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Identification and expression analysis of IL-4/13 receptors in grass carp *Ctenopharyngodon idella*

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ABSTRACT

Interleukin (IL)-4 and IL-13 are T helper 2 (Th2) cytokines with pleiotropic functions. IL-4 interacts with two receptors consisting of IL-4Rα/γ chain receptor (γC) and IL-4Rα/IL-13Rα1. In contrast, IL-13 binds to IL-13Rα2 but also shares the receptor complex containing IL-4Rα/IL-13Rα1. In fish, two IL-4/13 homologs have been identified but their phylogenetic relationships with IL-4 and IL-13 are ambiguous. In this study, we identified six putative IL-4/13 receptor homologs in grass carp, including γC1, γC2, IL-4Rα1, IL-13Rα1, IL-13Rα2 and a soluble form of IL-4Rα2. Comparative sequence analyses revealed that these receptors possess conserved characteristic domains and the genes encoding them share conserved gene synteny with their human counterparts. All six receptors contain a cytokine binding homology domain (CHD) and two fibronectin type III (FNIII) like domains, with IL-13Rα1 and IL-13Rα2 harbouring an extra Ig-like domain preceding the CHD domain. Interestingly, grass carp IL-13Rα1 and IL-13Rα2 lack the characteristic WSXWS motif, a typical feature of mammalian type I cytokine receptors. The IL-4/13 receptor genes are differentially expressed in tissues and primary leukocytes of head kidney and can be modulated by *Flavobacterium cloumnare (F. cloumnare)*, suggesting they are involved in immune response against *F. cloumnare* infection.

1. Introduction

Interleukin (IL)-4 and IL-13 were among the first cytokines described in the early 1980s. In mammals, IL-4 and IL-13 are pleiotropic cytokines mainly secreted by polarized Th2 T cells, granulocytes and monocytes/macrophages [1,2]. They are structurally and functionally related and are involved in many aspects of physiological processes including immune regulation, pregnancy, fetal development, mammary gland development and lactation, and brain functions such as memory and learning [3].

Most cells in mammals have the potential to respond to IL-4 or IL-13, or both. In the context of the immune responses, IL-4 and IL-13 trigger Th2 T cell differentiation, M2 macrophage polarization, MHCII expression and B cell and plasma cell differentiation. IL-4 and IL-13 exert functions via activating heterodimeric receptors consisting of 4 possible subunits including γC, IL-4Rα, IL-13Rα1 and a decoy receptor, IL-13Rα2 known to modulate the functions of IL-13. The four receptor chains form three types of receptor complexes to interact with IL-4 and IL-13, eliciting distinct cellular signals. IL-4 binds to two hetrodimeric receptors, IL-4Rα/γC and IL-4Rα/IL-13Rα1, with high binding affinity with IL-4Rα followed by recruitment of either γC or IL-13Rα1. Hence, the availability of γC and IL-13Rα1 on the cell surface is crucial to determine the signaling pathways activated within the responding cell [4]. In addition to serving as a subunit of IL-4 receptor, IL-13Rα1 is the binding receptor for IL-13, but with moderate affinity [5,6]. Consequently, the availability of IL-4 and IL-13 in the extracellular environment is an important determinant of signaling elicited by the IL-4Rα/IL-13Rα1. IL-13Rα2 is closely related to IL-13Rα1 and binds to IL-13 with

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high affinity, but not IL-4. The function of IL-13Rα2 is not fully understood. It is generally accepted that the primary role of IL-13Rα2 is to act as a decoy receptor antagonising IL-13 functions [7,8].

All the four receptor subunits are class I cytokine receptors, also known as hematopoietin receptors. They are type I membrane proteins containing a signal peptide, an N-terminal extracellular region, a transmembrane region and a C-terminal intracellular region. The extracellular region comprises a cytokine receptor homology domain (CHD) which has two characteristic tandem fibronectin type III (FNIII) domains of approx. 200 aa (designated as D1 and D2 domains) and contains distinctive motifs that can distinguish class I from class II cytokine receptor family [9]. The CHD of class I receptors is arranged in a pattern of CX-$_{(9\text{-}10)}$-CXWX-$_{(26\text{-}32)}$-CX-$_{(10\text{-}15)}$—C within the first FNIII domain where the two pairs of conserved cysteines form disulfide bonds. The second FNIII domain contains a relatively well conserved WSXWS motif at its C-terminal region [10]. The extracellular region of mammalian IL-4Rα and γC contains a single CHD which is sufficient to facilitate binding of IL-4, whilst the IL-13Rα1 and IL-13Rα2 have an extra Ig-like domain proceeding the CHD [6,11]. Compared with the extracellular region, the cytoplasmic region of type I cytokine receptors is less conserved but displays sequence similarities in Box 1 and 2 which are close to the transmembrane region and are involved in Janus Kinase (Jak) docking [9,12,13]. IL-4Rα has a longer intracellular tail than IL-13Rα1 [14]. As a decoy receptor, IL-13Rα2 has a much shorter intracellular region where signaling motifs such as Box 1 are absent [15].

The IL-4/13 receptors have recently been described in several fish species. For example, two γC genes are now known to be present in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) [16–18]. In trout, the two γCs share 89% aa identity, whilst the two zebrafish γCs have only 28% aa identity. Phylogenetic analyses indicate that the two trout γC genes are orthologs of zebrafish γC1 [17]. Like γC genes, two paralogs of IL-4Rα (IL-4Rα1 and IL-4Rα2) are also found in trout. They can be up-regulated in RTS-11 cells by polyinosinic-polycytidilic acid (polyI:C), LPS and recombinant IFNγ, with IL-4Rα2 being more responsive [19]. Regarding IL-13Rα2, the two trout IL-13Rα2 (IL-13Rα2a and b) paralogs share 79% aa identity and 31% aa identity with human IL-13Rα2 respectively and are highly expressed in gills, spleen and head kidney. The IL-13Rα1a but not IL-13Rα1b possesses an N-termimal Ig-like domain [19].

In the present study, we report the identification and expression analysis of six IL-4/13 receptor subunits in grass carp. They include γC1, γC2, IL-4Rα1, IL-13Rα1, IL-13Rα2 and a soluble form of IL-4Rα2. The receptors were shown to be differentially expressed in tissues of healthy fish and could be modulated by poly(I:C), or infection of *Flavobacterium cloumnare (F. cloumnare)*.

2. Materials and methods

2.1. Fish and challenge experiments

Grass carp (*Ctenopharyngodon idella*) (120 ± 12 g) used in this study were purchased from a local fish farm in Shanghai, China. Fish were acclimatized to laboratory conditions for 10 days in aerated freshwater at 25 ± 2 °C and fed with commercial diet twice daily until 24 h before challenge experiment. Fish were anaesthetized with MS-222 prior to experimental procedures. All experiments were conducted according to the local guidelines of use of animals for research.

To examine tissue expression distribution, tissues including muscle, brain, liver, head kidney, spleen, gills, intestine and skin were sampled from healthy fish. Total RNA was extracted using TRlzol Reagent (Ambion, USA) according to the manufacture's protocol for real time qPCR analysis.

The pathogenic G_4 strain of *F. cloumnare* was provided by Dr Haixie Xie, the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) [20]. *F. cloumnare* was cultured in Shieh medium at 28 °C for 48 h with constant shaking (200 rpm). Bacteria were collected by centrifugation at 4200 × *g* for 4 min, washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS buffer. The bacterial suspension was adjusted to 1×10^7 colony-forming units (CFU)/mL. The experimental fish were randomly divided into two groups (16 fish per group). For the challenge group, fish were injected intraperitoneally (i.p.) with bacterial suspension (1 μL/g body wieght). The control fish were i.p. injected with PBS (1 μL/g body weight). At 12, 24, 48 and 72 h after infection, head kidney and spleen tissue were sampled for total RNA extraction.

Poly(I:C) (Sigma, USA) was dissolved in sterile PBS. The experimental fish were randomly divided into two groups (12 fish per group). For treatment group, fish were i.p. injected with 100 μL poly(I:C) (5 mg/mL). Meanwhile, the control fish were injected with 100 μL PBS. At 12, 24 and 48 h after infection, spleen were sampled for total RNA extraction.

2.2. Expression analysis of receptor genes in primary head kidney cells

Head kidney tissue was collected from healthy fish, placed on a 70 μm nylon mesh and gently pushed through the mesh with constantly dripping of cold L-15 medium containing 0.1% FBS, 0.1% heparin, 100 U/mL penicillin and 100 U/mL streptomycin. The cell suspension was centrifuged at 400 × *g* for 30 min (without breaking) on a 45% Percoll gradient. Cells at the medium-percoll interface were collected and washed twice with the medium by centrifugation at 400 × *g* at 4 °C for 10 min. The cells were seeded into 6 well cell culture plates at 1.5×10^6 cells/well and cultured at 28 °C in a 5% CO_2 incubator for 8 h. The attached cells and those in suspension were collected for RNA preparation and real-time PCR analysis.

2.3. Sequencing of IL-4/13 receptor genes

Total RNA was extracted from head kidney of healthy grass carp using TRlzol Reagent (Ambion, USA) according to the manufacture's protocol. The RNA concentration was quantitated with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The First-strand cDNA was synthesized from total RNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. The synthesised cDNA was stored at −20 °C until use.

The partial cDNA sequences of grass carp γC1, γC2, IL-13Rα1, IL-13Rα2, IL-4Rα1 and soluble IL-4Rα2 were obtained from the whole-genome sequence database of grass carp (http://www.ncgr.ac.cn/grasscarp/) [21]. To obtain the full-length cDNA sequences, rapid amplification of cDNA ends (RACE) was performed. Based on the partial cDNA sequences, the gene-specific primers (GSP) for RACE PCR were designed (Supplement Table 1). For the 3′ RACE-PCR, the first strand cDNA was synthesised with primer Oligo(dT)$_{16}$ (Thermo Scientific, USA) as template. The 3′ end region of six receptors was amplified by PCR using F1 primers and adaptor primer APT for the first round PCR, followed by the nested PCR with primers F2 and adaptor primer AP using the first round PCR products (diluted 1:50, v/v) as template. For the 5′ RACE PCR, the cDNA samples were purified with the DNA purification kit (OMEGA, USA), tailed with poly(C) using terminal deoxynucleotidyl transferase (TdT) (TaKaRa, Japan). The PCR amplification was performed using R1 primers and adaptor APG for the first round PCR. The nested PCR was performed as in the 5’ RACE using primers R2 and AP. The PCR cycling conditions are: initial denaturing at 94 °C for 2 min, followed by 35 cycles of 94 °C/30 s, 55–64 °C/30 s, 72 °C/2 min, and a final extension at 72 °C for 10 min.

All the PCR products with expected molecular size were purified and sequenced. The DNAMAN (8.0) software program was used to assemble the full length cDNA sequence. The full length coding sequences were confirmed by a pair of primers located at the 5′ and 3′ untranslated region (UTR) (Supplement Table 1). The sequences were analysed using the BLAST program and termed *Ci*γC1, *Ci*γC2, *Ci*IL-4Rα1, s*Ci*IL-4Rα1,*Ci*IL-13Rα1 and *Ci*IL-13Rα2.

Table 1
Characteristics of IL-4/13 receptor subunits.

Features	Human γC	Grass carp γC 1	Grass carp γC2	Human IL-4Rα	Grass carp IL-4Rα1	Grass carp sIL-4Rα2	Human IL-13Rα1	Grass carp IL-13Rα1	Human IL-13Rα2	Grass carp IL-13Rα2
Extracelluar domain	Two FNIII domain	Two FNIII domain	Two FNIII domain	Two FNIII domain	Two FNIII domain	Two FNIII domain	One Ig and two FNIII domain	One Ig and two FNIII domain	One Ig and two FNIII domain	One Ig and two FNIII domain
Intramolecular disulfide	C^{62}–C^{72} C^{102}–C^{115}	C^{28}–C^{38} C^{63}–C^{76}	C^{33}–C^{43} C^{68}–C^{81}	C^{34}–C^{44} C^{54}–C^{84} C^{74}–C^{86}	C^{30}–C^{40} C^{50}–C^{80} C^{70}–C^{82}	C^{30}–C^{40} C^{50}–C^{80} C^{70}–C^{82}	C^{134}–C^{144} C^{173}–C^{185}	C^{167}–C^{180}	C^{145}–C^{155} C^{184}–C^{197}	C^{151}–C^{161} C^{190}–C^{203}
N-linked glycosylation sit	N^{24}, N^{71}, N^{75}, N^{84}, N^{159}, N^{249}	N^{37}, N^{48}, N^{97}, N^{119}, N^{135}, N^{158}, N^{211}	N^{48}, N^{53}, N^{76}, N^{104}, N^{125}, N^{134}, N^{142}, N^{168}, N^{217}	N^{53}, N^{98}, N^{128}, N^{134}, N^{176}, N^{209}, N^{481}	N^{56}, N^{111}, N^{176}	N^{111}, N^{136}	N^{37}, N^{61}, N^{105}, N^{138}, N^{157}, N^{235}, N^{265}, N^{293}, N^{392}, N^{341}	N^{24}, N^{34}, N^{62}, N^{90}, N^{118}, N^{131}, N^{142}, N^{198}, N^{202}, N^{240}, N^{264}	N^{115}, N^{215}, N^{290}, N^{299}	N^{67}, N^{121}, N^{221}, N^{342}
Functional motif	WSXWS and Box1and 2 motif	WSXWS and Box1 motif	WSXWS and Box1 motif	WSXWS and Box1 and 2 motif	LSXQS and Box1 motif	LSXQS motif	WSXWS and Box1 motif	KSXLS and Box1 motif	WSXWS motif	WSXWS motif

2.4. Sequence analysis

The nucleotide sequences were assembled and analysed using the DNAMAN programme (8.0). Homology identification was conducted using the BLAST program at the NCBI website (http://blast.ncbi.nlm.nih.gov). Programmes at the Expasy website (http://www.expasy.org/tools) [22] were used to analyse protein sequences. Protein domains were predicted using the SMART programme (http://smart.embl-heidelberg.de) and the Dompred program [23]. Signal peptide was predicted using the SignalP 3.0 program [24] and the transmembrane domain was predicted using the TMpred program [25]. Multiple sequence alignment was generated using the ClustalW2 program [26]. Phylogenetic trees were constructed by the Neighbour-Joining method using the MEGA 5.0 program [27] based on the CLUSTAL multiple alignment of the selected receptor sequences and bootstrapped for 10,000 times. The JTT matrix-based method was chosen for tree construction. All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (Pairwise deletion).

2.5. Real-time PCR quantification of gene expression

The first-strand cDNAs were synthesised using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfert Real Time) (TaKaRa, Japan) for quantitative real-time PCR and stored at −20 °C until use. qRT-PCR was carried out using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) according to the manufacturer's instructions and the LightCycler 96 Real Time PCR System (Roche, Switzerland). Each sample was loaded in triplicate on the PCR plate. The primers used for real-time PCR analysis were given in Supplement Table 1 and the elongation factor-1α (EF-1α) was used as the internal control to normalise gene expression. For comparison of the relative expression level, a standard curve was established using a mixture of equal amounts of purified PCR products amplified from cloned plasmids. A serial 10 fold dilutions of the standard template were run and served as a reference for quantification. The expression levels of each gene were calculated as arbitrary units normalised to the expression level of EF-1α. Fold changes of expression were calculated by comparing the average expression level of experimental group with that of the corresponding control group.

2.6. Statistical analysis

The real-time quantitative PCR data were analysed using the SPSS package 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance and the LSD post hoc test was used to analyse the expression data of challenge experiment, with $p < 0.05$ between treatment groups and control groups considered significant.

3. Results

3.1. Sequence analysis of γC1 and γC2

Two grass carp γC genes, designated as *Ci*γC1 and *Ci*γC2, were obtained and predicted to translate into proteins of 360 aa and 335 aa respectively (Fig. 1). The *Ci*γC1 contained a predicted signal peptide of 19 aa, an extracellular region of 201 aa, a predicted transmembrane domain of 23 aa and an intracellular region of 117 aa. The *Ci*γC2 molecule contained a predicted signal peptide of 26 aa, an extracellular region of 196 aa, a predicted transmembrane domain of 23 aa and an intracellular region of 90 aa. The extracellular region of both molecules consisted of two tandem fibronectin type III (FNIII) like domains (D1 and D2 domain). The *Ci*γC2 molecule lacked one of the seven predicted β-strands in the D2 (Supplement Fig. 2). In the D1 domain, four conserved cysteine residues were seen in both molecules with a signature of C-X$_{9}$.-C-X-W-X$_{22}$C-X$_{12}$-C similar to that of human γC (C-X$_{9\text{-}10}$-C-X-W-X$_{26\text{-}32}$C-X$_{10\text{-}15}$-C). The D2 domain of both receptors contained a WSXWS motif and two conserved cysteine residues (Table 1). In the cytoplasmic region, a Pro-Val-Val-Pro (PVVP) signature sequence and a cluster of hydrophobic amino acids (WWxxxPxP, designated as Box 1 motif) were present in both receptors [28] (Supplement Fig. 1 and 2). Seven and nine potential N-glycosylation sites were predicted in the extracellular region of *Ci*γC1 and *Ci*γC2, respectively (Supplement Fig. 1 and 2).The *Ci*γC1 molecule shared 36% and 73% aa identity with the *Ci*γC2 and a γC1 from closely related zebrafish (*Danio rerio*). However, the *Ci*γC2 shared relatively lower aa identity (62%) with zebrafish γC2. The *Ci*γC1 and *Ci*γC2 shared low identity with the γC molecules from mammals (28–32%).

3.2. Sequence analysis of IL-4Rα1 and soluble IL-4Rα2

The predicted *Ci*IL-4Rα1 protein contained a signal peptide of 20 aa, an extracellular region of 214 aa, a transmembrane domain of 23 aa and an intracellular domain of 322 aa (Fig. 2). Two tandem FNIII like domains (D1 and D2) were seen, each comprising seven predicted β-strands (Supplement Fig. 5). In the D1 domain, six cysteine residues were present, forming the conserved signature of class I cytokine receptor family [18]. The D2 domain lacked cysteine residues and the WSXWS motif was absent (Table 1). However, two proline residues located in the flexible hinge region between the D1 and D2 domains were conserved. In the cytoplasmic region, a putative Box 1 motif and four tyrosine residues could be identified (Supplement Fig. 5). Key residues within the Box 1 motif are well conserved. Homology analysis showed that *Ci*IL-4Rα1 shared low identity with their mammalian counterparts (21–24%) and relatively higher aa identity (50%) with zebrafish IL-4Rα1.

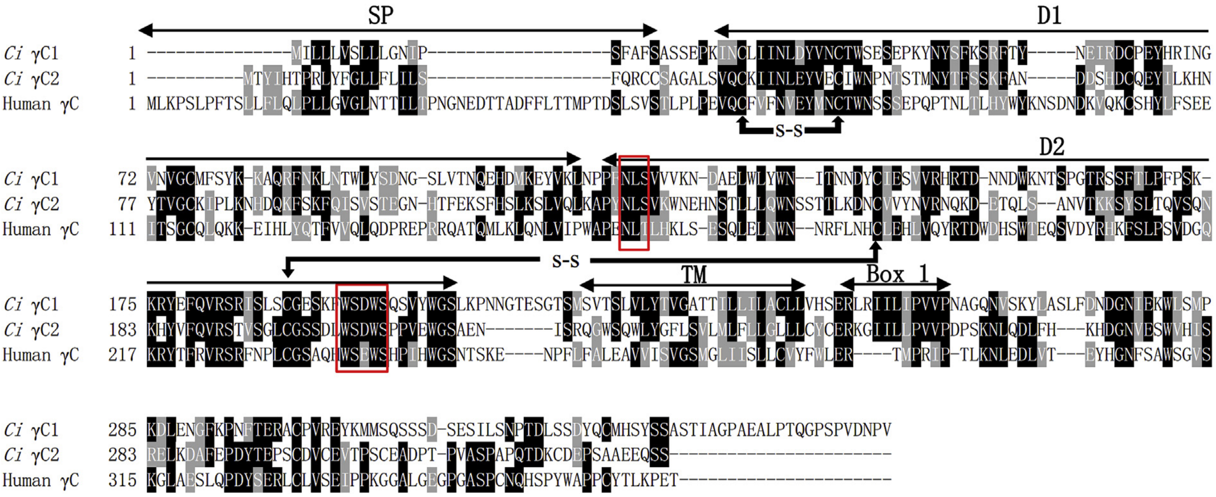

Fig. 1. Multiple alignment of deduced amino acid sequences of *Ci*γC1, *Ci*γC2 and human γC. Multiple alignment was generated using ClustalW2 (V3.21) and conserved amino acids shaded in black. Signal peptide (SP), D1 and D2 domain, transmembrane domain (TM) and the Box 1 motif are indicated by solid lines above the alignment. The four conserved cysteine residues in the D1 and D2 domain that form two intramolecular disulphide bonds are indicated. Other highlighted features include the WSXWS motif and one conserved potential N-glycosylation site.

A soluble variant of *Ci*IL-4Rα2 (s*Ci*IL-4Rα2) was also sequenced (Fig. 2). The predicted s*Ci*IL-4Rα2 protein contained a signal peptide of 20 aa and a secreted protein of 232 aa. Two tandem FNIII like domains (D1 and D2) were predicted and each contained seven predicted β-strands (Supplement Fig. 6).

3.3. Sequence analysis of IL-13Rα1

The grass carp IL-13Rα1 gene was predicted to translate into a protein of 409 aa (Fig. 3). It contained a putative signal peptide of 22 aa, an extracellular region of 295 aa, a transmembrane domain of 23 aa and an intracellular region of 69 aa. Compared with mammalian IL-13Rα1s [7,29], the *Ci*IL-13Rα1 had an Ig-like domain proceeding the CHD (D1 and D2) domain. However, the IL-13Rα1 had six predicted β-strands in the Ig-like and D2 domain, respectively. There were two proline residues present in the linker region between Ig-like and D1 domain. There were four, three and four cysteine residues in the Ig-like, D1 and D2 domains, respectively. Ten potential N-glycosylation sites were predicted in the extracellular region. A proline-rich motif (Box 1) and a tyosine residue in the intracellular domain were also present (Supplement Fig. 3). The *Ci*IL-13Rα1 molecule shared 24–56% and 21–22% sequence identity with homologs from other fish species and mammals, respectively. However, similarity between *Ci*IL-13Rα1 and *Ci*IL-13Rα2 was low (25%).

To compare the aa sequence of *Ci*IL-13Rα1 and IL-13Rα1 from other species, a multiple alignment was generated (Fig. 3). All the IL-13Rα1s had similar features including a signal peptide, an extracellular region consisting of an Ig-like domain, D1 and D2 domains, a transmembrane domain and an intracellular region. The four signature cysteine residues of class I cytokine receptors were located in the first FNIII domain of CHD and were conserved except for the trout IL-13Rα1a, *Ci*IL-13Rα1 and zebrafish IL-13Rα1 where the first cysteine residue was absent. In the D1 domain, most molecules had four cysteine residues with three of them well conserved whilst the other one (the second cysteine in the D1 domain) was conserved within fish but not in mammals. The linker sequence between the D1 and D2 domain were remarkably proline rich. Interestingly, the WSXWS motif, which is conserved in all the mammalian IL-13Rα1s, is not present in any of fish IL-13Rα1s. In the intracellular region, the proline-rich Box 1 and the tyrosine residue important for signaling are identifiable among all the IL-13Rα1s sequences (Fig. 3) [14] (see Fig. 2).

3.4. Sequence analysis of IL-13Rα2

The cDNA sequence of *Ci*IL-13Rα2 gene is 1846 bp in length with a canonical polyadenylation signal (ATTAAA) upstream of the poly A tail (Supplement Fig. 4). The translated protein consisted of 416 aa containing a signal peptide of 28 aa, an extracellular region of 331 aa, a transmembrane domain of 23 aa and an intracellular region of 34 aa (Fig. 4). The extracellular region contained three domains (Ig-like, D1 and D2), each with seven predicted β-strands as seen in mammals [29], but D2 domain had a predicted β-strand missing. The WSXWS motif was present in *Ci*IL-13Rα2 (Table 1).

There were two and one proline residues in the linker region between Ig-like and D1, and between D1 and D2, respectively. Four potential N-glycosylation sites were predicted in the extracellular region of *Ci*IL-13Rα2. Two tyrosine residues are present in the intracellular domain of *Ci*IL-13Rα2 (Supplement Fig. 4). The *Ci*IL-13Rα2 protein shared 41–77% and 31–34% aa identity with homologs from fish and mammals, respectively. Of the four potential N-glycosylation sites found in the *Ci*IL-13Rα2 molecule, the first two sites were conserved among most vertebrate species (Fig. 4).

3.5. Phylogenetic analysis

To confirm the phylogenetic relationships of six grass carp IL-4/13 receptors with homologs from other vertebrates, an unrooted tree was constructed. The protein sequences of γC, IL-4Rα, IL-13Rα1 and IL-13Rα2 from fish and vertebrate species were selected for analysis. Fig. 5 showed that both *Ci*γC molecules grouped closely with the γCs from fish species and formed a clade with tetrapod γCs with a bootstrap value of 86%. Within the fish clade, the two *Ci*γCs with their respective zebrafish homologs were well separated, indicating two separate γC groups had diverged in teleosts. It was apparent that the two rainbow trout γCs recently described were orthologs of fish γC1 [21]. In contrast, the two IL-4Rα1 genes found in rainbow trout, zebrafish and grass carp did not split into distinct clades of IL-4Rα1 and Rα2 within teleosts. Within the IL-13Rα clade, the grouping confidence of homologs from fish and mammals remained weak, with a relatively low bootstrap value (52%) seen between fish and mammalian IL-13Rα1s. The bootstrap values of the IL-13Rα clade were generally low. The gene synteny of receptor genes are conserved among human, zebrafish and human except for the γC1 gene which showed a different panel of neighboring genes (Fig. 6).

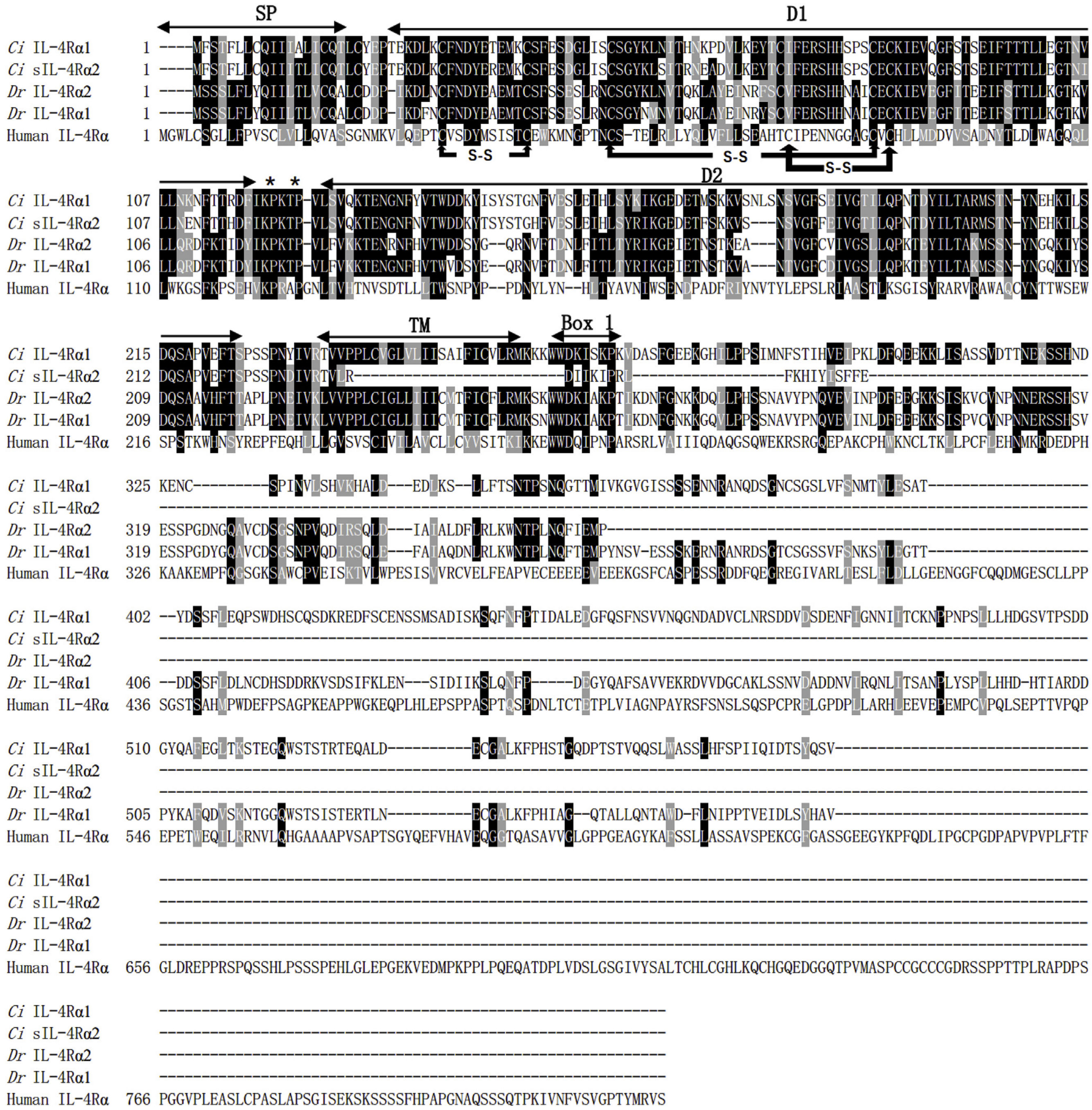

Fig. 2. Multiple alignment of the deduced amino acid sequence of *Ci*IL-4Rα1 and s*Ci*IL-4Rα2 with homologs from zebrafish and human. Multiple alignment was generated using ClustalW2 (V3.21) and conserved amino acids shaded in black. Signal peptide (SP), D1 and D2 domain, transmembrane domain (TM) and Box 1 motif are indicated by solid lines above the alignment. The six conserved cysteine residues in the D1 domain that form three intramolecular disulphide bonds are indicated. The two conserved proline residues in the linker region between the D1 and D2 domain are indicated by "*".

3.6. Tissue distribution

Quantitative RT-PCR analysis was used to quantify the gene expression of receptor subunits in various tissues. As shown in Fig. 7, all the receptor subunits were constitutively expressed in the eight tissues analysed but the expression levels varied considerably among the tissues. The highest level of expression of *Ci*γC1 and *Ci*γC2 was found in spleen and brain. Comparably, the *Ci*γC1 expression was significantly higher than that of *Ci*γC2 in spleen but lower in brain (Fig. 7a). Head kidney, thymus and gills also expressed high levels of *Ci*γC1, whilst for *Ci*γC2, high levels of expression were detected in liver, gills, head kidney and skin.

The *Ci*IL-4Rα1 was constitutively expressed in all the tissues examined. The expression of *Ci*IL-4Rα1 was significantly higher than that of s*Ci*IL-4Rα2 in spleen but lower in liver where the highest expression of s*Ci*IL-4Rα2 was detected (Fig. 7b).

The expression of *Ci*IL-13Rα1 was higher than *Ci*IL-13Rα2 in most tissues including instestine, gills, liver, spleen, skin and brain. The highest expression of *Ci*IL-13Rα1 and *Ci*IL-13Rα2 was detected in the intestine and head kidney respectively (Fig. 7c).

3.7. Gene expression in head kidney leukocytes

Primary head kidney leukocytes were isolated for investigating the

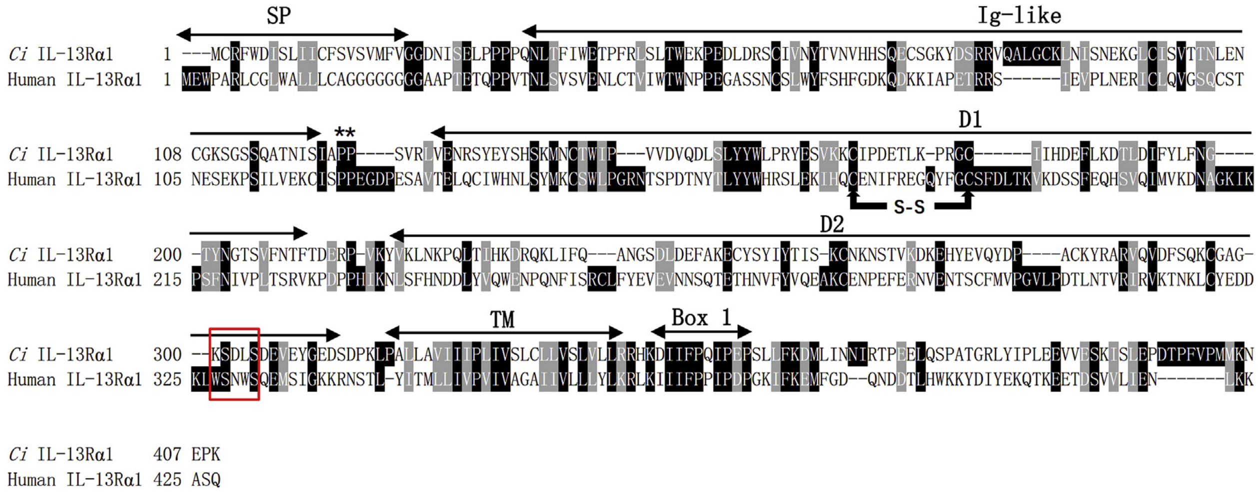

Fig. 3. Multiple alignment of the deduced amino acid sequences of CiIL-13Rα1 with known IL-13Rα1 molecules of human. The multiple alignment was produced using ClustalW2 and conserved amino acids shaded using BOXSHADE (V3.21). The signal peptide (SP), the Ig-like, D1 and D2 domains, the transmembrane domain (TM) and Box 1 motif are indicated by solid lines above the alignment. The two conserved cysteine residues in the D1 domain that form one intramolecular disulphide bonds are also indicated. The highlighted features indicated the WSXWS motif in the mammalian molecules. The two conserved proline residues in the linker region between the Ig-like and D1 domains are indicated by "*" above the alignment. The accession numbers for sequences used in this analysis are given in Fig. 5.

receptor gene expression in immune cells. The cells were cultured for 8 h at 28 °C and adherent cells and cells in suspension collected for qPCR analysis. The adherent cells contained enriched macrophages whilst suspension fraction comprised mostly lymphocytes, neutrophils and monocytes. The two γC receptors were highly expressed in both fractions whilst the transcript levels of other IL-4/13 specific receptor chains were relatively low (Fig. 8). There was no significant difference of gene expression between the adherent and suspension fractions for most of the receptor genes with one exception that the expression level of *Ci*IL-13Rα1 was higher in the suspension fraction than that in the adherent fraction.

3.8. Gene expression in response to F. cloumnare or poly(I:C)

The modulation of IL-4/13 receptors expression in response to *F. cloumnare* infection or poly(I:C) was investigated. The *Ci*γC1 and *Ci*γC2 were significantly up-regulated ($p < 0.05$) in head kidney and spleen at 24 h post infection (hpi) (Fig. 9). In spleen, the expression of *Ci*IL-4Rα1 and s*Ci*IL-4Rα2 was also increased at 24 and 12 hpi respectively (Fig. 9). In contrast, head kidney showed down-regulation of *Ci*IL-4Rα1 at 24, 48 and 72 hpi and s*Ci*IL-4Rα2 at 12 and 24 hpi. There was no apparent pattern of expression modulation of *Ci*IL-13Rα1 and *Ci*IL-13Rα2 after infection of *F. cloumnare*. Following early up-regulation at 12 hpi, the *Ci*IL-13Rα1 transcripts decreased significantly at 24 hpi and returned to normal level in spleen. Head kidney showed decrease of *Ci*IL-13Rα1 transcripts. For the *Ci*IL-13Rα2, its expression was largely unaltered after infection except for the down-regulation at 24 hpi in spleen and up-regulation at 48 hpi in head kidney. Compared with *F. cloumnare*, poly(I:C) had no apparent effect on the expression of all receptor genes except the *Ci*IL-13Rα2 (Fig. 10).

4. Discussion

In this report, six IL-4/13 receptor genes were described in grass carp. They include *Ci*γC1, *Ci*γC2, *Ci*IL-4Rα1, s*Ci*IL-4Rα2, *Ci*IL-13Rα1 and *Ci*IL-13Rα2. They possess characteristic features of class I cytokine receptor family such as the presence of a cytokine binding homology domain (CHD) and two fibronectin type III (FNIII) like domains in the extracellular region. The grass carp IL-13Rα1 and IL-13Rα2 lack the WSXWS motif seen in mammalian type I cytokine receptors (Table 1). The IL-4Rα has a long cytoplasmic region whilst the γCs, IL-13Rα1 and IL-13Rα2 have a relatively short cytoplasmic region [18,30]. The

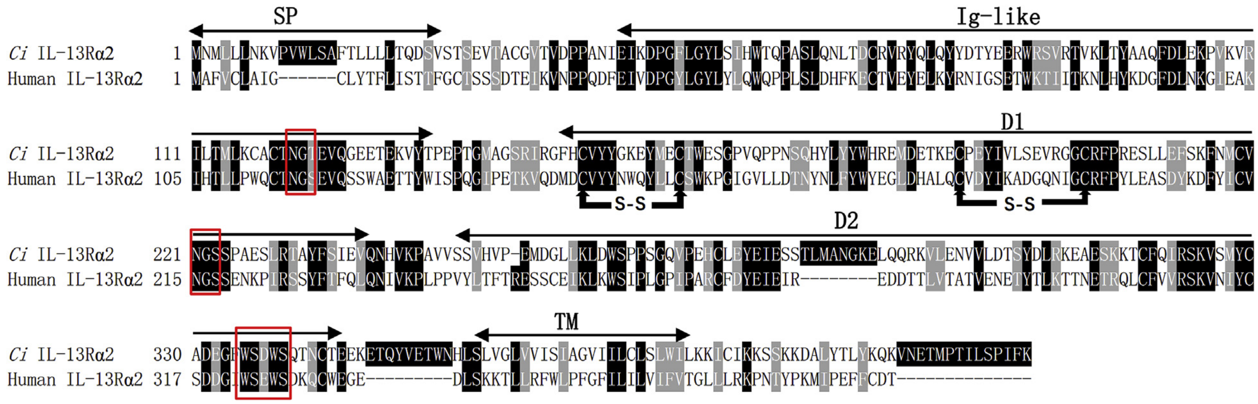

Fig. 4. Multiple alignment of the deduced amino acid sequence of CiIL-13α2 with known IL-13Rα2 molecules of human. The multiple alignment was produced using ClustalW2 and conserved amino acids shaded using BOXSHADE (V3.21). The signal peptide (SP), the Ig-like, D1 and D2 domains and the transmembrane domain (TM) are indicated by solid lines above the alignment. The four conserved cysteine residues in the D1 domain that form two intramolecular disulphide bonds are also indicated. The highlighted features include two conserved potential N-glycosylation sites and the WSXWS motif. The accession numbers for sequences used in this analysis are given in Fig. 5.

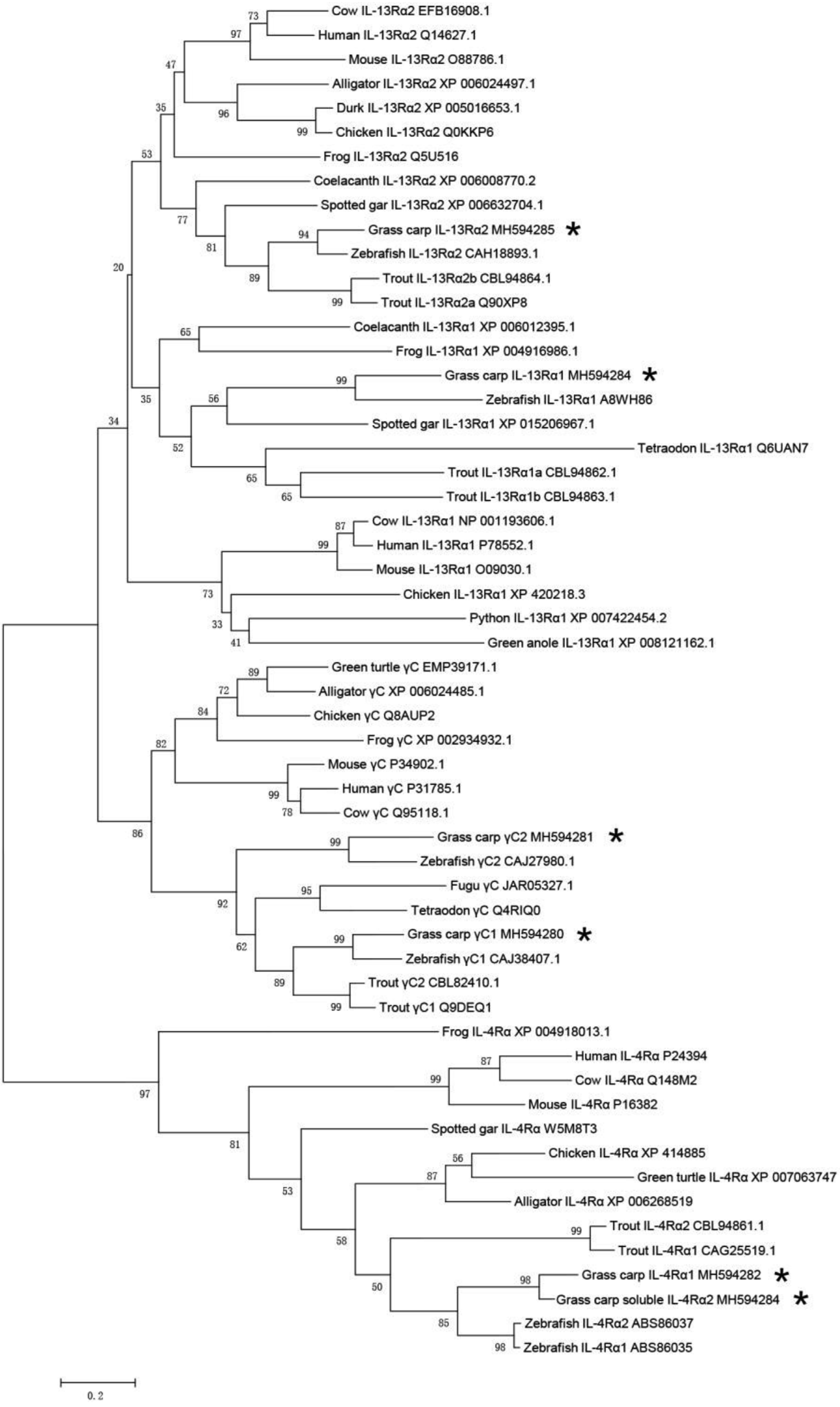

Fig. 5. Neighbour joining (NJ) phylogenetic tree of IL-4/IL-13 receptors. The tree was constructed using the NJ method within the MEGA5 program. The JTT matrix-based model using pair-wise deletion option was chosen. The percentage of bootstrap values is shown next to the branches based on 10,000 bootstrap replications. The grass carp sequences are indicated by asterisks.

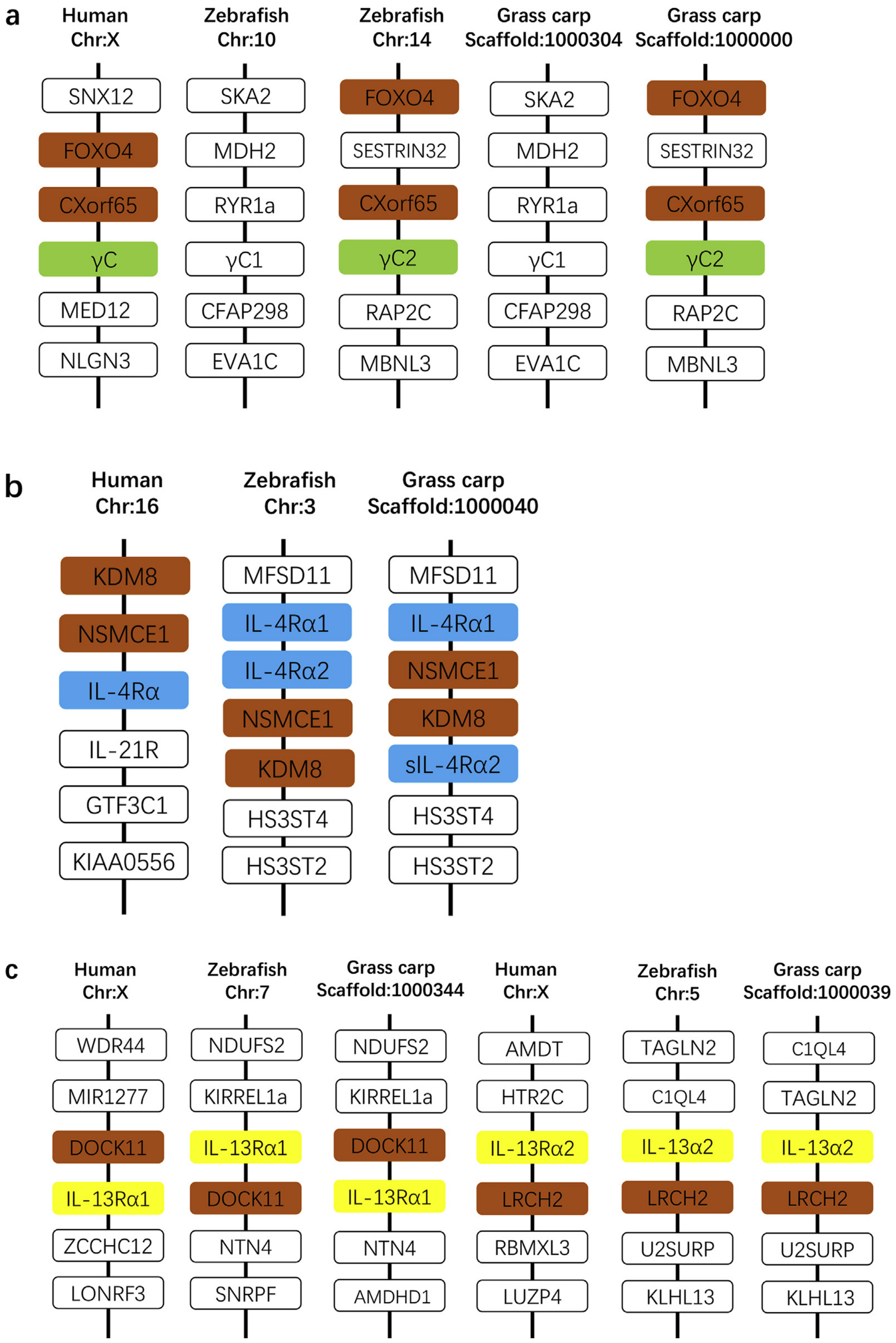

Fig. 6. Gene synteny analysis of IL-4/13 receptors. The synteny information for human and zebrafish genes was obtained from the Ensembl database.

receptor genes could be modulated in tissues after stimulation with poly (I:C) or infection of *F. cloumnare*.

Multiple potential N-glycosylation sites were predicted in all the grass carp IL-4/13 receptors (Supplement Figs. 1-6). Some of these potential N-glycosylation sites are conserved across different species (Figs. 1 and 4). In mammals, N-glycosylation of IL-13Rα2 has been shown to be required for inhibitory activity of IL-13 [31]. N-glycosylation is the most ubiquitous protein co-translational modification in the endoplasmic reticulum and serves as the primary determinant for specific molecular recognition as well as protein folding and stability, which is a common protein modification pathway in eukaryotic cells [32].

The WSXWS motif is conserved in all known mammalian class I cytokine receptors including γC, IL-4Rα, IL-13Rα1 and IL-13Rα2. However, in fish this motif is present only in γCs and IL-13Rα2 and no canonical WSXWS motif is found in any known fish IL-4Rα and IL-

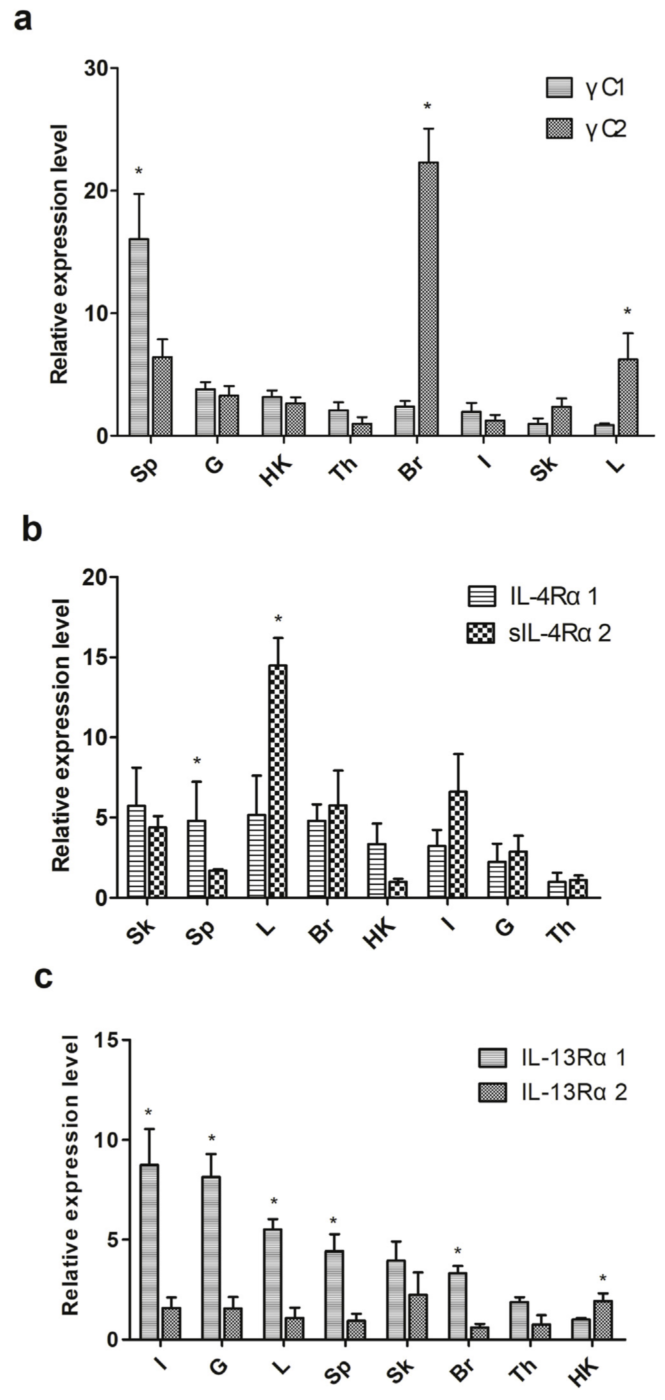

Fig. 7. Expression of *Ci*IL-4/13 receptors in tissues. The transcript expression levels of *Ci*γC1, *Ci*γC2, *Ci*IL-4Rα1, s*Ci*IL-4Rα2, *Ci*IL-13Rα1 and *Ci*IL-13Rα 2 were determined by real-time RT–PCR. M: muscle, Br: brain, L: liver, HK: head kidney, Sp: spleen, G: gills, I: intestine, Sk:skin. The relative expression levels of receptors were expressed as arbitrary units that were normalised against the expression levels of EF-1α. The LSD post hoc test was performed after one-way analysis of variance between the two receptors in the same tissue and "*"$p < 0.05$ considered significant, N = 4.

13Rα1 molecules. The WSXWS motif plays a critical role in ligand binding and signal transduction in human and other mammalian IL-4/13 receptors [33,34]. However, in fish, the WSXWS motif is modified to FSDWS in trout IL-4Rα1 and IL-4Rα2 [20] and to LSDQS in grass carp IL-4Rα1 and soluble IL-4Rα2.

In this report, a gene encoding soluble IL-4Rα2 has been sequenced. Soluble IL-4Rα variants are common in mammals and are transcribed by alternative splicing from the same gene that gives rise to the membrane bound IL-4Rα2 [35,36]. In most cases, a stop codon is introduced in the alternatively spliced mRNA, resulting in the deletion of transmembrane region and intracellular region. Soluble receptors are typically decoy receptors that antagonise ligand functions. In zebrafish, two IL-4Rα genes (IL-4Rα1/IL-4Rα and IL-4Rα2/IL-4Rα-iso) are found and both could be translated into membrane and soluble receptors [36], with IL-4Rα1/IL-4Rα showing inhibitory activity in B cell proliferation and IgM production. Interestingly, the membrane bound zebrafish IL-4Rα2/IL-4Rα-iso has a much shorter cytoplasmic region than trout IL-4Rα1 and IL-4Rα2 and lacks several important signal motifs such as STAT5 and STAT6 phosphorylation site [36]. Nevertheless, it is unclear from the present study whether a membrane bound *Ci*IL-4Rα2 exists in grass carp. Additionally, further studies are required to determine the molecular mechanism leading to the generation of soluble *Ci*IL-4Rα2. .

The expression of IL-4/13 receptor genes have been examined in several fish species. It has been shown that they are constitutively expressed in tissues but expression levels can vary (Fig. 7). The γC receptor is shared by several members of IL-2 cytokine family such as IL-2, IL-4, IL-7, IL-9 and IL-13, and exists as a single copy gene in mammals. In trout, 2 copies of γC genes are found and the transcript levels of γC1 are generally higher than γC2 in most tissues. However, in the present study, the expression levels of *Ci*γC1 and *Ci*γC2 are comparable in most tissues except for spleen, brain and liver where their expression levels were significantly different. Besides, both receptors were highly expressed in fractioned primary head kidney leukocytes, enriched macrophages and cells in suspension (Fig. 8). This suggests that the γC receptors may play a role in regulating the functions of their ligands in immune homeostasis. It is common that during evolution duplication of gene copies results in sub- or neo-functionalisation to diversify gene functions. Whether the two γC receptors have any binding preference with the IL-2 cytokine family members such as IL-4/IL-13A and IL-4/IL-13B remains to be determined.

The expression of specific binding receptors for IL-4/13 was investigated in tissues of healthy grass carp. Similar to the two γCs, the transcript levels of *Ci*IL-4Rα1 and s*Ci*IL-4Rα2 were found to be different in spleen and liver but not other tissues (Fig. 7). This is in contrast to the findings in zebrafish where membrane bound IL-4Rα2/IL-4Rα-iso but not IL-4Rα1/IL-4Rα was highly expressed in tissues and sIL-4Rα2/sIL-4Rα-iso transcripts were expressed only in brain and muscle [36]. In primary leukocytes, the *Ci*IL-4Rα1 expression was higher than s*Ci*IL-4Rα2 (Fig. 8). However, in rainbow trout monocyte/macrophage cell line (RTS-11), IL-4Rα2 was more abundant than IL-4Rα1 [19]. The *Ci*IL-13Rα1 showed relatively higher expression than *Ci*IL-13Rα2 in most tissues with the only exception of thymus. It must be noted that the IL-13Rα1 is a signaling receptor for IL-4/13 whilst the IL-13Rα2 serves as the decoy receptor to antagonise ligand functions.

Expression of IL-4/13 receptors can be modulated in fish by immunostimulants or pathogen infection. PHA, a known mitogen, was shown to up-regulate expression of γC1, γC2, IL-4Rα1 and IL-4Rα2 in trout head kidney leukocytes but down-regulate IL-13Rα1 and IL-13Rα2 [17,19]. Bacterial and viral infection also leads to alteration of expression of IL-4/13 receptors in fish tissues. Challenge of large yellow croaker and grass carp with *Aeromonas hydrophila* increased transcript levels of IL-4Rα1 in spleen [37,38]. A salmon macrophage/dendritic like cell line, when infected with salmonid alpha virus, exhibited an increase of IL-4Rα expression but inhibition of IL-13Rα1 expression [39]. In agreement of these studies, both *Ci*γC1 and *Ci*γC2 genes were induced in spleen and head kidney 24 h after infection of *F. cloumnare* (Fig. 9). In grass carp spleen, the expression of *Ci*IL-4Rα1 and s*Ci*IL-4Rα2 was up-regulated at early stage of infection but subsequently inhibited. IFNγ, a typical Th1 cytokine, was also shown to differentially modulate expression of IL-4/13 receptors in primary trout head kidney leukocytes [20].

Two IL-4/13 homologs have been identified and functionally characterised in fish. It has been shown that they possess similar functions

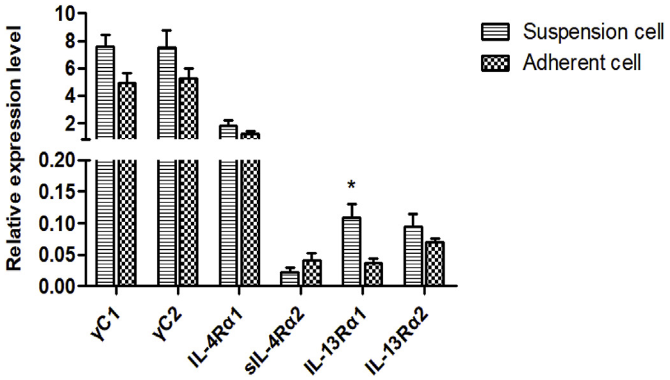

Fig. 8. Expression in fractioned primary head kidney leukocytes. Primary cells were separated from head kidney and seeded in 35-cm cell culture plates for 8 h. The suspension cells and adherent cells were collected for RNA extraction.The expression levels was normalised to that of EF-1α and used for statistical analysis. The results represent the average +SEM from 4 replicates. The LSD post hoc test was performed after one-way analysis of variance between the two receptors in the same tissue and "*"$p < 0.05$ considered significant.

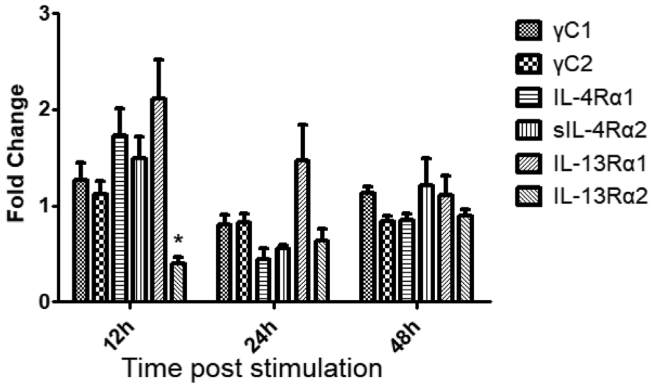

Fig. 10. Expression modulation of IL-4/13 receptor expression in spleen after injection with poly(I:C). Fish were i.p. injected with 100 μL PBS or poly(I:C) (5 mg/mL). Spleen was collected at different time points after injection. The expression levels were normalised to that of EF-1α. Fold change was calculated by comparing the average level of expression of infected tissues with that of corresponding control groups. The LSD post hoc test was performed after one-way analysis of variance and "*"$p < 0.05$ considered significant, N = 4.

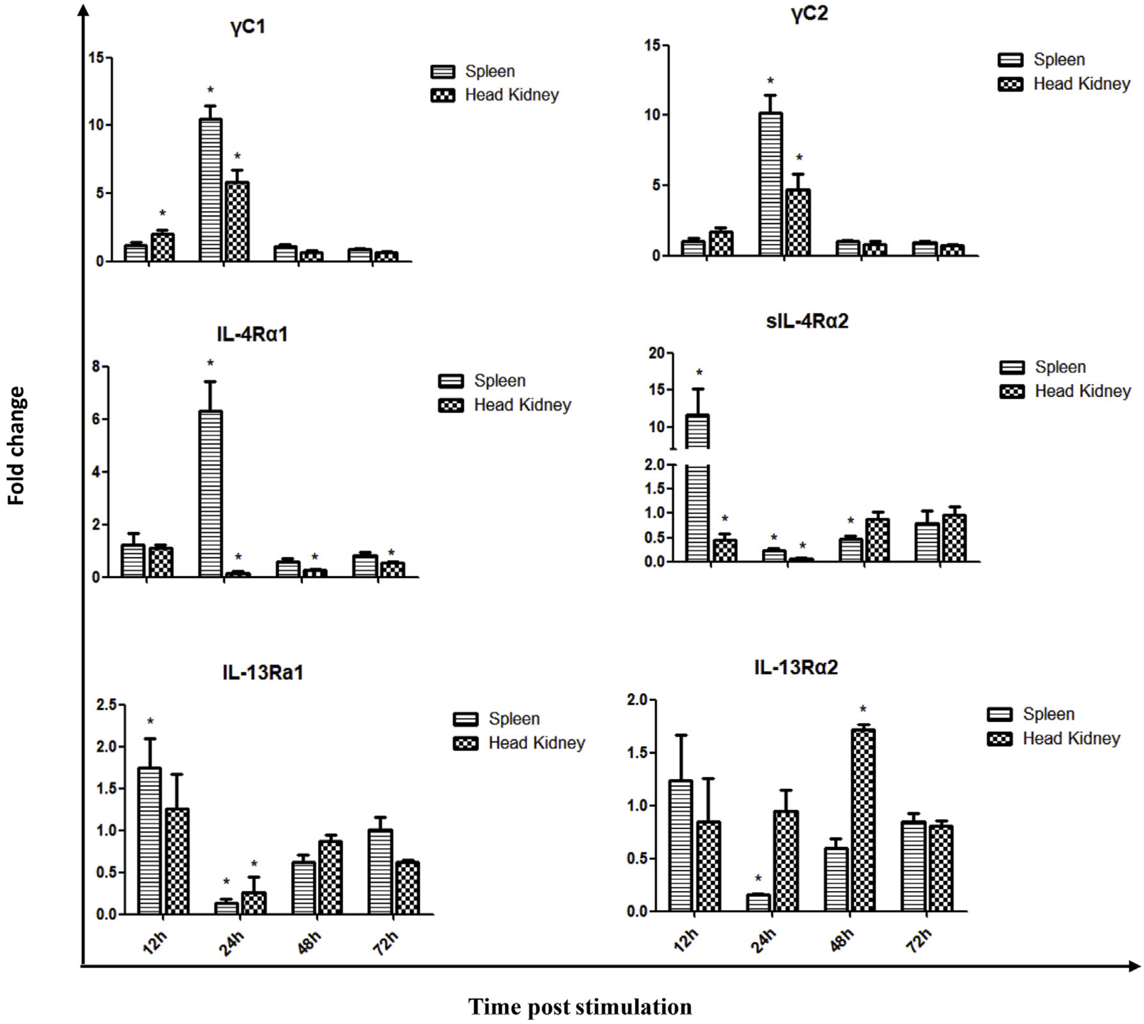

Fig. 9. Expression modulation of IL-4/13 receptors after infection with *F. cloumnare*. Spleen and head kidney were collected at different time points after infection. The expression levels were normalised to that of EF-1α and used for statistical analysis. Fold change was calculated by comparing the average level of expression of infected tissues with that of corresponding control groups. The LSD post hoc test was performed after one-way analysis of variance and "*"$p < 0.05$ considered significant, N = 4.

with mammalian IL-4 and IL-13 homologs. Recombinant trout IL-4/13A and IL-4/13B exert similar activities and are able to induce expression of antibacterial genes such as hepcidins and cathelicidins in primary head kidney cells but inhibit IFNγ expression [40]. Besides, the recombinant trout IL-4/13A has been shown to promote proliferation of CLEC4T1+ cells which express high levels of MHCII [41]. In zebrafish, administration of fusion GST-IL-4 significantly promotes proliferation of mIgM + B cells in vivo [36]. Nevertheless, the ligand/receptor interaction of IL-4/13 in fish is yet fully understood. It seems that the cellular signaling pathways activated by IL-4/13 ligands could be more complex in fish than in mammals since fish have more putative IL-4/13 receptors.

In conclusion, six IL-4/13 receptor genes were identified in grass carp. All the receptors were constitutively expressed in most of tissues of healthy fish, and their expression could be modulated by infection of *F. cloumnare*. The results suggest that the functions of IL-4/13 ligands could be regulated by modulation of expression of individual receptors and decoy receptors. Further studies are needed to fully elucidate the ligand/receptor interactions of IL-4/13 cytokines in fish and their impact on the immune responses to pathogen infections.

Acknowledgement

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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.2019.01.009.

References

[1] A.N. Mckenzie, H. Spits, G. Eberl, Innate lymphoid cells in inflammation and immunity, Immunity 41 (2014) 366–374.

[2] E. Hams, P.G. Fallon, Innate type 2 cells and asthma, Curr. Opin. Pharmacol. 12 (2012) 503–509.

[3] S.P. Gadani, J.C. Cronk, G.T. Norris, J. Kipnis, IL-4 in the brain: acytokine to remember, J. Immunol. 189 (2012) 4213–4219.

[4] I.S. Junttila, R.J. Creusot, I. Moraga, D.L. Bates, M.T. Wong, M.N. Alonso, M.M. Suhoski, P. Lupardus, M. Meierschellersheim, E.G. Engleman, Redirecting cell-type specific cytokine responses with engineered interleukin-4 superkines, Nat. Chem. Biol. 8 (2012) 990–998.

[5] A.L. Andrews, J.W. Holloway, S.T. Holgate, D.E. Davies, IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets, J. Immunol. 176 (2006) 7456–7461.

[6] T. Ito, S. Suzuki, S. Kanaji, H. Shiraishi, S. Ohta, K. Arima, G. Tanaka, T. Tamada, E. Honjo, K.C. Garcia, Distinct structural requirements for interleukin-4 (IL-4) and IL-13 binding to the shared IL-13 receptor facilitate cellular tuning of cytokine responsiveness, J. Biol. Chem. 284 (2009) 24289–24296.

[7] S.O. Rahaman, P. Sharma, P.C. Harbor, M.J. Aman, M.A. Vogelbaum, S.J. Haque, IL-13Rα2, Adecoy receptor for IL-13 acts as an inhibitor of IL-4-dependent signal transduction in glioblastoma cells, Cancer Res. 62 (2002) 1103–1109.

[8] P.J. Lupardus, M.E. Birnbaum, K.C. Garcia, Molecular basis for shared cytokine recognition revealed in the structure of an unusually high affinity complex between IL-13 and IL-13Rα2, Structure 18 (2010) 332–342.

[9] J.F. Bazan, Structural design and molecular evolution of a cytokine receptor superfamily, P. Natl. Acad. Sci. USA 87 (1990) 6934–6938.

[10] C.J. Bagley, J.M. Woodcock, F.C. Stomski, A.F. Lopez, The structural and functional basis of cytokine receptor activation: lessons from the common beta subunit of the granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3), and IL-5 receptors, Blood 89 (1997) 1471–1482.

[11] S.L. Laporte, Z.S. Juo, J. Vaclavikova, L.A. Colf, X. Qi, N.M. Heller, A.D. Keegan, K.C. Garcia, Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system, Cell 132 (2008) 259–272.

[12] J.N. Ihle, Cytokine receptor signalling, Nature 377 (1995) 591–594.

[13] T. Kishimoto, T. Taga, S. Akira, Cytokine signal transduction, Cell 76 (1994) 253–262.

[14] P.L. Orchansky, R. Kwan, F. Lee, J.W. Schrader, Characterization of the cytoplasmic domain of interleukin-13 receptor-alpha, J. Biol. Chem. 274 (1999) 20818-10825.

[15] M. David, D. Ford, J. Bertoglio, A.L. Maizel, J. Pierre, Induction of the IL-13 receptor alpha2-chain by IL-4 and IL-13 in human keratinocytes: involvement of STAT6, ERK and p38 MAPK pathways, Oncogene 20 (2001) 6660–6668.

[16] T. Wang, C.J. Secombes, Cloning and expression of a putative common cytokine receptor gamma chain (gamma C) gene in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 11 (2001) 233–244.

[17] T. Wang, W. Huang, M.M. Costa, C.J. Secombes, The gamma-chain cytokine/receptor system in fish: more ligands and receptors, Fish Shellfish Immunol. 31 (2011) 673–687.

[18] C. Liongue, A.C. Ward, Evolution of Class I cytokine receptors, BMC Evol. Biol. 7 (2007) 120–135.

[19] T. Wang, W. Huang, M.M. Costa, S.A. Martin, C.J. Secombes, Two copies of the genes encoding the subunits of putative interleukin (IL)-4/IL-13 receptors, IL-4Rα, IL-13Rα1 and IL-13Rα2, have been identified in rainbow trout (*Oncorhynchus mykiss*) and have complex patterns of expression and modulation, Immunogenetics 63 (2011) 235–253.

[20] H.X. Xie, P. Nie, B.J. Sun, Characterization of two membrane-associated protease genes obtained from screening out-membrane protein genes of Flavobacterium columnare G4, J. Fish. Dis. 27 (2010) 719–729.

[21] Y. Wang, Y. Lu, Y. Zhang, Z. Ning, Y. Li, Q. Zhao, H. Lu, R. Huang, X. Xia, Q. Feng, Erratum: the draft genome of the grass carp (*Ctenopharyngodon idellus*) provides insights into its evolution and vegetarian adaptation, Nat. Genet. 47 (2015) 625–631.

[22] E. Gasteiger, C. Hoogland, A. Gattiker, S.E. Duvaud, M.R. Wilkins, R.D. Appel, A. Bairoch, Protein identification and analysis tools on the ExPASy server, in: J.M. Walker (Ed.), The Proteomics Protocols Handbook, Humana Press, 2005, pp. 571–607.

[23] R.L. Marsden, L.J. Mcguffin, D.T. Jones, Rapid protein domain assignment from amino acid sequence using predicted secondary structure, Protein Sci. 11 (2010) 2814–2824.

[24] O. Emanuelsson, S. Brunak, H.G. Von, H. Nielsen, Locating proteins in the cell using TargetP, SignalP and related tools, Nat. Protoc. 2 (2007) 953–971.

[25] K. Hofmann, W. Stoffel, TMbase - a database of membrane spanning proteins segments, Biol. Chem. Hoppe-Seyler 374 (1993) 166.

[26] R. Chenna, H. Sugawara, T. Koike, R. Lopez, T.J. Gibson, D.G. Higgins, J.D. Thompson, Multiple sequence alignment with the Clustal series of programs, Nucleic Acids Res. 31 (2003) 3497–3500.

[27] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0, Mol. Biol. Evol. 24 (2007) 1596–1599.

[28] M. Murakami, M. Narazaki, M. Hibi, H. Yawata, K. Yasukawa, M. Hamaguchi, T. Taga, T. Kishimoto, Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family, P. Natl. Acad. Sci. USA 88 (1991) 11349–11353.

[29] K. Arima, K. Sato, G. Tanaka, S. Kanaji, T. Terada, E. Honjo, R. Kuroki, Y. Matsuo, K. Izuhara, Characterization of the interaction between interleukin-13 and interleukin-13 receptors, J. Biol. Chem. 280 (2005) 24915–24922.

[30] J.L. Boulay, J.J. O'Shea, W.E. Paul, Molecular phylogeny within type I cytokines and their cognate receptors, Immunity 19 (2003) 159–163.

[31] M. Kioi, S. Seetharam, R.K. Puri, N-linked glycosylation of IL-13R alpha2 is essential for optimal IL-13 inhibitory activity, FASEB J. 20 (2006) 2378–2380.

[32] A. Yan, W.J. Lennarz, Unraveling the mechanism of protein N-glycosylation, J. Biol. Chem. 280 (2005) 3121–3124.

[33] T. Miyazaki, M. Maruyama, G. Yamada, M. Hatakeyama, T. Taniguchi, The integrity of the conserved 'WS motif'common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction, EMBO J. 10 (1991) 3191–3197.

[34] D.E. Quelle, F.W. Quelle, D.M. Wojchowski, Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization, Mol. Cell Biol. 12 (1992) 4553–4561.

[35] J.O. Sunyer, Fishing for mammalian paradigms in the teleost immune system, Nat. Immunol. 14 (2013) 320–326.

[36] L.Y. Zhu, P.P. Pan, W. Fang, J.Z. Shao, L.X. Xiang, Essential role of IL-4 and IL-4Rα interaction in adaptive immunity of zebrafish: insight into the origin of Th2-like regulatory mechanism in ancient vertebrates, J. Immunol. 188 (2012) 5571–5584.

[37] Y. Mu, F. Ding, P. Cui, J. Ao, S. Hu, X. Chen, Transcriptome and expression profiling analysis revealed changes of multiple signaling pathways involved in immunity in the large yellow croaker during *Aeromonas hydrophila* infection, BMC Genomics 11 (2010) 1–14.

[38] Y. Ying, Y. Hui, L. Hua, A. Wang, Transcriptome profiling of grass carp (*Ctenopharyngodon idellus*) infected with *Aeromonas hydrophila*, Fish Shellfish Immunol. 51 (2016) 329–336.

[39] X. Cheng, Ø. Evensen, H.M. Munang'Andu, A de novo transcriptome analysis shows that modulation of the JAK-STAT signaling pathway by salmonid alphavirus subtype 3 favors virus replication in macrophage/dendritic-like TO-cells, BMC Genomics 17 (2016) 1–17.

[40] T. Wang, P. Johansson, B. Abós, A. Holt, C. Tafalla, Y. Jiang, A. Wang, Q. Xu, Z. Qi, W. Huang, First in-depth analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of expression and modulation but overlapping bioactivities, Oncotarget 7 (2016) 10917–10946.

[41] P. Johansson, T. Wang, B. Collet, Y. Corripiomiyar, M.M. Monte, C.J. Secombes, J. Zou, Identification and expression modulation of a C-type lectin domain family 4 homologue that is highly expressed in monocytes/macrophages in rainbow trout (*Oncorhynchus mykiss*), Dev. Comp. Immunol. 54 (2016) 55–65.