Identification, characterization and expression of a novel cytokine M17 homologue (MSH) in fish

Jee Youn Hwang 1, Mudjekeewis D. Santos, Hidehiro Kondo, Ikuo Hirono, Takashi Aoki*

Laboratory of Genome Science, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku, Tokyo 108-8477, Japan

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Abstract

Members of the interleukin 6 (IL6)-cytokine subfamily of proteins are involved in numerous physiological processes including cellular development, inflammatory function, and acute phase and immune responses. Previously, a cytokine-like gene named M17, which is closely associated with the IL6 subfamily, has been identified in fish with no apparent orthologue in higher vertebrates. Here, we cloned a novel cDNA from Japanese flounder, Paralichthys olivaceus, which had significant identity but exhibited contrasting expression with fish M17s, named here as M17 Homologue (MSH). With subsequent in silico search and full annotation of the M17 orthologue in zebrafish (Danio rerio), MSH orthologues in tiger puffer (Takifugu rubripes), green spotted pufferfish (Tetraodon nigroviridis) and stickleback (Gasterosteus aculeatus), as well as structural, synteny comparisons and phylogenetic analysis with known IL6-cytokines, we determined the novelty of the fish MSH. Japanese flounder MSH was observed to be highly expressed in immune-related tissues and are induced by immune stimulants, lipopolysaccharide (LPS), polyI:C and peptidoglycan (PG) in vitro suggesting that it is involved in fish immunity particularly against viral and bacterial agents, a functional feature exhibited by previously reported fish cytokines.

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1. Introduction

The IL6-cytokine subfamily have pro- and anti-inflammatory properties and are major players in hematopoiesis, as well as in acute phase and immune responses. These cytokines have the same four α-helix bundle motif and share a common signal transducing receptor component, glycoprotein 130 (gp130) [1,2]. They include interleukin 6 (IL6), interleukin 11 (IL11), leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2) and ciliary neurotrophic factor (CNTF). While granulocyte colony-stimulating factor (CSF3) is not classified as an IL6-cytokine because it doesn’t utilize gp130, its protein domain is well conserved with IL6 forming

* Corresponding author. Tel.: +81 3 5463 0556; fax: +81 3 5463 0690.
E-mail address: aoki@kaiyodai.ac.jp (T. Aoki).

1 Present address: Division of Fish Pathology, Fisheries Research and Development Institute, 408-1 Gijang, Busan 619-0902, Korea.
the IL6/CSF3/MGF protein family [3]. IL6 is a pleiotropic cytokine involved in numerous biological functions e.g. oncogenesis, inflammation, immune regulation and hematopoiesis [4]. IL11 is a multifunctional cytokine that stimulates hematopoietic progenitor cells and exerts a series of important immunomodulatory effects [5]. LIF is a pleiotropic cytokine based on its growth and differentiation activities on hematopoietic cells [6]. OSM is a multifunctional cytokine produced by activated T lymphocytes and monocytes and shares properties with all the members of this family of proteins [7]. OSM is structurally and functionally very similar to LIF, which suggests that the two genes arose by duplication [7,8]. CSF3 influences neutrophil life cycle in mammals [9].

Some members of the IL6-cytokine subfamily have been identified and characterized in fish including IL6, IL11, and M17 [2,10]. An M17 from goldfish (Carassius auratus L.), which induces goldfish macrophage differentiation and nitric oxide production has been recently reported [11]. However, in spite of this information and compared to their mammalian counterparts, information on fish IL6-cytokine subfamily orthology and function is very limited and unclear.

Here, we cloned a cDNA in Japanese flounder (Paralichthys olivaceus) that resembles M17 in general structure but not in mRNA expression in tissues, which we named as M17 homologue (poMSH). Using in silico analysis and comparison to Japanese flounder MSH, we were able to fully annotate the MSH orthologues from fugu (Takifugu rubripes), pufferfish (Tetraodon nigroviridis), and stickleback (Gasterosteus aculeatus) as well as the M17 orthologue from zebrafish. From this, we determined that MSH is a novel gene based on comparative structural and expression analysis. We also showed that Japanese flounder MSH is involved in fish immunity, and thus exhibits a cytokine-like function, as it is constitutively expressed in immune-related tissues/cells and is significantly induced in primary kidney cell culture by bacterial agents lipopolysaccharide (LPS) and peptidoglycan (PG) and by the interferon inducer, polyinosinic:polycytidylic acid (polyI:C).

2. Materials and methods

2.1. Cloning

A previously constructed cDNA library [12] from Japanese flounder kidney cells stimulated with ConA/PMA was screened using a cDNA fragment homologous to carp M17. Hybridization was performed as described previously [13]. The nucleotide sequences were determined using fluorescently labeled M13 primers on an automated DNA sequencer (Li-cor, USA). The sequences were compared with all reported sequences in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

2.2. In silico analysis

Determined nucleotide sequences were characterized using GENETYX WIN Ver. 7.0 software (SDC Software Development) and the SignalP program was used to predict the cleavage site (http://www.cbs.dtu.dk/services/SignalP/). “tBLASTn” search algorithm was employed to search the zebrafish, tiger pufferfish, green spotted pufferfish and stickleback genomic databases accessed through Ensembl (http://www.ensembl.org/). Predicted homologous sequences were fully annotated using Japanese flounder MSH as reference.

2.3. Phylogenetic analysis

The amino acid sequences of M17, MSH and other reported members of the IL6 family were retrieved from the Swissprot, EMBL and GenBank databases and analyzed for their evolutionary relationship using the Neighbor-joining (NJ) algorithm employing the Poisson correction method [14] packaged in MEGA 3.1 software (http://www.megasoftware.net/) at 1000 bootstrap values. The bootstrap consensus tree was shown.

2.4. Semi-quantitative RT-PCR

Tissues from 3 Japanese flounder samples (2 adults and 1 juvenile) were dissected for RNA extraction and cDNA synthesis including brain, eyes, gills, heart, intestine, kidney, liver, muscle, skin, spleen and stomach. Peripheral blood leukocytes (PBLs) were likewise isolated using density-gradient centrifugation. The primers used for RT-PCR were
IL-forward 5'-TCCGATTTGCCTGAGATACC-3', and IL-reverse 5'-TGGGAAGAGGCTCTGTTAGA-3'. The β-actin gene was used as an internal control [15]. PCR was performed with an initial denaturation step for 2 min at 95 °C followed by 30 cycles each of 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C and 30 s of extension at 72 °C. The PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide. Resulting bands were photographed with a densitometer (Atto) and were semi-quantitatively assessed for their relative expression following Lindenström et al. [16]. ImageJ software was used to measure light intensity [17].

2.5. Quantitative real-time PCR

Head kidney was dissected from Japanese flounder and then mash-filtered using a sterile mesh net and a syringe plunger in a 24-well Petri dish containing RPMI 1640 (NIPRO, Japan). This was done in 3 apparently healthy fish. The concentrations of stimulants used were: polyI:C (1 μg/ml), lipopolysaccharide, LPS (1 μg/ml) and peptidoglycan, PG (1 μg/ml). Cells were harvested after 0, 1, 3, and 6 h post-stimulation. Absolute copy number of the Japanese flounder M17 following immunostimulation was determined using real-time PCR. Primer sets were IL-RT-F; 5'-CGTCTTCAACAGAAGGTCTTCTG-3' and IL-RT-R; 3'-AAGGTGGCTGGTACATCAAT-5'; IL-RT2-F; 5'-TGGCGGTCTTAAGACCCATT-3' and IL-RT2-R; 3'-AAGAGCAAAGTCTGCAAGGGG-5' and beta-actin primers [15] were used for a real-time PCR assay. The assay was carried out using SYBR Green PCR master mix (PE Biosystems) following the manufacturer’s protocol.

3. Results

3.1. Japanese flounder M17 homologue (MSH)

We identified a complete cDNA sequence of a cytokine (AB280428) having close homology (41%) with carp M17 from the screening of a Japanese flounder head kidney cDNA library (Fig. 1). It consisted of 1006 bp encoding 215 amino acid residues with two putative N-glycosylation sites (arginine, N81 and arginine, N138). The first 28 amino

| GTCGACCCACGCCTTCCGATTTCGACTCGAGATACCGGAGAGGAGAATGAAUGCCTTATGTTATGAAAG | 60 |
| AGAATGAGTTTTCACAAGATTATGAACTGACAACAAACGTTACTCTCTCTCGCTCGGT | 120 |
| ATGCGCTGGAGTTTCACAGGACGTGCAGGAGCAGCAGCTGTTGAAATTCT | 180 |
| M A V D S T R T V A S G S Q Q G N S | 240 |
| GTGACAGAGCTTTAAAGACCTCAGAGACTCTTTAAAGAGAATGTGTTGACCTCATCAGAA | 300 |
| V Q Q T L K R T L L K K E S V D L I E | 360 |
| ACATAAGCCTTCAAGGAGATGTGAGACAGGCTCTCAGATTTTGCTGCTGAAAC | 420 |
| T Y K A S Q G G S E D L L | 480 |
| ATCCCTCCAACACACTCTGCCTGAGACCGAGATCAGTACGCAAGGACACAGCAGTTCGAG | 540 |
| H L Q A F L T H F K R V Y E Q Q T D L Q | 600 |
| TTACATCGAGCGGCTGGCTGAGGCTTCCACATTCTGTCGAGACCCGGCAGGATCTG | 660 |
| L P S N P L L A E L T N S S T R S R N L | 720 |
| GCTATTCTACAAACAAGCTCTCAGGATTTCTCCAGAACCCTGGTTTCATCTCAGGAC | 780 |
| A S L I N S F Y Q S L F P N L P M P E P | 840 |
| GCAGGAGGCCCCAGCAGCAGCTCTCCCGTGATCTGACAGAACAGCTCTCAGGACAGC | 900 |
| A G G P T T L P P P Q N V F Q Q K V Y G | 960 |
| TGCCGCTGCTCTCAGGACAGCTTACTAGGATGCAATCTGTCATGGCTAGAAG | 1006 |

Fig. 1. Full length of Japanese flounder MSH cDNA sequence. The putative signal peptide is underlined and N-linked glycosylation sites are boxed. In the 3'UTR the RNA instability motifs (ATTAAA) are in bold and the polyadenylation signal (ATTAAA) is in bold underlined.
acids, cleaved between alanine (A) and valine (V), were predicted to constitute a signal sequence by the software SignalP. The 3' untranslated region had 5 AUUUA mRNA destabilizing motifs and contains the consensus polyadenylation signal ATTAAA. Since the molecule has significant identity with carp M17, we named it as Japanese flounder (P. olivaceus) M17 homologue (poMSH).

3.2. In silico search, alignment and identity

By searching available fish genomic databases using poMSH as query sequence and doing comparisons, we located and fully annotated the orthologue of MSH in tiger pufferfish (trMSH)-CAF92247, green spotted pufferfish (gaMSH)-Ensembl code: ENSGACT0000022426, while we correctly annotated the orthologue of M17 in carp (ccM17), goldfish M17 (caM17) and zebrafish (dnM17-NW_634687) (Fig. 2(A)). Alignment of these 6 fish cytokine amino acid sequences showed 4 conserved cysteine (C) residues and that the carp, goldfish and Japanese flounder MSH sequences showed 4 conserved cysteine (C) residues and that the carp, goldfish and zebrafish MSH sequences showed 4 conserved cysteine (C) residues.

**Fig. 2.** (A) Multiple alignment of carp, goldfish and zebrafish M17, and Japanese flounder, tiger pufferfish, green spotted pufferfish and stickleback MSH. Signal sequences are boxed-gray, conserved cysteine residues are indicated by boxed-black, cysteine residues for M17 only are with asterisks (*) and conserved motifs are with boxed. (B) Identities among fish M17s, MSHs, and with mouse CT-2 and chicken CNTF (filled-gray).
zebrafish M17s possessed 2 additional conserved cysteine (C) residues. This alignment also showed 4 conserved amino acid motifs (boxed regions), parallel to the conserved protein domains detected by the ProDom server that could potentially pertain to the 4 α-helices.

The signal peptides of the M17s and MSHs were predicted to be 32 a.a. and 29 a.a., respectively, using Hidden Markov and Neural network models (Fig. 2(A)). These signal peptides (including the additional long N-terminal sequence predicted in zebrafish [11] not shown in this study) were composed of numerous methionine (M) residues that are potential start codons.

We did BLAST search and identity analysis for M17 and MSH and found that these genes did not show any counterpart in higher vertebrates. The closest identity this BLAST search produced was with chicken CNTF and mouse CT-2, hence M17 and MSH appeared to be present only in fish and not in higher vertebrates (Fig. 2(B)). Furthermore, only the M17 group showed some level of homology (22–26%) to mouse CT-2 and chicken CNTF while the MSH group did not, except for poMSH, which has a 21% homology to mouse CT-2. Such pattern of homologies supported a separate orthology for M17 and MSH.

3.3. Genomic structures and synteny

Our analysis of the genomic structures in existing fish genome sequences revealed that zebrafish M17 (drM17), tiger pufferfish (trMSH) and stickleback (gaMSH) were composed of 3 exons and 2 introns, where the 3rd exon at the 3' end was considerably longer than the 1st and 2nd exons, similar to the published carp M17 (data not shown). The green spotted pufferfish (tnMSH), although it had a 4 exon–3 intron architecture, possessed essentially the same pattern with zebrafish, tiger pufferfish and stickleback because its 3rd exon, 3rd intron and 4th exon appeared to constitute the long 3rd exon in the latter species. This means that at the genomic organization level, M17 and MSH were similar.

Genome analysis revealed that the drM17 cluster of zebrafish included tescalcin, zinc finger matrin type 5, myosin heavy polypeptide 2, myosin heavy chain 4, frequenin homologue A, and the actin related 2/3 complex-subunit 5-like, while the trMSH, tnMSH and gaMSH genes were determined to be conservatively flanked by myosin IC, sushi domain containing 2, serine/threonine-protein kinase, tescalcin, calcium-binding protein 7 and zinc finger and matrin type 5. Comparative synteny between the M17 and MSH loci showed difference in their gene composition, again supporting an evolutionary separation between M17 and MSH (Fig. 3).

3.4. Neighbor-joining analysis of IL6-cytokine subfamily

A neighbor-joining (NJ) analysis based on the amino acid sequences of the fish M17s, MSHs and other related cytokines showed that fish M17s and MSHs clustering was supported by high bootstrap value at 99%. In addition,
the M17/MSH grouped with CT-1/CT-2/CNTF, and this M17/MSH/CT-1/CT-2/CNTF cluster significantly separated with an OSM/LIF cluster at 77% bootstrap values. This suggests that M17/MSH is more closely related phylogenetically to CT-1/CT-2/CNTF than to OSM/LIF (Fig. 4). A tree constructed with the maximum parsimony showed similar pattern to the NJ tree (data not shown).

Fig. 4. Neighbor-joining tree of the amino acid sequences of M17, MSH and other members of the IL6 subfamily of cytokines. Accession numbers of the genes include; M17: carp (AAM52337), zebrafish (XP_684795), goldfish (DQ861993); MSH: Japanese flounder (AB280428), tiger pufferfish (CAF 99247); green spotted pufferfish (O62728); stickleback (Ensemble: ENSGACT00000022426); CT-1: mouse (Q60753), human (NP_001321), rat (NP_058825); CT-2: mouse (NP_942155), dog (XP_547035); CNTF: chicken (Q02011), human (P26441), pig (002732), rat (P20294); OSM: human (P13725), cow (NP_783644), mouse (P53347); LIF: human (P15018), mouse (P09056), pig (CAC14463), cow (Q27956); IL11: human (P20809), mouse (P47873), zebrafish (BN000713), tiger pufferfish (BN000714), green spotted pufferfish (BN000714), green spotted pufferfish (AY374548), catfish (CAJ57446), trout (AJ535687), carp (AJ632159) Japanese flounder (AB299205); CSF3: Japanese flounder (AB200968), Tiger and green spotted pufferfish [3]; and IL6: human (P05231), pig (P26893), tiger pufferfish (NM_001032722). Branch numbers correspond to percentage of bootstrap values at 1000 resampling.
3.5. Constitutive expression of poMSH

The Japanese flounder poMSH was constitutively expressed in all the tissues examined although at varying levels using a 30 cycle PCR reaction. It was highly expressed in gills, heart, kidney and peripheral blood leukocytes (PBLs), moderately expressed in spleen, stomach, skin, eyes and intestine, and almost not expressed in brain, liver and muscle (Fig. 5).

3.6. Stimulated expression of poMSH

Quantitative PCR revealed that Japanese flounder poMSH gene was significantly induced in kidney cells by polyI:C at 1 h post-stimulation. The gene was likewise up-regulated at very high levels by bacterial components, LPS and PG at 6 h after induction. Interestingly, the copy number of poMSH transcripts induced by PG is more than the LPS (Fig. 6).

4. Discussion

M17 has already been reported in carp, goldfish, zebrafish, green spotted pufferfish and tiger pufferfish [2]. In this study, we isolated a cDNA molecule in Japanese flounder that is homologous to M17. However, we subsequently characterized it as a novel gene unique from M17 named as M17 homologue (poMSH) using comparative structural and expression analysis. With the characterization of poMSH, we were able to show that tiger pufferfish, green spotted pufferfish and stickleback M17s are MSH orthologues, while zebrafish, carp and goldfish M17s are M17 orthologues.

MSH was found to be unique from M17. Structurally, MSH has 4 cysteine residues while M17 has 6 suggesting a possible difference in their protein folding since cysteine (C) residues are known to form di-sulfide bridges. MSH does not possess significant identity with the chicken CNTF (except for poMSH with mouse CT-2) while carp, zebrafish and goldfish M17 have, and the MSH loci cluster is not well conserved with the M17 loci. Phylogenetically, MSH and M17 are significantly separated at 99% bootstrap values. Constitutive expression also showed a striking difference between MSH and M17. poMSH is clearly expressed in all the tissues examined except in the brain, while M17 as shown previously in carp and goldfish was detected in very high quantities in the brain and to a lesser extent in kidney and blood leukocytes. Such differential expression suggests that MSH performs other and/or additional functions than M17. In addition, these data suggests that MSH is a gene closely related but separate from M17. Such gene evolution is not uncommon within the IL6 subfamily. For example OSM and LIF, considered to be genes with different functions, have high amino acid similarity and are thus thought to be a product of gene duplication [2]. M17 itself has been reported to be of 2 types both located in chromosome 5 differing only by 2 amino acids [11]. Because of the close similarity of the 2 genes and of the widely believed genome duplication in teleosts, we actually tried to investigate whether MSH is the duplicate copy of M17. Using trMSH and drM17, we did a “tBLASTn” search of the fugu and zebrafish genomic databases, respectively, but we could not find a “homologue” either with significant E-value, identity and alignment nor did we find a cluster similar to the trMSH and drM17 clusters.

Since it is known that zebrafish and carp (Cypriniformes) are more primitive than the Japanese flounder (Pleuronectiformes), tiger pufferfish and green spotted pufferfish (Tetraodontiformes), and stickleback (Gasterosteiformes) [18], it follows that M17 is more primitive than MSH. Thus, it is tempting to speculate that MSH may have arisen from M17 by gene duplication similar to OSM and LIF. MSH could also be an evolutionary selected form of M17 since we could not find the duplicate copy of MSH in the available genomic databases. More information on these genes could clarify these issues.

The characteristic of fish M17 reported so far is confusing at best. It has been previously shown in carp to be structurally more related to OSM/LIF than CNTF because of the presence of signal peptides, position of the cysteines and similarity in exon/intron configuration. But it was at the same time said to be more similar to CNTF than OSM/LIF in terms of high expression in the brain [10]. In goldfish, it was observed to share similar sequences (except the presence of signal peptide) and expression with CNTF but exhibited LIF-like functions [11]. We now have reason to believe that at the evolutionary standpoint, M17/MSH is actually more related to CNTF than to OSM/LIF.

The MSH orthologues appear to be secreted molecules because they contain signal peptides and are likely to be cytokine-like molecules because they show the classic cytokine motif i.e. having the mRNA-destabilizing pentamer

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AUUUA [19] and the consensus polyadenylation signals (ATTAAA) [20]. As mentioned, the Japanese flounder MSH is ubiquitously and constitutively expressed in numerous tissues except in the brain suggesting its involvement in various physiological processes in fish. In particular, it is highly expressed in tissues/cells that are involved in immuno-hematopoietic functions such as gills, kidney and PBLs. Such expression pattern is similar to LIF/OSM rather than M17/CNTF. LIF and OSM exert numerous effects on the regulation, proliferation and differentiation of cells of various origins including macrophages, embryonic stem cells, blastocytes and neuronal cells, and induce acute phase protein production in hepatocytes [2]. CNTF is a neurotrophic factor that influences the growth and survival of cells in the neuronal lineage [21], and can also induce acute phase protein production in hepatocytes [2]. M17, which is expressed in brain, kidney and blood leukocytes in goldfish, and expressed only in brain and PBLs in carp, has been found to induce production of nitric oxide in macrophages, stimulate proliferation of macrophages, and induce differentiation of monocytes to macrophages [10,11].

Also, poMSH is stimulated by polyI:C, an interferon inducer, by bacterial LPS and PG suggesting that the gene has a role against viral and bacterial infections in fish. Induction of poMSH by viral and bacterial agents, as well as its constitutive expression in immuno-hematopoietic tissues, indicates that the molecule has cytokine-like function. A number of Japanese flounder cytokines reported so far exhibit the same properties including Tumor Necrosis Factor (TNF), Interleukin-1β (IL-1β), CSF3 and a novel CC chemokine JFCCL3 [3,13,22,23]. PolyI:C has been reported to

![Fig. 5. RT-PCR expression of Japanese flounder MSH gene in tissues of apparently healthy Japanese flounder. Values are relative expression of the MSH to β-actin.](image)

![Fig. 6. Quantitative real-time PCR analysis of Japanese flounder MSH in head kidney cells induced by (A) polyI:C (1 μg/ml), (B) LPS (1 μg/ml) and (C) peptidoglycan (1 μg/ml) sampled at 0 h, 1 h, 3 h and 6 h post-induction.](image)
induce IL6\textsuperscript{[24]} while LPS and PG can highly induce chemokines and cytokines\textsuperscript{[25–28]}. Studying further the function of MSH in relation to fish hematopoiesis and immunity could prove to be interesting.

It is quite interesting that MSH and M17 genes are likely to be present only in fish. In addition to this, MSH expression is more similar to OSM and LIF even though structurally it is more homologous to M17 and to CNTF except for the presence of a signal peptide. This structural and functional attribute of MSH further confirm the pleiotropic and redundant nature of cytokines particularly of the IL6-cytokine subfamily.

This likewise adds to the complexity of this group of molecules, which in fish appears to be more diverse than in higher vertebrates, additional evidence supporting the “more genes in fish” theory\textsuperscript{[29]}. The identification of MSH also complicates the suggestion in previous reports that M17 is an ancestral molecule to LIF and OSM, and that its function is ancestral to the divergence of CNTF and LIF, issues that require further studies.

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