregulated Ras signals lead to severe diseases, for example cancers, RASopathies and psychiatric disorders [8].

Ras protein responds to various extracellular factors such as cytokines, hormones and growth factors, and plays pivotal roles in immune defense of animals [2]. Three immune signaling pathways downstream of Ras, Raf-Mek-Erk, PI3K-Akt and RalGDS-Ral, have been illustrated, and among which h-Ras-Raf-Mek-Erk is a well characterized axis for lymphocyte-mediated adaptive immunity [9–13]. For T or B lymphocyte immune responses, after initiation of the TCR/BCR signaling, H-Ras can be rapidly and magnificently activated by PLCγ1 or PLCγ2, and then recruits c-Raf to plasma membrane to initiate the subsequent tertiary MAPK cascade [14–16]. Followed that activated transcriptional factors are responsible for the gene transcription, cytokine production, clonal expansion and function [17,18]. In T cells, normal immune responses need proper regulation of Ras activation. It has been reported that the expression of a constitutively active H-Ras in T cells can activate Raf-Mek-Erk cascade and transcriptional factors to induce IL-2

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production, coupled with a calcium pathway and calcineurin activation, whereas the dominant negative H-Ras lessens Erk activation and activity of NFAT reporter [19–23]. Moreover, based on current knowledge, the TCR-Ras induced Raf-Mek-Erk signaling pathway is required for Th cell differentiation, although the results are still inconsistent and controversial. Some study suggested MAPK/ERK signaling was indispensable for Th1 cell differentiation and IFN-γ producing [24], while other study revealed such signaling is important for the opposite direction Th2 cell differentiation and IL-4 production [25]. In B cells, Ras activation is also associated with proliferation and activation. Blockade of Ras cascade inhibited upregulation of Egr-1 (early response product), CD69 (activation marker) and BCR-induced proliferation in mature B lymphocytes [26]. Among the Ras subfamily, the three Ras isoforms, H-Ras, N-Ras and K-Ras, appear to be coordinately activated. N-Ras and K-Ras were initially recognized to predominate in lymphocytes and different isoforms were assumed redundant in signal transduction [27]. However, further researches supported distinct consequences resulted from different isoform activation such as various ability to activate raf-1 or PI3K [28,29]. Precise regulation of T lymphocyte may also due to expression, localization and properties of distinct Ras isoforms [30].

Although regulatory mechanism of lymphocyte mediated adaptive immunity has been well clarified in mammals, corresponding studies in early vertebrates especially the teleost are just in infancy. In the latest decade, several suspected T cell subsets such as $CD3^+$, $CD4\text{-}1^+$, $CD4\text{-}2^+$ or $CD8^+$ leukocytes have been identified from different teleost species [31–37]. And these cells might exert similar functions as those in mammals, for example undergoing antigen-specific proliferation, performing cell-mediated cytotoxicity and exerting antibacterial responses [31,33,34,38,39]. Meanwhile, IgM^+, IgD^+ or IgT^+ B cells have also been identified from teleost and proven to play significant roles in adaptive immunity [40–42]. However, the knowledge about regulatory mechanism of lymphocyte mediated adaptive immune responses in teleost is just a drop in the bucket. In previous study, we have illustrated that c-Raf that is downstream of Ras was involved in Nile tilapia adaptive immune response via controlling lymphocyte activation [43]. Recently, we found Nile tilapia encode two isoforms of Ras, H-Ras and K-Ras, but lack of N-Ras, in the genomic sequence on NCBI GenBank. Here, we studied the regulatory role of H-Ras on regulating adaptive immune response of Nile tilapia. In the present study, we analyzed the sequence properties of H-Ras from Nile tilapia *Oreochromis niloticus*, detected its distribution in lymphoid tissues, illustrated its potential involvement in adaptive immune response, and investigated its regulatory role on lymphocyte activation. Our works aimed to provide novel evidences for regulatory mechanism of lymphocyte-mediated adaptive immunity of the teleost.

2. Materials and methods

2.1. Animals

We purchased Nile tilapia *O. niloticus* with body length of 8 to 10 cm from an aquatic farm in Guangzhou, Guangdong Province, China. We kept the Nile tilapia in aerated fresh water with constant temperature at 28 °C for two weeks before experiments.

2.2. Sequence analysis

The cDNA and amino sequences of On-H-Ras with accession number XM_019361943 and XP_019217488 were acquired from GenBank and Genpept, respectively. We perform the multiple sequence alignment with ClustalX 1.83 and displayed the result by sequence manipulation suite (SMS) and WebLogo sequence alignment tool. The tridimensional structures of the proteins were predicted by SWISS-MODEL [44,45] and the models was exhibited and modified with PyMOL software. The phylogenetic tree was constructed by MEGA4.

2.3. Transcriptional profile of On-H-Ras in lymphoid tissues

The tissues including spleen, head kidney, trunk kidney, blood and liver, were isolated from six healthy Nile tilapia parallelly. We extracted the total RNA of these tissues by TRIzol reagent (Invitrogen) in accordance with manufacture's protocol. After DNA degradation, the RNA was used as template for synthesis of cDNA with oligo (dT) -adapter primer catalyzed by M-MLV reverse transcriptase. The synthesized cDNA was 1:50 diluted for SYBR Green fluorescent real-time quantitative PCR (RT-PCR). The amplification of On-H-Ras was carried out with a pair of gene-specific primers (AAGCAGGTTGTGATAGACGGGGA and AGACTTGGTATTGTTGATGGCGAA) to synthesize a 144 bp fragment. The other pair of primers (CGGAATCCACGAAACCACCTA and CCAGACGGAGTATTTACGCTCA) was designed to synthesize β-actin as internal control. The RT-PCR reaction was performed in a CFX Connect Real-Time System (BIO-RAD). After confirming the dissociation curve, the mRNA level was analyzed with $2^{-\triangle\triangle Ct}$ method [46].

2.4. Inducible expression of On-H-Ras during bacteria induced adaptive immune response

The concentration of *A. hydrophila* cultivation was adjusted to 3.6×10^6 CFU mL^{-1} with PBS. One-tenth milliliter of bacterial suspension was intraperitoneally (*i.p.*) injected to each Nile tilapia for bacteria challenge, while PBS instead of bacterial suspension was used in control group. Four individuals were randomly sampled in the challenge and control group on day 5, 8 and 16 after stimulation, respectively. The spleens of Nile tilapia were collected for real-time RT-PCR to determine the transcriptional level of On-H-Ras according to the above method.

2.5. Leukocyte isolation

Leukocytes of Nile tilapia were collected from spleen with slightly modified protocol based on previous reports [36,47,48]. In brief, we grinded the spleen to cell suspension in Leibovitz's L-15 medium (Gibco) with 2% FBS. After centrifugation at 800*g*, 4 °C for 5 min, the cells were harvested, resuspended in L-15 with 2% FBS, and put onto 34% (v/v) and 52% density-gradient Percoll (GE Healthcare). The middle cell layer between 34% and 52% Percoll composed of leukocytes were collected after centrifugation at 500*g*, 25 °C for 30 min with the lowest acceleration and deceleration.

2.6. mRNA transcript level of On-H-Ras after PHA stimulation

The leukocytes (1×10^7 cells) were cultivated at 28 °C with 5% CO_2, and stimulated with phytohaemagglutinin (PHA, Sigma) on final concentration of 5 μg mL^{-1} diluted in DMEM medium (with 10% FBS and 1% penicillin/streptomycin). The leukocytes in control group were treated by identical medium in absence of PHA. At 5th and 10th h after stimulation, the leukocytes were harvested and used to extract RNA for real-time RT-PCR. The transcript of On-H-Ras was tested according to the above method.

2.7. Western-blot assay of H-Ras protein after PMA and ionomycin stimulation

The isolated leukocytes of Nile tilapia were suspended in D-PBS containing Ca^{2+} and Mg^{2+} (BBI Life Sciences) for 30 min at 28 °C to rest. We stimulated the rested cells with mixture of 50 ng mL^{-1} Phorbol myristate acetate (PMA, Sigma) and 500 ng mL^{-1} *ionomycin* (P + I), and terminated the stimulation with pre-cooled D-PBS. The resting cells without P + I treatment were served as control. All the samples were harvested at 5th and 15th min after stimulation, and then lysed in NP40 buffer (Sigma) on ice for 30 min. The lysate was centrifuged at 13000 g, 4 °C for 10 min to acquire supernatant protein.

The samples were separated by 12% SDS-PAGE and electrophoretically transferred onto 0.22 μm nitrocellulose membranes (Pall) at 100 V for 2 h in a waterish system. After blocked with 4% non-fat milk powder for 1 h at 25 °C, the membranes were respectively incubated with mouse *anti*-H-Ras (Santa Cruz Biotechnology, 1:500 diluted in 2% BSA) and rabbit *anti*-β-actin antibodies (Cell Signaling Technology, 1:1000 diluted in 2% BSA) at 4 °C with gentle shaking, overnight. We then incubated the membranes with goat anti-mouse IgG H&L Alexa Fluor 680 (Abcam) and goat anti-rabbit IgG H&L Alexa Fluor 790 (Abcam) with 1:10000 dilution in 4% non-fat milk powder for 1 h at room temperature. Thrice rinses with PBST were followed each incubation. Finally, the membranes were scanned by an Odyssey CLx Image Studio.

2.8. Immunofluorescence assay of H-Ras in leukocytes post P + I stimulation

The isolated leukocytes were harvested at 30 min after P + I stimulation and resting cells were served as control. Then the leukocytes were spun onto slides with Cytospin and fixed in methyl alcohol for 5min, prepared at −20 °C for use. After block with 1% BSA (BBI Life Sciences) for 1 h at 25 °C, the slides were incubated with 1:50 diluted mouse *anti*-H-Ras (Santa Cruz Biotechnology) and 1:200 diluted rabbit *anti*-β-actin as primary antibodies or incubated with PBS as negative staining control. The mixture of 1:1000 diluted Alexa Fluor 488-conjugated goat anti-mouse IgG (Abcam) and 594-conjugated goat anti-rabbit IgG (Abcam) was added to the slides to detect the binding. Each incubation was carried out in an immunohistochemical wet box for 1 h at 37 °C and terminated by three washes with PBST. Finally, the slides were mounted with antifade solution containing DAPI (Solarbio) and observed with a fluorescence microscope.

2.9. Lymphocyte activation determination in absence of Ras activity

Nile tilapia was intraperitoneally injected with 5 mg/kg weight/day of Ras inhibitor FTI-277 (MCE MedChemExpress) diluted in PBS for consecutive two days, before the leukocytes were isolated and stimulated with 5 μg mL^{-1} PHA in the presence of 10 μM additional FTI-277 for 10 h. Leukocytes isolated from PBS injected fish was unstimulated or stimulated with PHA for controls. The mRNA expression level of Nile tilapia CD122 (NCBI, XM_019360337) in leukocytes of these group was examined by Real-time RT-PCR as described above. The specific primer for CD122 were GGAGGTGTGTTTGGCAAGTT and CTGAGTCAGGATG AGGGCAT.

2.10. Statistical analysis

The results were presented as mean ± SE (n = 4–6). The data was subjected to two-tailed *t*-test to define significance (*: $p < 0.05$, **: $p < 0.01$).

3. Results

3.1. Sequence characteristics of On-H-Ras

The 570 bp CDS of On-H-Ras encodes a peptide composed of 189 amino acid residues with predicted molecular weight of 21.45 kDa. The Blastp shows that amino acid of On-H-Ras is highly similar with H-Ras from *Homo sapiens* (CAG38816) and *Caenorhabditis elegans* (NP_502213) with similarity of 96% and 78% respectively. The multiple sequence alignment also shows that On-H-Ras shares a high similarity with its homologues from other species except the C-terminal hypervariable regions (Fig. 1). The two switch regions, switch-I (D30-D38) and switch-II (A59-E76), are highly conserved from invertebrates to mammals. Moreover, the phosphate-binging loop G10-S17 (also named P-loop, a GXXXXGKS/T motif) and nucleotide-binding loop N116-R123 (harboring N/TKXD motif) are well conserved as well, while the cysteine residues (C182, 185 and 187) on C-terminal that can be modified with lipid chains are conserved in vertebrates (Fig. 1).

3.2. The tertiary structure of On-H-Ras

Since the amino acid sequence of On-H-Ras is highly conserved compared to other species, we further analyze its structural features with homologous modeling method. On-H-Ras is a mono-domain protein containing a single Ras domain without signal peptide or transmembrane region based on prediction of simple modular architecture research tool (SMART) and TMHMM. The result predicted by SWISS-MODEL exhibits that tertiary structures of On-H-Ras and human H-Ras (Hs-H-Ras) are highly similar. The overall structure of On-H-Ras is composed of 5 α-helixes and 6 β-sheets, adopting two typical switch regions (switch-I and switch-II), and a P-loop located nearby (Fig. 2a). Both On-H-Ras and Hs-H-Ras can exist in homologous tetramer according to prediction (Fig. 2b).

3.3. Phylogenetic characteristics of On-H-Ras

We constructed the phylogenetic tree to clarify the evolutionary relationships among vertebrate H-Ras proteins. The total amino acid sequences of On-H-Ras and its 41 homologues, were used to develop the rooted tree, among which the H-Ras from *Drosophila melanogaster* was defined as outgroup. Three different algorithms, neighbor-joining (NJ), minimum evolution (ME) and maximum parsimony (MP), all supported the same phylogenetic topology (Fig. 3). The organisms from five classes of vertebrate, teleost, amphibian, reptile, avian and mammal, were clustered well and each class was monophyletic, forming single clade. In the teleost cluster, the Nile tilapia H-Ras presented the closest relationship with that of *Paralichthys olivaceus* (Fig. 3).

3.4. mRNA expression pattern of On-H-Ras in lymphoid tissues

To unveil the potential role of On-H-Ras in adaptive immune response, we first detected its expression profile in lymphoid tissue of Nile tilapia by real-time RT-PCR. The mRNA of H-Ras is expressed in all the lymphoid tissues detected with comparative level, including spleen, head kidney, blood, trunk kidney and liver (Fig. 4).

3.5. Induced expression of On-H-Ras during adaptive immune response

To further confirm the involvement of On-H-Ras in adaptive immune responses, we infected the Nile tilapia with pathogenic microbe *A. hydrophila*, and examined its transcriptional level at the adaptive immune phase. Because *i.p.* injected bacteria caused a systemic infection, thus the spleen organ that represent systemic immunity was selected for analysis. The On-H-Ras mRNA did not show significant differences between PBS control and infection group on day 5, but it was significantly ($p < 0.01$) induced on day 8 post infection with more than 4-fold upregulation compared to control (Fig. 5). However, the On-H-Ras mRNA expression declined to initial level 16 days after infection (Fig. 5). This fluctuation of On-H-Ras transcriptional level suggested its potential involvement in primary response in the adaptive immunity of Nile tilapia.

3.6. Upregulation of H-Ras during lymphocyte activation

Since the expression of On-H-Ras was significantly induced during primary adaptive immune response, its potential involvement in the lymphocyte activation was determined. The isolated splenic leukocytes were stimulated with PHA, a T lymphocyte-specific mitogen, to induce T lymphocyte activation. The On-H-Ras mRNA level was significantly ($p < 0.05$) upregulated at 5 h post stimulation compared to control in absence of PHA (Fig. 6).

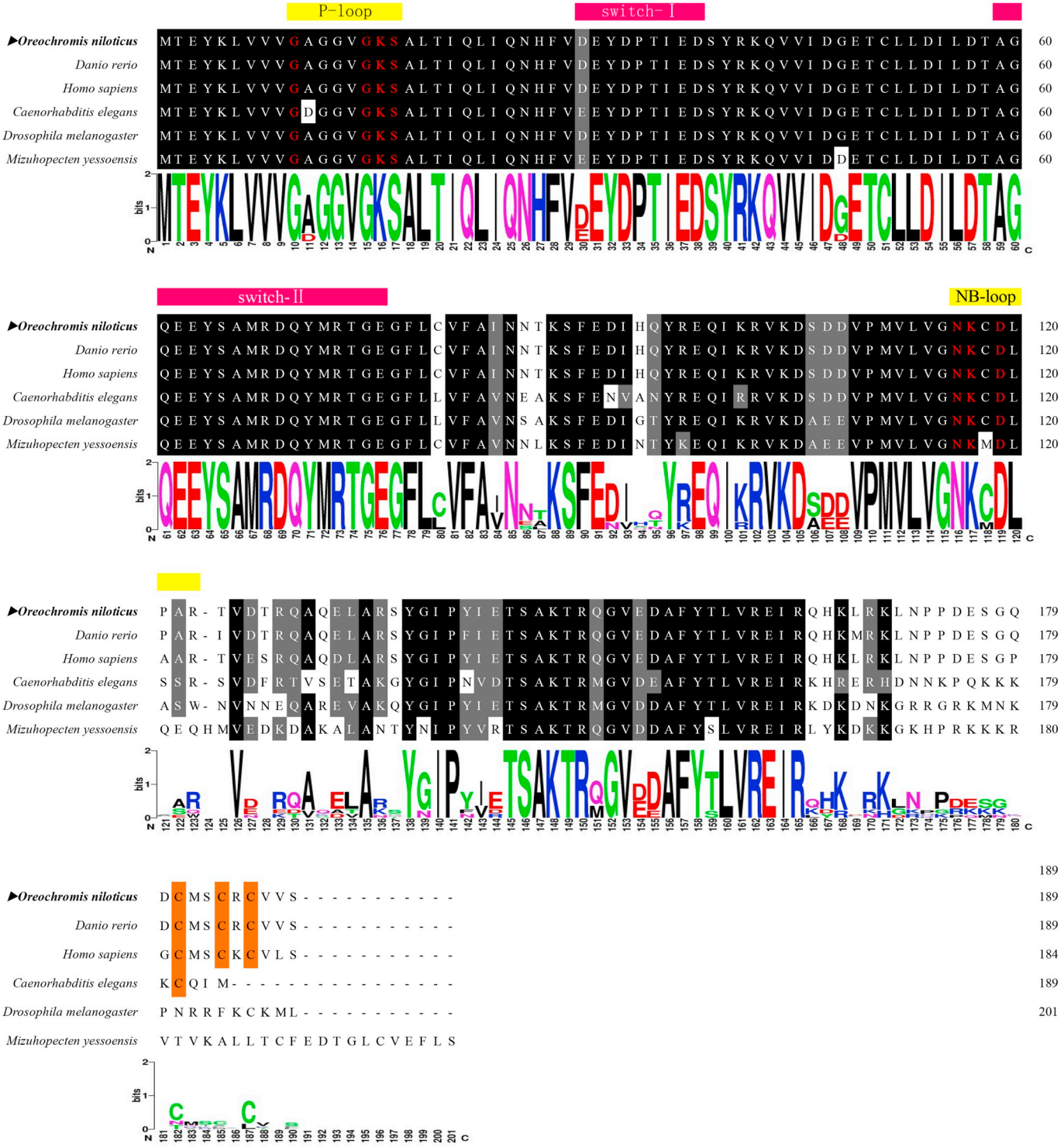

Fig. 1. Multiple sequence alignment. The whole amino acid sequences of H-Ras from Nile tilapia and other species were aligned. The black-colored residues are 100% conserved and gray-shaded residues are similar. The fragments labeled with yellow bars represent phosphate-binging loop (P-loop) and nucleotide-binding loop (NB-loop), and magenta bars represent switch regions. The cysteine residues that can be modified with lipid chains are shaded in orange. The selected homologues are from *Oreochromis niloticus* (XP_019217488), *Homo sapiens* (CAG38816), *Danio rerio* (NP_001017623), *Drosophila melanogaster* (AAF54388), *Mizuhopecten yessoensis* (OWF54200), and *Caenorhabditis elegans* (NP_502213). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To further elucidate the engagement of On-H-Ras in lymphocyte signaling, its protein level was detected upon leukocytes were activated by lymphocyte activation agonist PMA and Ionomycin (P + I) in vitro. The western-blot assay clearly showed that protein level of H-Ras was significantly and promptly enhanced at 5 min and 15 min after P + I stimulation compared to resting group (Fig. 7). Meanwhile the obvious augmentation of H-Ras was also observed in cytoplasm of leukocytes at 30 min after stimulation and no positive signal was observed in the PBS control revealed by immunofluorescence assay (Fig. 8). These results indicated that Nile tilapia H-Ras engaged in the regulation of

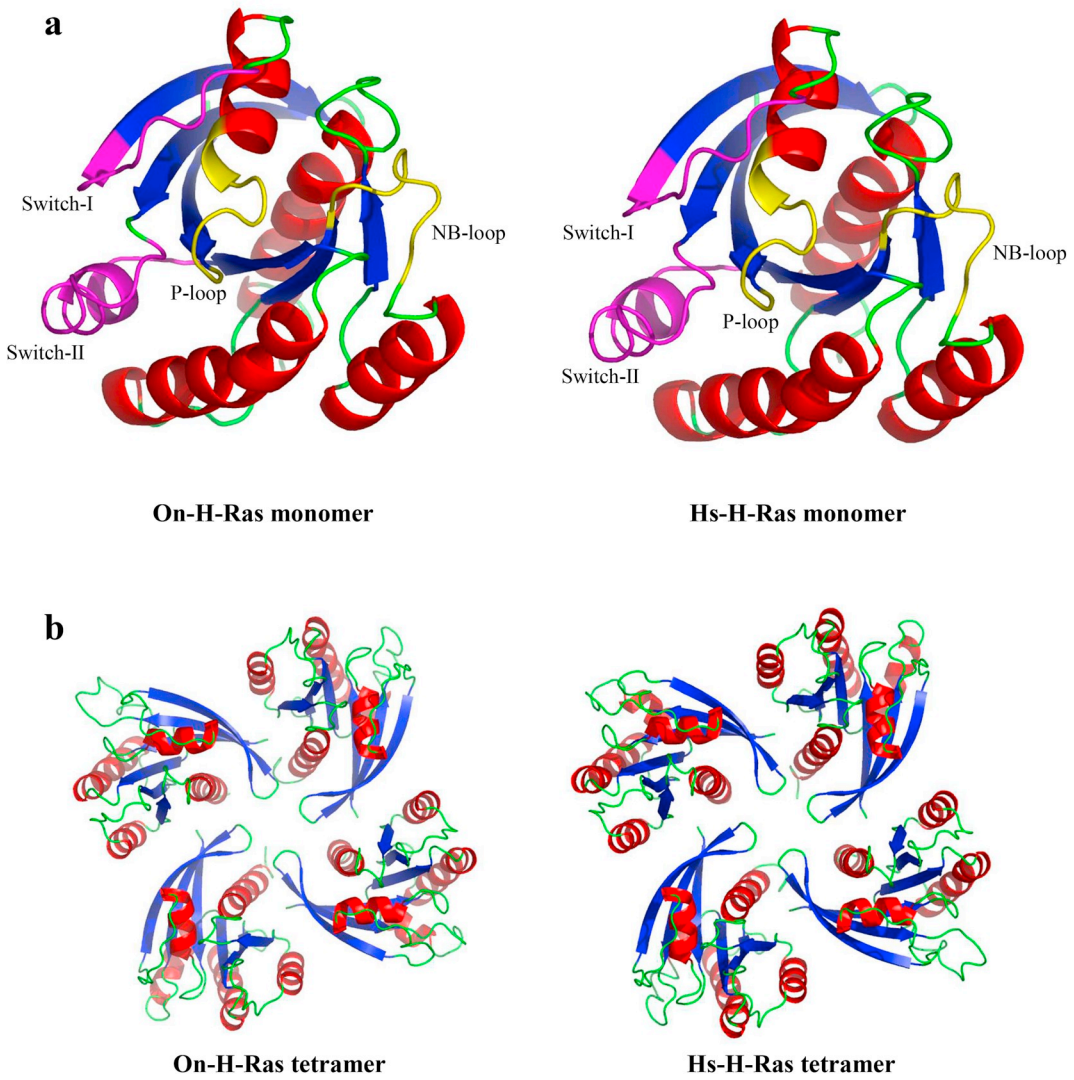

Fig. 2. Tertiary structures of H-Ras. The models are constructed based on H-Ras from *O. niloticus* (On-H-Ras) and *H. sapiens* (Hs-H-Ras), displaying the predicted monomer (a) and tetramer (b) of H-Ras. Different colors represent: red, α-helix; blue, β-strand; green, loop; yellow, P-loop and NB-loop; magenta, switch region. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

lymphocyte activation signaling.

3.7. Impairment of lymphocyte activation in absence of Ras activity

To further investigate the regulatory role of Ras to lymphocyte activation, we treated the fish with Ras inhibitor, and examined the expression level of lymphocyte activation marker CD122 after leukocytes were stimulated with PHA in vitro. The result show that after lymphocytes were activated by PHA, mRNA expression of CD122 was significantly up-regulated (Fig. 9). However, once Ras activity was inhibited, the up-regulation of CD122 was obviously impaired (Fig. 9), suggesting the potential involvement of Ras in the lymphocyte activation.

4. Discussion

H-Ras is a small G protein that responds to multiple intra-cellular stimulation and thereby turns on diverse downstream signaling pathways [49]. As an important molecular switch controlling variable cell signaling, abundant studies have been carried out on H-Ras in mammals, however relevant studies in early vertebrates, especially the teleost is almost blank. In the present study, a highly conserved H-Ras was identified from Nile tilapia *O. niloticus*, and its potential regulatory role on lymphocyte-mediated adaptive immunity was then illustrated. To our knowledge, this study represents the first description revealing that H-Ras regulates lymphocyte-mediated adaptive immunity in a fish species.

According to multiple sequence alignment, the amino acid of On-H-Ras is highly conserved compared to selected Ras sequences ranging from nematode to human. The activity of H-Ras depends on cycle between GTP-bound and GDP-bound states, and these two switch regions play an important role in the state alteration by a loaded-spring conformational change [8,50]. The H-Ras also has a universal G-domain consisting of 6 β-sheets and 5 helixes and thus is suspected to function as a nucleotide-binding protein. The most critical elements that contribute to binding are two motifs, the N/TKXD motif and GXXXXGKS/T motif that locate on NB-loop and P-loop, respectively [50–52]. In Nile tilapia H-Ras, both switch regions and functional loops are well conserved with H-Ras from other selected species, and so does the tertiary structure. Ras biology occurs in membrane, so modification of cysteine residues with farnesyl or palmitoyl on hypervariable C-terminal is essential for its membrane anchoring [53,54]. The cysteine residues on C-terminal of On-H-Ras are conserved compared to human and zebrafish H-Ras but not in invertebrates, implying the potential functional

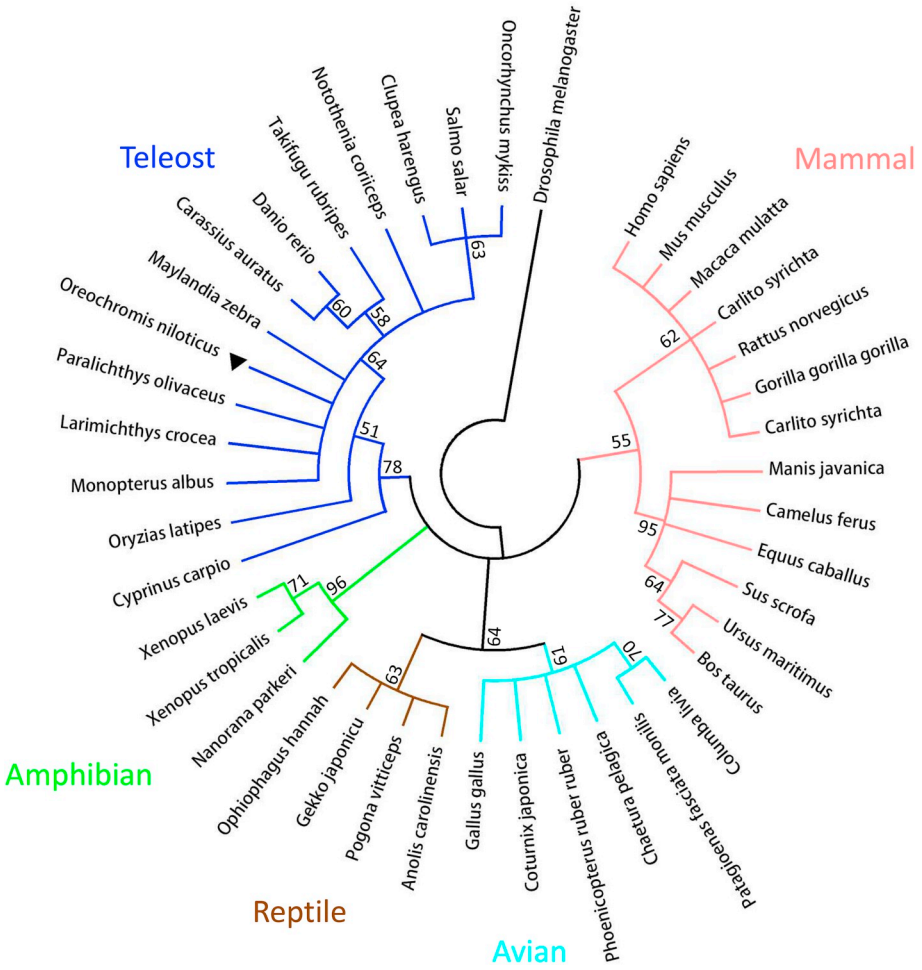

Fig. 3. Phylogenetic tree of H-Ras. The neighbor-joining (NJ), minimum evolution (ME) and maximum parsimony (MP) algorithms all supported the same phylogenetic topology. The species of each class are colored uniformly. The amino acid sequences from vertebrates in the phylogenetic tree included H-Rass from *Oreochromis niloticus* (XP_019217488), *Homo sapiens* (CAG38816), *Danio rerio* (NP_001017623), *Mus musculus* (NP_032310), *Rattus norvegicus* (XP_017457824), *Paralichthys olivaceus* (XP_019957742), *Oncorhynchus mykiss* (XP_021428664), *Sus scrofa* (XP_020938212), *Xenopus laevis* (NP_00 1084278), *Gallus* (NP_990623), *Ophiophagus hannah* (ETE73456), *Bos taurus* (NP_001229275), *Macaca mulatta* (NP_001253350), *Takifugu rubripes* (XP_011 605574), *Gekko japonicus* (XP_015268610), *Pogona vitticeps* (XP_020639780), *Xenopus tropicalis* (NP_001 017003), *Phoenicopterus ruber* (KFQ85779), *Chaetura pelagica* (XP_009994725), *Coturnix japonica* (XP_015 719230), *Larimichthys crocea* (XP_010731032), *Monopterus albus* (XP_020474230), *Notothenia coriiceps* (XP_010772454), *Patagioenas fasciata monilis* (OPJ7 3730), *Oryzias latipes* (XP_023811968), *Salmo salar* (NP_001135104), *Equus caballus* (XP_023510505), *Cyprinus carpio* (XP_018935439), *Nanorana parkeri* (XP_018411539), *Columba livia* (XP_005509117), *Anolis carolinensis* (XP_008106814), *Carlito syrichta* (XP_008046914), *Oryctolagus cuniculus* (pir||A43816), *Gorilla gorilla* (XP_004050407), *Maylandia zebra* (XP_004563448), *Clupea harengus* (XP_012692822), *Carassius auratus* (XP_026058875), *Ursus maritimus* (XP_008697816), *Manis javanica* (XP_017534296), and *Camelus ferus* (XP_006181097). The *Drosophila melanogaster* (AAF54388) is defined as outgroup. Support for each node was tested with 1000 bootstrap replicates.

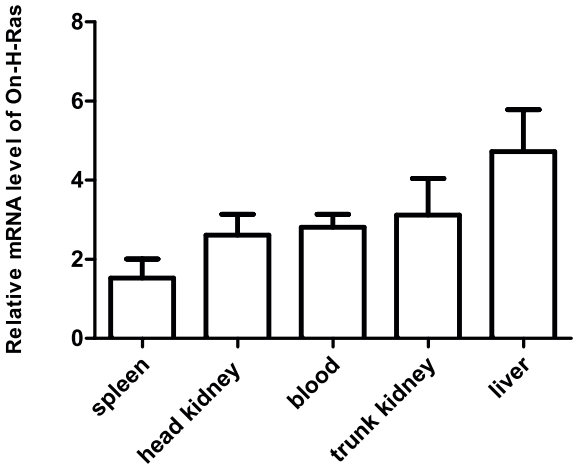

Fig. 4. The tissue expression of On-H-Ras detected by real-time RT-PCR. The On-H-Ras expression at the mRNA level in head-kidney, spleen, blood, trunk kidney and liver were normalized to that of spleen. Data represent the mean ± SE (n = 4-6).

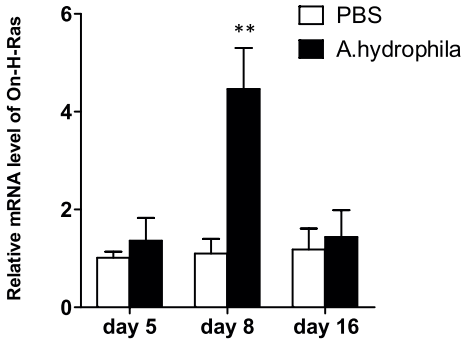

Fig. 5. Inducible expression of On-H-Ras responding to *A. hydrophila* stimulation. The changes of On-H-Ras mRNA expression on day 5, 8 and 16 after stimulation was monitored by real-time RT-PCR. Data represent the mean ± SE (n = 4).

differentiation between vertebrates and invertebrates. Some component in Ras-Raf-Erk axis, such as Raf is activated through dimerization [55], however the activation way requires further illustrate for Ras protein. Although there are evidences supporting the Ras dimer model [56–58], and even nanocluster model composed of 5–10 monomers, interaction among monomers is undefined [59]. According to SWISS-MODEL prediction, On-H-Ras protein could polymerize like human H-Ras, however further study is still needed to illustrate the precise activation way. The GTPase Ras superfamily comprises more than 150 members in human, whose orthologs are also identified in *D. melanogaster, C. elegans, Saccharomyces cerevisiae, Dictyostelium* and plants [60], and in our phylogenetic analysis, the H-Rass clustered well in different classes of vertebrates. Altogether, the conservations of On-H-Ras in sequence, structure and evolutionary profiles suggest its potential similar biological functions with its homologues of higher vertebrates.

Expression pattern is closely associated with physiology function, so we detected the expression of On-H-Ras in lymphoid tissues to better understand its potential role in adaptive immunity. According to Bgee database, H-Ras is expressed in many organs of mouse including liver,

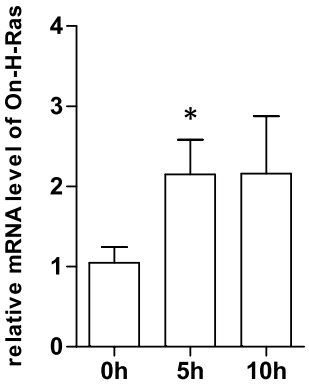

Fig. 6. Upregulation of On-H-Ras post PHA stimulation. The transcript of On-H-Ras in leukocytes was detected by real-time RT-PCR at 0, 5 and 10 h post stimulation. Data represent the mean ± SE (n = 4).

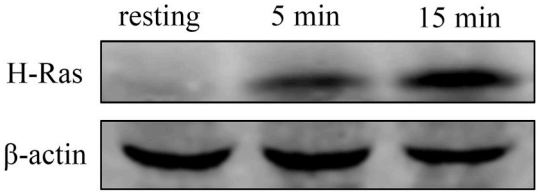

Fig. 7. Enhanced H-Ras after P + I stimulation. The protein of H-Ras in Nile tilapia leukocytes was detected by western-blot assay at 5 and 15 min, respectively post P + I stimulation. The resting cells without P + I treatment were served as control. The data shown are representative of two independent experiments.

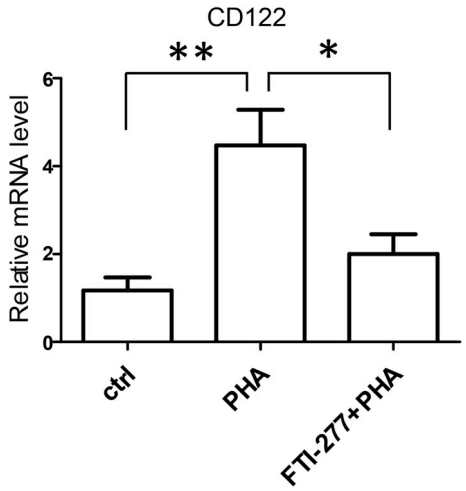

Fig. 9. Expression of Nile tilapia CD122 post PHA stimulation. Nile tilapia individuals were treated with Ras inhibitor FTI-277 for two consecutive days before leukocytes were stimulated with PHA for 10 h. mRNA expression of CD122 was examined by real-time RT-PCR assay. Data represent the mean ± SE (n = 6).

kidney, spleen and blood. In teleost, kidney and spleen are two major lymphoid organs, and lymphocyte is an important subpopulation of blood leukocytes [61], so expression of Ras in these tissues suggested its potential function in immune system. Nowadays, the interaction between thyroid follicles and immune system has been verified, and liver is also an organ where heterotopic thyroid follicles locate in [62]. As Ras responds to multiple extracellular factors including hormones and growth factors, expression of Ras in liver suggested its multifunction.

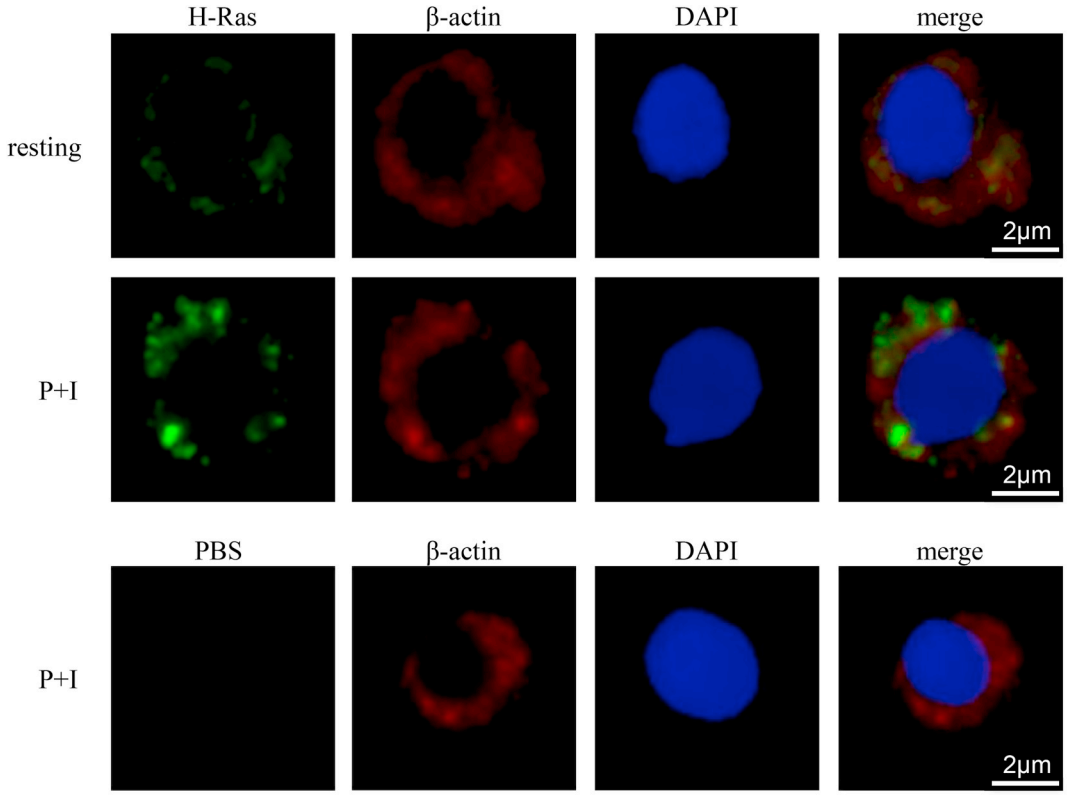

Fig. 8. Augmentation of H-Ras in leukocyte after P + I stimulation. The stimulated cells were harvested at 30 min and resting cells served as control. The stimulated cells were also incubated with PBS instead of *anti*-H-Ras antibody for the negative staining control. Green: H-Ras, red: β-actin, blue: DAPI. The data shown are representative of two independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

However, the tissue expression pattern still requires further studies to illustrate.

To further investigate the involvement of On-H-Ras in adaptive immunity, we infected Nile tilapia with pathogenic bacteria *A. hydrophila* and detected the mRNA expression of On-H-Ras during adaptive immune response stage. As *i.p.* bacterial injection induces a systematic infection, thus the spleen, which is a representative systemic lymphoid organ, was harvested from infected fish for real-time RT-PCR detection. For lymphocyte-mediated adaptive immune response, lymphocytes are activated by antigen beyond 72–96 h after infection, and then cells undergo a prompt clonal expansion that is accompanied by differentiation of effector lymphocytes, which are responsible for infection clearance. Following clearance of pathogen, most majority of effector lymphocytes come to die by apoptosis, but a small percentage of these cells survive to become memory lymphocytes [63–65]. Our data reveals a significant induction of On-H-Ras upon bacterial challenge during the primary response of adaptive immunity (day 5–8), whereas the expression returns to baseline 16 days post infection. This expression pattern of On-H-Ras suggested its potential involvement in the primary response of lymphocyte-mediated adaptive immunity of Nile tilapia.

Ras and its tightly controlled signals play pivotal roles in regulation of lymphocyte-mediated adaptive immunity. Once TCR activated, Ras transiently activated by transforming into active GTP-bound form [66]. Ras activation is important for induction of Erk activity and IL-2 expression during T cell activation; besides, increase of Ras activity might also mobilize activation of calcineurin [19,49,67,68]. To figure out the involvement of H-Ras in lymphocyte activation of Nile tilapia, we activated lymphocytes in vitro and monitored its expression during this process. In our study, the reagents PHA, PMA and ionomycin were used for lymphocyte signaling research. PHA is an exclusive T cell mitogen whose effect is mild so it is usually applied for proliferation study and mRNA expression test [69]. While PMA is a diacylglycerol (DAG) mimicry which can activate protein kinase C (PKC), and ionomycin is an ionophore inducing a bulk of calcium influx. Combination of PMA and ionomycin caused intracellular activation of lymphocytes through a receptor-independent manner, triggering a drastic and prompt activation, and facilitates lymphocyte signaling study [70]. In the present study, we stimulated splenic leukocytes with PHA for expression detection. Our results revealed that the mRNA of On-H-Ras significantly upregulated upon activation, indicating H-Ras took part in the adaptive immune regulation mediated by T cells in Nile tilapia. We further activated the splenic leukocytes with combination of PMA and ionomycin, and found the protein level of On-H-Ras promptly enhanced at 5 min and 15 min post stimulation detected by western-blot. The immunofluorescence analysis also showed augmented of H-Ras in cytoplasm at 30 min post stimulation. Upon the inhibition of Ras activity in Nile tilapia by specific inhibitor FTI-277, the up-regulation of lymphocyte activation marker CD122 was significantly impaired after leukocytes were activated by PHA in vitro, suggesting the involvement of Ras in the lymphocyte activation. Altogether, our results illustrated that On-H-Ras regulated activation of lymphocyte on both mRNA and protein level. In the MAPK cascade, activated Ras causes Raf activation, and ultimately leads Erk entering nuclear to promote gene expression [14]. In previous study, we have proved that Nile tilapia c-Raf could regulate lymphocyte activation by increasing phosphorylation [43]. Here, our works further demonstrated the regulatory roles of H-Ras, which is upstream of c-Raf, to lymphocyte immunity of Nile tilapia. Together, our results tend to suggest that teleost MAPK cascade might function similarly to that in mammals during lymphocyte activation, however, the detailed regulation involving other components of this axis, such as Mek and Erk, remains to be further studied.

In summary, the GTPase H-Ras from teleost *O. niloticus* was characterized in structure, evolution, expression pattern and regulatory role in lymphocyte immunity. Our study represents the first demonstration that teleost uses a conserved H-Ras to regulate the adaptive immune responses meditated by lymphocytes. These results advance the knowledge about regulatory mechanism of teleost adaptive immunity, and thus shed new light to the evolution of adaptive immune system.

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