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Cloning, Characterization and Expression Analysis of Interleukin-10 from the Zebrafish (*Danio rerion*)

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Cytokines are proteins produced by many different cells of the immune system and play a significant role in initiating and regulating the inflammatory process. In this research, an important cytokine, interleukin-10 (IL-10) gene, has been identified and characterized from zebrafish (*Danio rerio*) genome database. Zebrafish IL-10 is located within a 2690 bp fragment and contains five exons and four introns, sharing the same organization with mammalian IL-10 genes. An open reading frame of 543 bp was found to encode a putative 180 amino acid protein with a signal peptide of 22 amino acids, which shares 29.7-80.9% homology with amino acid sequences of other known IL-10. The signature motif of IL-10 is also conserved in zebrafish IL-10. The predicted transcript was finally confirmed by sequencing of cDNA clones. Multi-tissue reverse transcriptase PCR (RT-PCR) was performed to examine the tissue distribution and expression regulation of this gene in seven organs of normal and lipopolysaccharide (LPS) stimulation zebrafish. The results demonstrated that this gene was expressed slightly in normal kidney, gill and gut, no expression was detected in other four tissues. The expression was clearly upregulated after LPS stimulation. Using the ideal zebrafish model, further study of IL-10 characterization and function may provide insight on the understanding of the innate immune system.

Keywords: Cytokine, Genomic analysis, Interleukin, Zebrafish

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Introduction

Cytokines are proteins produced by many different cells of the immune system, which act upon other cells, and play a significant role in initiating and regulating the inflammatory process (Herve *et al*., 2003; John *et al*., 2003). Interleukin-10 (IL-10) is a fascinating cytokine first identified as a cytokine synthesis inhibitory factor (CSIF) in mouse (Fiorentino *et al*., 1989; Moore *et al*., 1990). The recent data suggest that IL-10 not only is the limitation and termination of inflammatory responses and the regulation of differentiation and proliferation of several immune cells such as T cell, B cell and natural killer cell (Zdanov, 2004), but also mediates immunostimulatory properties that help to eliminate infectious and noninfectious particles with limited inflammation (Rousset *et al*., 1992; David *et al*., 2003; Asadullah *et al*., 2003).

IL-10 genes have been isolated and characterized from a number of mammalian species (Goodman *et al*., 1992; Dumoutier *et al*., 2000; Alexander, 2004), all known mammalian IL-10 genes are composed of five exons and four introns, and contain several mRNA instability motif ATTTA sequences in the 3' untranslated regions (UTRs) (Shaw *et al*., 1986; Brown *et al*., 1996). The expression and regulatory mechanisms of IL-10 have been studied widely in mammal (De Wall *et al*., 1991; Ding *et al*., 2000). However, only a few cytokines have been cloned and characterized in the lower vertebrates. Recently, IL-10 genes have been isolated and characterized from fugu (*Fugu rubripes*) (Zou *et al*., 2003), common carp (*Cyprinus carpio* L.) (Savan *et al*., 2003) and rainbow trout (*Oncorhynchus mykiss*) (Inoue *et al*., 2005) by comparative genomic analysis or by PCR-mediated homology cloning. Interestingly, unlike the mammalian IL-10, the rainbow trout IL-10 do not contain the mRNA instability motif ATTTA sequence in the 3'UTR. In normal tissues IL-10 expression patterns were different among above three kinds of fish species, but the reasons that induce this difference have been not known, to study the mechanism of this difference is

very interesting.

Zebrafish, *Danio rerio*, has recently emerged as an ideal model for the study of development, genetics and gene functions (Altmann *et al*., 2003). In this study, IL-10 gene was identified and characterized from zebrafish using bioinformatics methods, and the open reading frame (ORF) was validated by RT-PCR. Multi-tissue RT-PCR was performed to examine the tissue distribution and expression regulation of IL-10 in seven organs of normal and LPS stimulation adult zebrafish. The results demonstrated that this gene was expressed slightly in normal kidney, gill and gut, no expression was detected in other four tissues. The expression was clearly upregulated after LPS stimulation. Further study of IL-10 characterization and function may provide insight on the understanding of the innate immune system.

Materials and Methods

Identification and characterization of zebrafish IL-10 A stretch of zebrafish genomic sequence (GenBank accession no. BX321912) was obtained directly from GenBank database at the National Center for Biotechnology Information (NCBI). Subsequently, the DNA sequence was further analyzed for predicted transcripts using GenScan program (Burge et al., 1998) (http://genes.mit.edu/GENSCAN.html). Eleven transcripts were generated by GenScan program, one of which revealed high homology with known interleukin-10 molecules in N-terminal region, but the C-terminal region had little similarity to the known IL-10 and was approximately 130 aa longer. To determine the correct reading frame of each exon, BLAST program and DNASTAR software were used to maximize the in-frame translated sequence. Finally, the individual exon sequences was compiled into the full protein encoding sequence by manual adjustment.

Putative protein and phylogenetic analysis The molecular mass and isoelectric point of the putative zebrafish IL-10 were predicted using Compute pI/Mw tool at the ExPASy molecular biology WWW server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/) and the deduced signal peptide was predicted using SignalP (Bendtsen *et al*., 2004). Motif search was performed with eMOTIF Scan program (http://motif.stanford.edu/emotif/emotifscan.html). The homology between amino acid sequence of zebrafish IL-10 and other known IL-10s was analyzed using Clustal W (Higgins D *et al*., 1994). Multiple sequence alignment and phylogenetic tree construct were performed using Mega3 (Kumer *et al*., 2004). The genetic distance between species was calculated using ρ-distance method. The cladogram was generated using the neighbor-joining (NJ) method. In the analysis, the gaps were deleted, and a 1000 bootstrap procedure was used to test the robustness of the node on the trees.

cDNA cloning and muti-tissue RT-PCR RT-PCR was performed to clone and sequence zebrafish IL-10 cDNA. After LPS simulation, total RNA was extracted from kidney using Trizol reagent (Invitrogen, Carlsbad, USA) and the standard protocols recommended by the supplier. 400 ng of total RNA was used as the template for the synthesis of first strand cDNA by reverse transcriptase using AMV Reverse Transcription System (promega, Madison, USA). For PCR amplification, Primers were designed and synthesized based on the predicted cDNA sequence as follows: FW1 5'-GCTCATCTGTACATCTTCTCACTTG-3' and RV15'-CTGTCCAACCCAGCAACATCCTA-3'. PCR were carried out using 25 U Ex Taq polymerase (TaKaRa, Kyoto, Japan) in 50 μL reactions containing 250 μM of each dNTP, 0.4 μM of each primer, 5 μL 10× Taq buffer, 37.5 μL sterile H_2O and 1 μL cDNA template according to the standard protocol. PCR amplification was conducted under the following conditions: an initial cycle of denaturation step at 94°C for 2 min, 35 cycles of amplification at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. 5 μL product was size-fractioned by 1.2% (w/v) agarose gel electrophoresis and stained with ethidium bromide. Desired PCR products were isolated by gel elution (Qiagen, Chatsworth, USA), and ligated into pGEM-T vectors (Promega, Madison, USA). These were transformed into JM101 competent cells and plated on selective agar plates containing 100 μg/ml Ampicillin. Positive colonies were grown in overnight cultures and plasmid DNA extracted using affinity/elution method (QIA prep kit, Qiagen, Chatsworth, USA). Insert size was determined by agarose gel electrophoresis after digestion with *Eco*R 52 in comparison to the 100 bp DNA Marker. Three of cDNA colonies were sequenced on an ABI 377 instrument, respectively.

To reveal the tissue distribution and expression regulation of zebrafish IL-10, total RNA was extracted from seven organs of normal and LPS stimulation adult zebrafish respectively. The first strand cDNA was synthesized from each sample by reverse transcriptase as described previously. The gene specific primers FW2 (5'-ACGCTTCTTCTTTGCGACTG-3') and RV2 (5'-CACCATATCCCGCTTGAGTT-3') were designed to amplify 342 bp fragment of IL-10. Positive control primers were designed according to zebrafish β-actin (GenBank accession no. AF057040) as Actin-FW: 5'-CAGACTACCTGATGAAGATCCTGAC-3' and Actin-RV: 5'-GTGTTGGCATACAGGTCCTTACG-3' to amplify 336 nucleotide fragment. Multi-tissue RT-PCR was performed according to the method described by Laing (Laing *et al*., 2001).

Results

Identification and characterization of the zebrafish IL-10 gene The genomic analysis methods was used to identify zebrafish IL-10, which has been submitted to GenBank (GenBank accession no. AY887900). Trace sequences representing exon 1 and partial exon 3 were first identified by GenScan program. Then, the other exons and the exact exon/intron boundaries were identified based on the known IL-10s using BLAST and Clustal W program. The compiled zfIL-10 gene was 2690 bp in length and contained a 543 bp ORF. Like most cytokine genes, its 3' UTR was AT (67.2%) rich and contained a typical polyadenylation signal AATAAA at nuceotide position 478 bp downstream from the translation stop codon, and four mRNA instability sequences ATTTA. Its 5' UTR contained a promoter TATA box at nucleotide position 90 bp

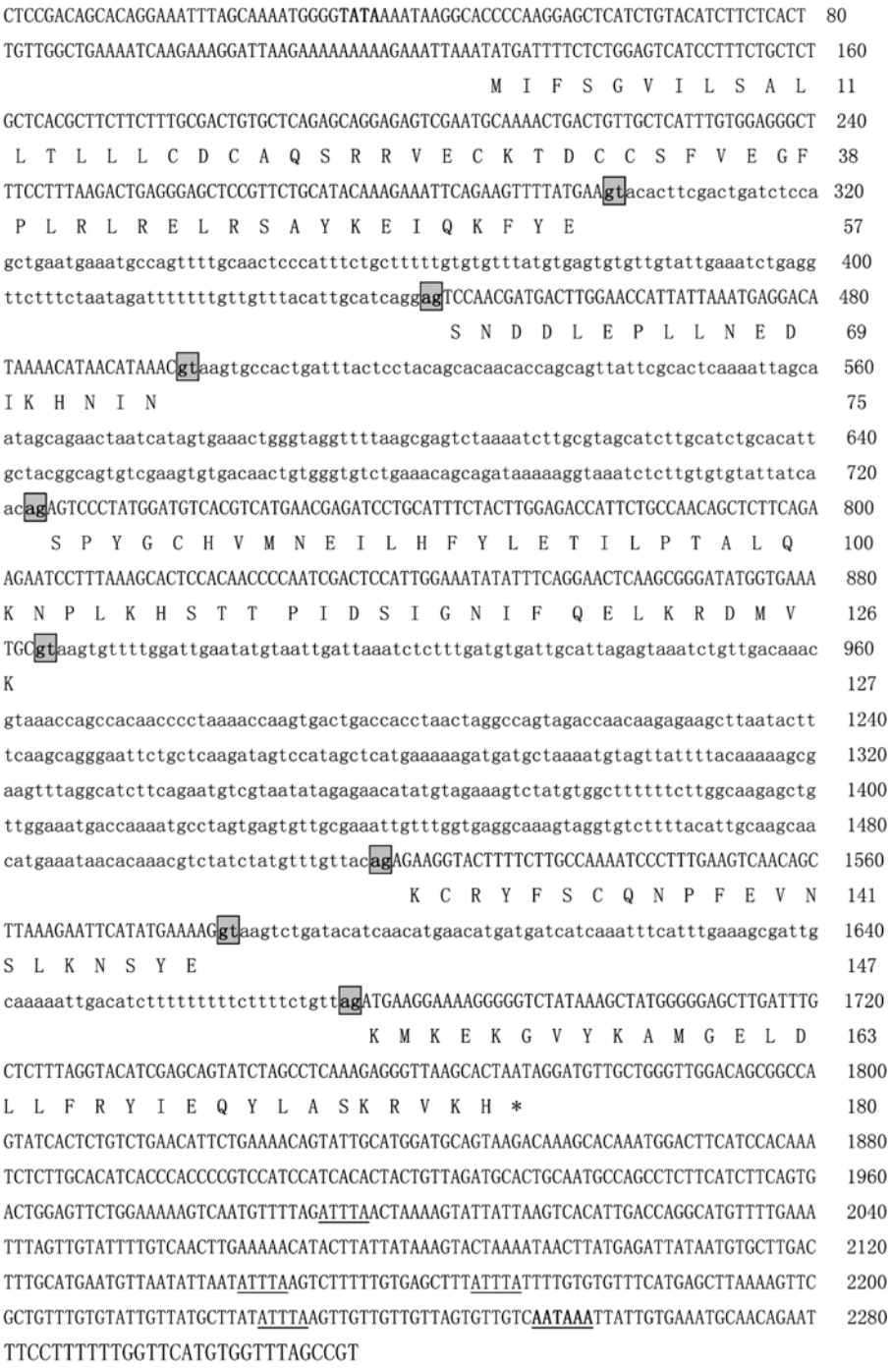

Fig. 1. The zebrafish IL-10 gene sequence and deduced amino acid sequence. The intron sequences are in *lower case* and the intron splicing consensus (gt/ag) are *boxed*. The promoter (TATA) and the poly (A) signal are in *bold* and *underlined*, whilst the mRNA instability motif in the 3' UTR is *underlined*. The deduced amino acid sequence is given *below* the nucleotides. The stop condon is represented with an *asterisk*.

upstream of the translaton initiation codon. Zebrafish IL-10 gene was composed of five exons and four introns, sharing the same exon structure with mammalian counterparts. The four introns were 144, 227, 433 bp and 94 bp in length respectively, similar to that of common carp and fugu, but more compact than that of human and rainbow trout. A typical intron splice motif (5' GT-intron-3' AG) was present at the 5' end and the 3' end of all the four introns (Fig. 1).

Analysis of the putative protein and phylogenetic tree Zebrafish IL-10 contains an open reading frame encoding 180 amino acids with a theoretical molecular weight of 20.98 kD α and the isoelectric point of 8.07 using ORF finder at NCBI and Compute pI/Mw tool at the ExPASy server. A putative signal peptide of 22-amino-acid (MIFSGVILSALLT-LLLCD

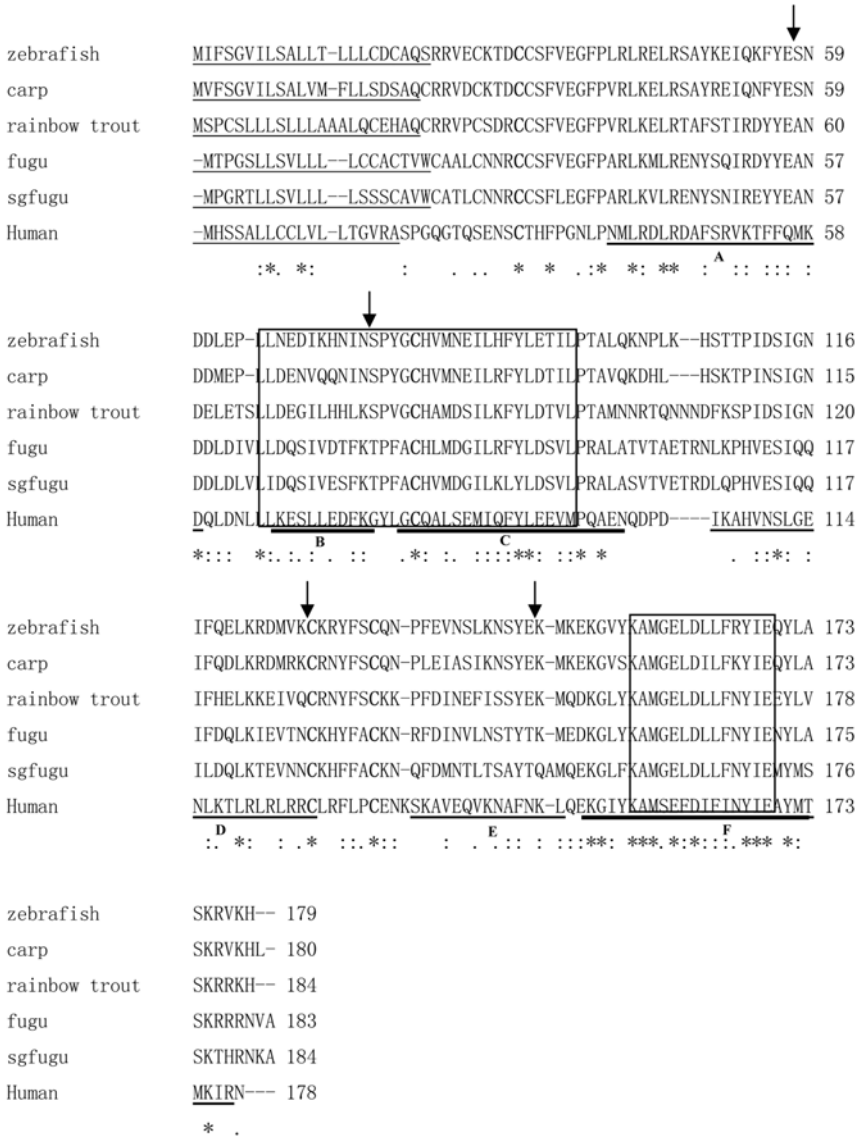

Fig. 2. Alignment of the deduced amino acid sequence of zfIL-10 with other homologues in fish and human counterparts. Identical (*) and similar (. or :) amino acid residues are indicated. The six α-helical domains predicted in human IL-10 are marked by the *bar below*. *Arrows* "↓" indicate the intron position in zfIL-10 gene. The IL-10 family signature motifs are *boxed*, and the cysteine residues involved in the formation of disulphide bridges are shown in *bold*. The signal peptide is *underlined*.

CAQS) was predicted at the N-terminus using the SignalP program. Two signature motifs were identified using the eMOTIF Scan program follows: LLNEDIKHNINSPYGCHV MNEILHFYLETIL and KAMGELDLLFRYIE belonging to the conserved IL-10 family signature sequence L-[FILMV]-X(3)-[ILV]-X(3)-[FILMV]-X(5)-C-X(5)-[ILMV]-[ILMV]-X(3)-L-X(2)-[IV]-[FILMV] and KA-X(2)-E-X-D-[ILV]-[FLY]-[FILMV]-X(2)-[ILMV]-[EKQZ] respectively. Four conserved cysteine residues in other known IL-10s were found in zebrafish IL-10. The amino acid sequence of zebrafish IL-10 was aligned (Fig. 2) and shared 29.7-80.9% homology with other known IL-10s.

Based on the genetic distances calculated from IL-10 amino acid sequence of 17 species with p-distance model, a cladogram was constructed using the N-J method with goldfish interferon (IFN) as the outgroup. As shown in Fig. 3, the in-group of 16 species was divided into two group. The first group all belonged to fish species. The second group also could be divided into two branches, one was composed of 10 species of mammal, another was avian. All the clades got very high bootstrap support values.The results implied that the phylogenetic tree based on the IL-10 gene were reliable, and generally revealed their evolutionary distances.

cDNA cloning and expression analysis RT-PCR was performed with total RNA extracted from kidney of LPS stimulation zebrafish. The predicted transcript was finally confirmed by sequencing of cDNA clones(data not shown).

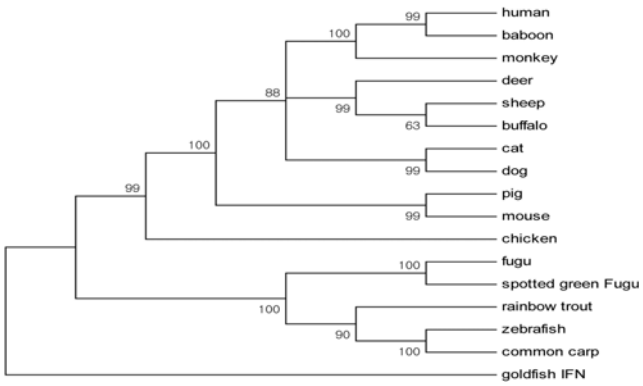

Fig. 3. A phylogenetic tree constructed by the neighbor-joining method (using Mega2 software) from the amino acid sequences of the IL-10 together with the zfIL-10 gene. The numbers indicate the bootstrap confidence values obtained for each nodes after 1000 replications. The IL-10 protein sequences were retrieved from the Swissprot database: human, CAG46825; baboon, AAV85009; monkey, P51496; deer, AAA85434; sheep, CAG38358; buffalo, AAQ94107; cat, P55029; dog, NP_001003077; pig, AAC83808; mouse, NP_034678; chicken, CAF18432; fugu, CAD62446; spotted green fugu, AAP57415; rainbow trout, BAD20648; common carp, BAC76885; goldfish interferon, AAR20886.

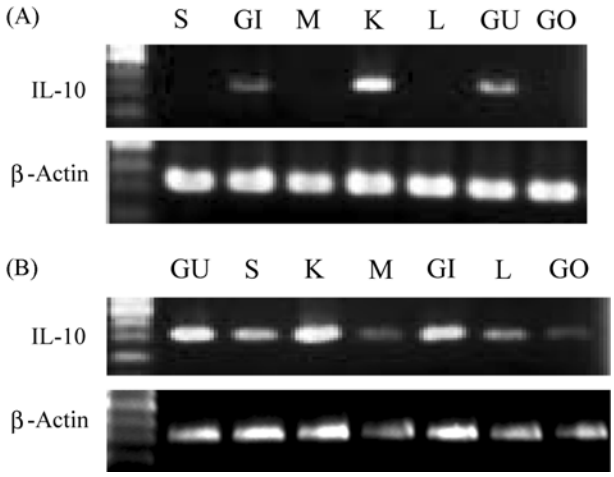

Fig. 4. Expression patterns of IL-10 gene in seven normal and LPS stimulation organs from zebrafish.Total RNA was extracted from seven organs respectively for muti-tissue RT-PCR using the IL-10 specific primers. A was normal tissues, and B was LPS stimulation tissues. Upper letters were: S. skill; GI. gill; M. muscle; K. kidney; L. liver; GU. Gut; GO. Gonad. The same samples were assayed for β-Actin mRNA expression as a positive control.

The results showed that there is only one nucleotide difference between the sequences of cDNA clones and the predicted transcript, which maybe is brought due to the difference between individuals.

Multi-tissue RT-PCR was performed to examine the tissue distribution and expression regulation of zebrafish IL-10 in seven organs of normal and LPS stimulation adult zebrafish. Slight amount of PCR product was detected using zebrafish IL-10 specific primers in normal zebrafish kidney, gill and gut. However, no visible PCR product was detected with cDNA derived from skin, muscle, gonad and liver. After 4 h following LPS stimulation, PCR product was detected in all seven organs, furthermore, the expression is clearly up-regulated in kidney, gill and gut (Fig. 4).

Discussion

IL-10 gene has been cloned in several vertebrates such as human (Vieira, 1991), mouse (Kim *et al*., 1992), chicken (Rothwell *et al*., 2004) and fish (Inoue *et al*., 2005), and demonstrated that the expression patterns of fish IL-10 were different from mammalian, as well as different among fish species. To further study the characteristic and distribution of this gene, IL-10 was identified and cloned from zebrafish, a ideal experimental model. Genomic analysis, an efficient and powerful approach for identifying new gene in various organisms, was used to identify zebrafish IL-10. zebrafish IL-10 amino acid sequence shares 29.7-80.9% homology with other known IL-10s. The homology between zebrafish and common carp is the most high up to 80.9%, and our recent study demonstrated that this gene is highly conserved in fish of the same family (unpublished). Phylogenetic analysis indicated that mammalian and avian IL-10s form distinct clades to the fish IL-10s. Furthermore, pairwise comparison of amino acid and nucleotide sequence identity and similarity also indicated similar degrees of divergence between fish IL-10s and all mammalian and avian IL-10s. These results indicated that the divergence of fish IL-10s from an ancestral IL-10 occurred prior to the divergence of mammalian and avian IL-10s.

Zebrafish IL-10 don't contain NXS/T motif needed for N-terminal glycosylation, which suggests that zebrafish IL-10 is not glycosylated. Mouse IL-10 has one potential glycosylation site, but which is not required for biological activity (Moore *et al*., 1993), so maybe the ancestor gene of IL-10 is not to be glycosylated to exert its functions. The putative mature peptide of zebrafish IL-10 contains four conserved cysteine residues (cys-31, 80, 128 and 134) which are critical to maintain the tertiary structure of IL-10s, two extra cysteine residues (cys-27 and 32) are also present in mature peptide, but it is not known that whether they are important for tertiary structure. IL-10 consists of six α-helices termed A, B, C, D, E and F, which tightly associate with the other monomer to form two interpenetrating domains (Walter *et al*., 1995), zebrafish IL-10 also possesses all six α-helices. Zebrafish IL-10 contains two typical IL-10 signature sequence motifs, which also confirms that the predicted gene is IL-10.

The previous study indicated that the expression patterns of IL-10 were different among fish species (Inoue *et al*., 2005). In normal tissues, common carp IL-10 was strongly expressed in head kidney and intestine, rainbow trout IL-10 was weakly

expressed in gill, puffer fugu IL-10 was weakly expressed in kidney and liver. In this study, zebrafish IL-10 was slightly in normal kidney, gill and gut tissues, which also was different from above three fish species, but why the difference induced has been not known. To further study the mechanism of this difference is interesting. After 4h LPS stimulation, zebrafish IL-10 expression was clearly up-regulated in kidney, gill and gut, and also detected in other four tissues. Therefore, it is concluded that this gene is associated with the innate immune system of zebrafish. The mechanism of expression regulation of IL-10 in zebrafish is under further research.

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