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Short communication

The atypical chemokine receptor 4 in red sea bream (*Pagrus major*): Molecular characterization and gene expression analysis

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ABSTRACT

Atypical chemokine receptor 4 (ACKR4) is regulated by cytokines, binds chemokines and regulates the chemokine gradient. We verified the cDNA sequence by confirming ACKR4 from red sea bream (PmACKR4) by next generation sequencing (NGS) and analysed the molecular characteristics and gene expression profile. In the analysis using the predicted amino acid sequence of PmACKR4, a highly conserved G protein-coupled receptor 1 region and two cysteine residues were identified and included in the ACKR4 teleost cluster in the phylogenetic analysis. In healthy red sea bream, PmACKR4 mRNA was expressed at the highest levels in head kidney and was upregulated in all immune -related tissues used in the experiment after challenges with *Streptococcus iniae* (*S. iniae*) and red sea bream iridovirus (RSIV). These results suggest that ACKR4 is highly conserved in red sea bream and may play an important role in the immune system as previously reported. It is thought that ACKR4 acts as a regulator of immune -related cells via immune reactions after pathogenic infection.

1. Introduction

Chemokines are chemotactic cytokines that are defined by the presence of four highly conserved cysteine residues that are important for cell migration, angiogenesis, neurological development, cell development and division [1]. They are classified into four groups (CXC, CC, XC and CX3C) based on their characteristic patterns in terms of two N-terminal cysteines, and can also be classified into three groups (inflammatory, homeostatic and dual function chemokines) according to their expression patterns and functions [2,3]. These functions play an important role in linking and activating the innate immune system and adaptive immune system by localizing cells. The biological function of chemokines is generally mediated through G protein-coupled chemokine receptors. A wide range of ligand interactions exist in the chemokine receptor family and many chemokines can bind to multiple receptors [4]. Unlike conventional chemokine receptors, atypical chemokine receptors do not mediate chemotactic or G protein signalling [5].

Atypical chemokine receptors (ACKRs) are currently reported to comprise six members (ACKR1–ACKR6), and it is predicted that all ACKRs except ACKR6 are composed of seven transmembrane domain structures [6]. They are structurally similar to existing chemokine receptors and bind high-affinity chemokines; however, unlike traditional chemokines, they cannot control cell migration [4]. Previous reports have indicated that, ACKRs are involved in chemokine system regulation, chemokine ligand internalization, intracellular degradation and inflammatory responses regulation [4,7], and they are reported to be expressed primarily in leukocyte and non-leukocyte cell types, such as erythrocytes, those constituting lymph nodes and endothelial cells [8,9].

Atypical chemokine receptor 4 (ACKR4), known as CCRL1, CCR11 or CCX-CKR, is regulated by cytokines and binds to homeostatic chemotactic chemokines (CCL19, CCL21 and CCL25) to regulate chemokine gradients [10–12]. In addition, ACKR4 has been reported to play an important role in regulating the trafficking of CCR7-dependent dendritic cells in mice [13]. In addition, ACKR4 is a negative regulator of breast cancer growth and metastasis, and has been shown to promote the progression of CCL21-mediated nasopharyngeal carcinoma in knockdown mice [11,14]. ACKR is not only a major component of the chemokine system in the innate immune system, but has also been reported to be essential for adaptive immunity [15,16]. Despite reports related to various important immunological functions, studies on

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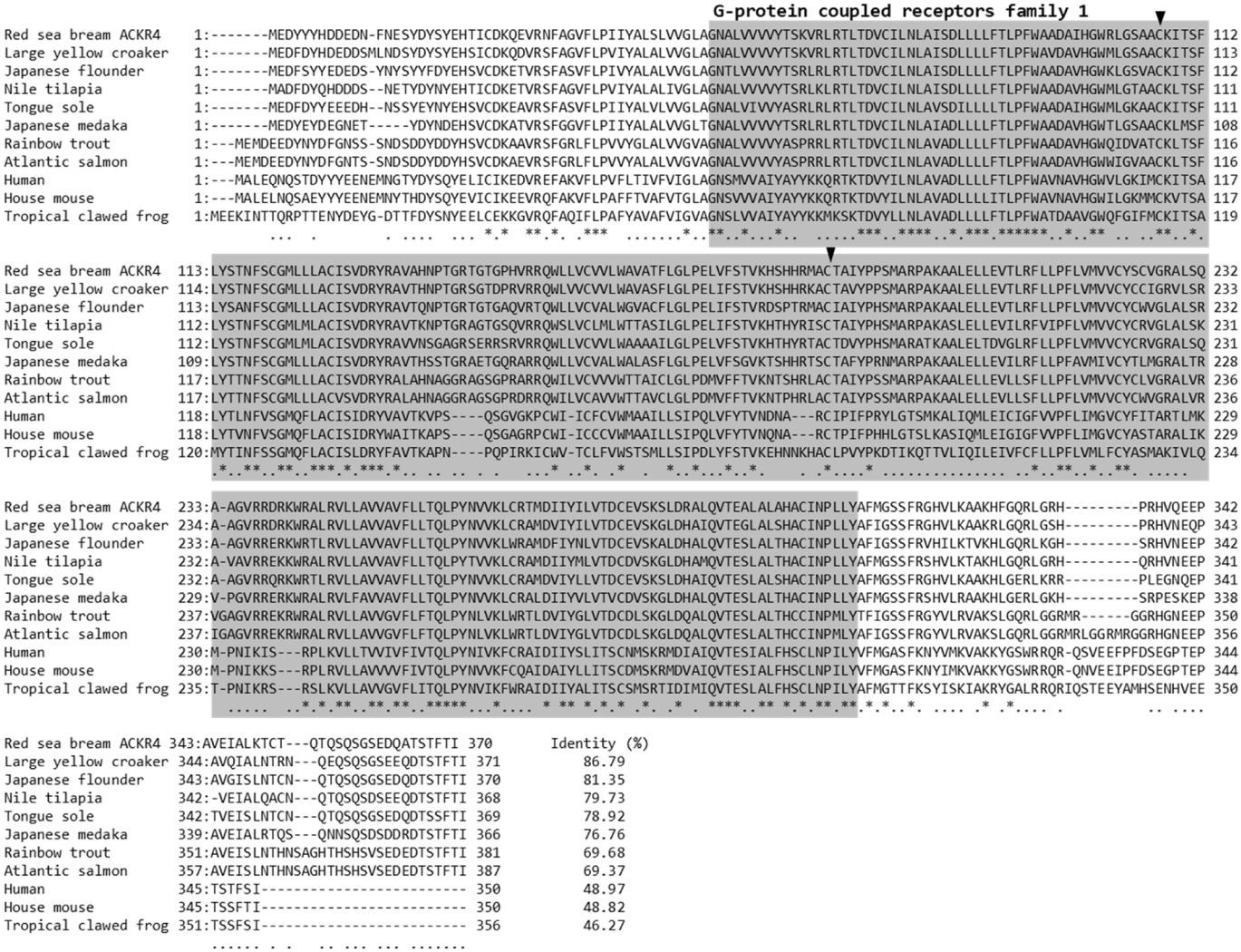

Fig. 1. Comparisons of the PmACKR4 amino acid sequence with other known ACKR4s sequences of different species. Amino acids that are identical to the red sea bream (*Pagrus major*) sequence are indicated by asterisks (*), similar amino acid residues are indicated by dots (.), and the G protein-coupled receptor 1 region and cysteine residues are indicated by the grey box and symbol (▼).

ACKR4 remain limited, especially in teleosts.

Red sea bream (*Pagrus major*) is one of the most important aquacultural species in the Republic of Korea and Japan because of its consistently high consumption by humans and high economic value. However, the economic damage caused by annual pathogenic disease continues. Research and efforts to prevent disease have continued, but they remain insufficient.

In this study, the sequence of ACKR4 (PmACKR4) was obtained from red sea bream to confirm molecular biological characteristics and gene expression patterns. These results suggest that PmACKR4 may play an important role in the immune system against pathogens.

2. Materials and methods

2.1. Fish

The red sea bream (weight: 173.2 ± 31.1 g, body length: 22.4 ± 0.9 cm) used in the experiment was supplied from the Gyeongsangnam-do Fisheries Resources Research Institute (Tongyeong, Republic of Korea) and was maintained at 22 ± 1 °C in an aerated seawater aquarium for two weeks. Commercial feed was provided twice a day, and clinical tests were performed before use to confirm health.

2.2. Sequence analysis

The PmACKR4 sequence was obtained from the livers of red sea bream infected with *Streptococcus iniae* (*S. iniae*) via next generation sequencing (NGS) analysis. Primers were designed based on the obtained open reading frame (ORF) sequence and subcloned into the pGEM T-easy vector (Promega, USA) to verify the ORF sequence of PmACKR4 by Sanger sequencing. The identified PmACKR4 cDNA sequence was predicted using the GENETYX software version 8.0 (SDC Software Development, Japan), and related amino acid sequences were searched using the BLAST algorithm of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The characteristic domain and motif of PmACKR4 were predicted by the PROSITE profile database (http://www.expasy.org/tools/scanprosite/). The deduced amino acid sequences were aligned using GENETYX software version 8.0, and a phylogenetic tree was built using the Molecular Evolutionary Genetic Analysis (MEGA) software version 6.0.

2.3. Tissue collection and cDNA preparation

Twelve tissues (trunk kidney, head kidney, liver, stomach, spleen, skin, muscle, intestine, eye, brain, heart and gills) were aseptically extracted for the profiling of mRNA expression of PmACKR4 from five

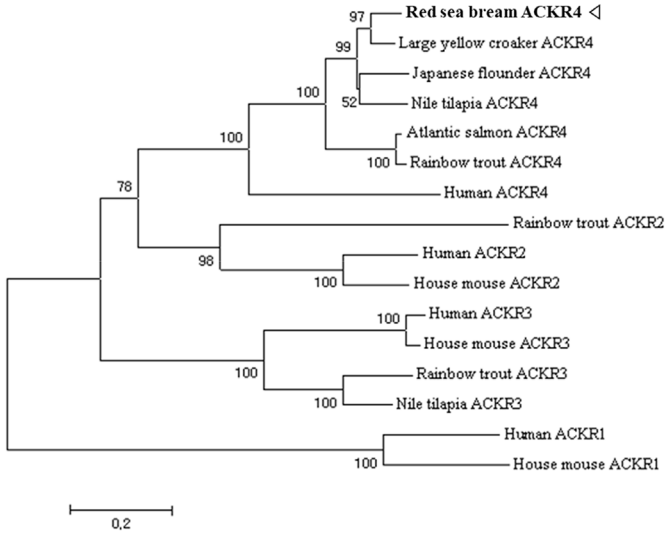

Fig. 2. Phylogenetic tree based on the amino acid sequence of ACKRs downloaded from GenBank. The phylogenetic tree was constructed by the neighbour-joining method. Bootstrap values shown at the nodes of the tree are based on 2,000 bootstrap replicates. The scale bar represents sequence divergence. GenBank accession number: Large yellow croaker ACKR4 (XP_010751862), Japanese flounder ACKR4 (XP_019942932), Nile tilapia ACKR4 (XP_003439264), Atlantic salmon ACKR4 (XP_014036192), Rainbow trout ACKR4 (XP_021418679), Human ACKR4 (NP_848540), Rainbow trout ACKR2 (XP_021469305), Human ACKR2 (NP_001287), House mouse ACKR2 (NP_001263648), Human ACKR3 (NP_064707), House mouse ACKR3 (NP_064707), Rainbow trout ACKR3 (XP_021453433), Nile tilapia ACKR3 (XP_003455483), Human ACKR1 (NP_001116423) and House mouse ACKR1 (NP_034175).

healthy red sea breams and stored at −80 °C until use for total RNA extraction. Total RNA was extracted from tissues using Trizol reagent (Takara, Japan) according to the manufacturer's instructions, and samples were treated with recombinant DNase I (Takara) to remove genomic DNA. The concentration and purity of the total RNA samples were measured with a NanoVue spectrophotometer (GE Healthcare, UK) and total RNA was subjected to cDNA synthesis using the 1st strand cDNA synthesis kit (Takara) according to the manufacturer's instructions. The quality of the synthetic cDNA was confirmed by PCR with the elongation factor-1α (EF-1α) primer and the PCR product was confirmed on agarose gel.

For the analysis of mRNA expression of PmACKR4 after pathogen infection, healthy red sea bream was artificially infected with *S. iniae* (10^5 CFU/fish) or red sea bream iridovirus (RSIV) (10^6 copies/fish). The strains (*S. iniae* and RSIV) that were used for the infection experiments were supplied from the Fish Pathology Division of the National Institute of Fisheries Science (Busan, Republic of Korea). Fish were randomly collected from each group at specific times (0, 1 and 12 h) and (1, 3, 5 and 7 days) post-infection, and tissues (whole kidneys, gills, liver and spleen) were collected aseptically. Total RNA extraction and cDNA synthesis were performed as described above.

2.4. Real-time PCR analysis

To measure the expression levels of PmACKR4 mRNA, RT-qPCR analysis was performed on a Thermal Cycler DICE Real-Time System (Takara) using TB Green™ Premix Ex Taq™ (Takara) and a specific primer set (Forward: 5′-TGACCGACTGTGAAGTCAGC-3′, Reverse: 5′-AAGTGTTTGGCAGCCTTGAG-3′). Relative expression levels of PmACKR4 were calculated using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with EF-1α as a control (Forward: 5′-CCTTCAAGTA CGCCTGGGTG-3′, Reverse: 5′-CTGTGTCCAGGGGCATCAAT-3′), and each sample was analysed in triplicate. Statistical analysis of PmACKR4 mRNA expression after pathogen challenge was performed using SPSS software 19.0 (IBM, USA) with one-way analysis (ANOVA) and Tukey's test (*P value $<$ 0.05 and **P value $<$ 0.01).

3. Results and discussion

The sequence of the verified PmACKR4 was registered with GenBank with the following number: MK593175. Multiple sequence alignment analysis of PmACKR4 with ACKR4 from other species showed that the G protein-coupled receptor 1 region and two cysteine residues (Cys 107 and 187) were well conserved (Fig. 1). These molecular characteristics have been reported to be potentially important for ligand binding, signalling and disulfide bonding [17]. This result also confirms the ACKR sequence that replaces the DRY motif (known as the DRYLAIV motif) located at the border of the second intracellular loop

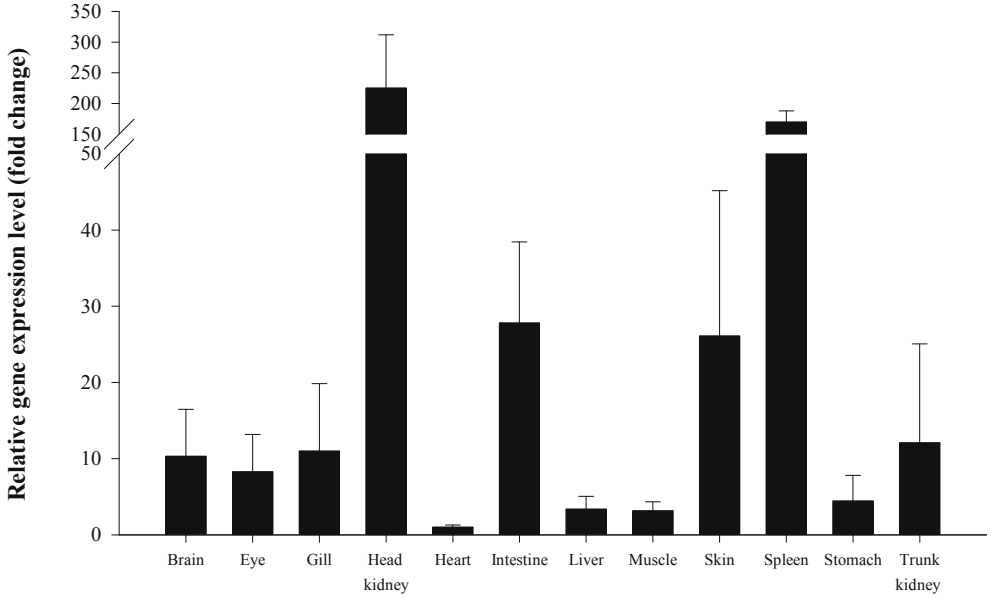

Fig. 3. Gene expression analysis of the PmACKR4 gene by RT-qPCR in various tissues of healthy red sea bream. The EF-1α gene was used to normalise the RT-qPCR results. The experiments were repeated five times and the data are presented as the means ± SD (*P $<$ 0.05, **P $<$ 0.01) versus the control (heart).

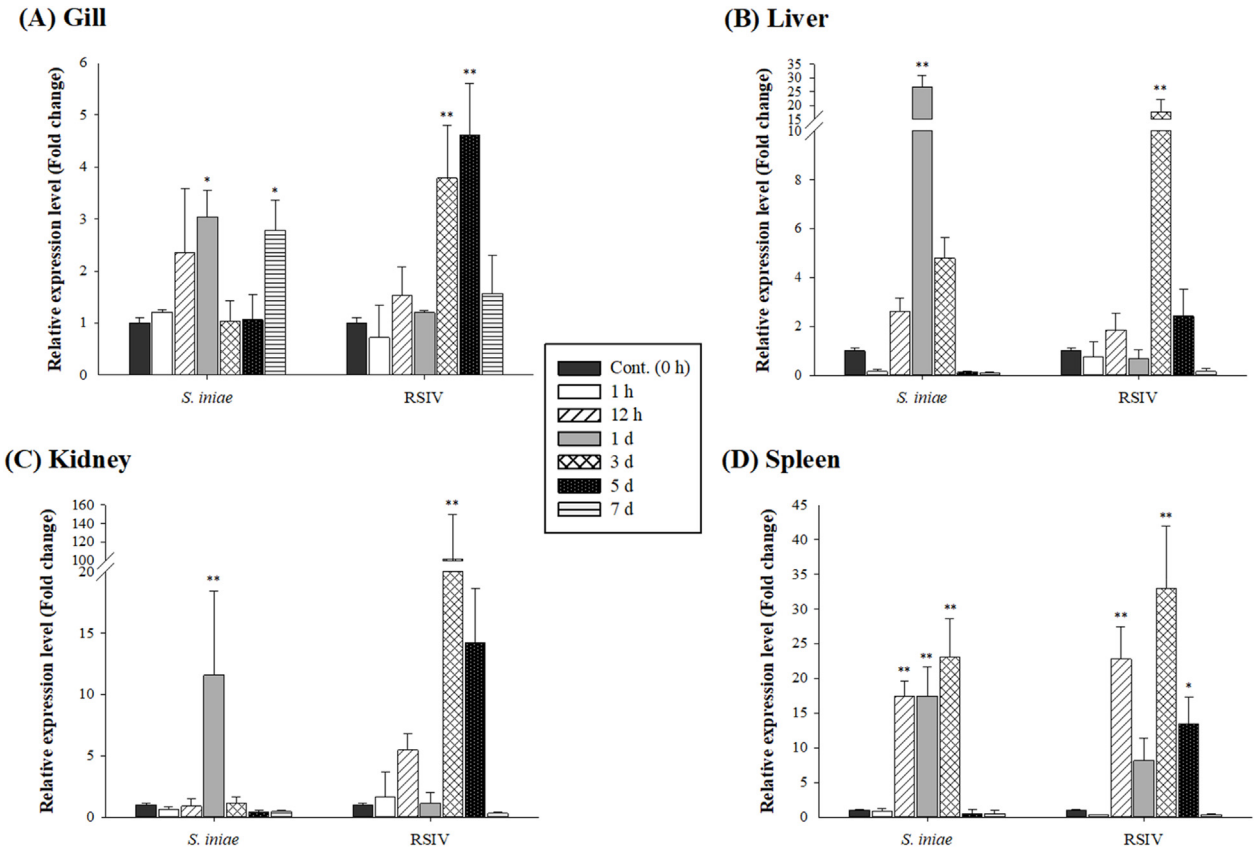

Fig. 4. Expression analysis of PmNKRF mRNA in kidney, spleen, gill and liver of red sea bream after infection with *S. iniae* or RSIV using RT-qPCR. PmNKRF was quantified relative to the EF-1α gene. Gene expression and its significance are represented as the mean ± SD (N = 5). Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$) versus the control (0 h).

and transmembrane domain 3, a typical feature of conventional chemokine receptors. Previous studies have reported that this motif is responsible for the G protein that binds to the receptor, and that this deficiency is associated with a lack of signalling capacity [18,19]. The homology of PmACKR4 and ACKR4s of other species was very high (46.27–86.79%), and showed the highest homology with the ACKR4 of the large yellow croaker (86.79%). Phylogenetic analysis also showed that PmACKR4 belongs to the group of fish ACKR4 and is most closely related to the ACKR4 of the large yellow croaker (Fig. 2). These results suggest that PmACKR4 is functionally similar to the ACKR4s of other species.

ACKR4 has been isolated from many species of fish, but reports on its expression characteristics and functional analysis are very limited. PmACKR4 mRNA was the most abundant in the head kidney and spleen in healthy red sea bream, while it was relatively low in the heart, muscle, liver and stomach (Fig. 3). In zebrafish, ACKR4 is widely distributed throughout embryonic developmental stages and in various tissues of the healthy adult fish, and the highest expression in the spleen and brain is observed in the adult fish [20]. Likewise, in humans and mice, ACKR4 mRNA is distributed in various tissues (especially in immune-related tissues and cells), and these results are similar to our results [21,22].

PmACKR4 mRNA expression was significantly increased in all analysed red sea bream tissues compared to the control (0 h) in pathogen challenge experiments (Fig. 4). In the liver and kidney, the PmACKR4 mRNA expression levels after *S. iniae* and RSIV infection were highest at 1 and 3 days, respectively (Fig. 4B and C). In the gill, PmACKR4 mRNA expression was significantly higher at 1 and 5 days after the *S. iniae* and RSIV challenge (Fig. 4A) and was significantly higher at 3 days in the spleen (Fig. 4D). Overall, PmACKR4 mRNA was relatively high in the kidney and spleen, which are major hemopoietic organs of fish, due to pathogen infection. In addition, mRNA expression levels were significantly upregulated at relatively late time points, indicating that these genes may play important roles as modulators of cellular immunity, which is a typical function of the ACKR. In previous studies, ACKR4 was shown to be essential for regulating the migration of CCR7-dependent antigen presenting cells and stromal cells and is also known to play an important role in regulating the differentiation of early activated B cells and the excessive response of Th17 cells [13,23–25]. As well, ACKR4 has been reported to be regulated by pro-inflammatory cytokines [11], and our additional experiments have also shown a significant increase in interleukin-1β (IL-1β) upon pathogen challenge in red sea bream (data not shown). However, further studies, such as testing gene knockouts or treatment with cytokines, should be conducted to confirm the correlation between IL-1β expression and PmACKR4 regulation. Our results do not demonstrate a clear function for PmACKR4, but suggest the possibility of immunological function during inflammatory response induced by the invasion of certain pathogens. Therefore, additional functional studies of ACKR4 may be required to understand the complex and dynamic cellular immune system during immune responses to disease and infection in teleosts.

In conclusion, we identified PmACKR4 from red sea bream to confirm its molecular characteristics and confirmed the mRNA expression of PmACKR4, which was upregulated by pathogenic infection. Although it is difficult to confirm this with our mRNA expression data alone, PmACKR4 is thought to play an important role in the immune response to pathogen infection in red sea bream.

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