ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(5): 327–334 **CN** 31-1940/Q

Molecular Cloning and mRNA Profile of Insulin-like Growth Factor Type 1 Receptor in Orange-spotted Grouper, *Epinephelus coioides*

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Abstract The insulin-like growth factor type 1 receptor (IGF-IR) belongs to the tyrosine kinase (TK) receptor family. Besides being mitogenic, IGF-IR plays a crucial role in cell survival, transformation and maintenance of the malignant phenotype. In this study, we cloned the cDNA from the hypothalamus of the orange-spotted grouper (*Epinephelus coioides*) using reverse transcription PCR (RT-PCR) and the rapid amplification of cDNA ends (RACE) method. The deduced amino acid sequence showed that the receptor comprises 1413 amino acid residues. It contains cysteine-rich domains in its α-subunit, and a conserved transmembrane domain and TK domains in its β-subunit. Comparison of the amino acid sequence with those of other species showed that the grouper IGF-IR shares 90.2%, 89.6%, 71.9% and 72% similarity with the IGF-IR of the Japanese flounder, turbot, zebrafish-a and zebrafish-b, respectively. When compared with its mammalian homologue, grouper IGF-IR contains a large insertion at its C-terminus. Phylogenetic analysis has revealed that the grouper IGF-IR belongs to the b-type IGF-IRs and has a higher similarity with flounder and turbot IGF-IR, and a lower similarity (<70%) with human, mouse and avian IGF-IR. Grouper IGF-IR transcripts were detected in the brain, peripheral tissues, embryos and early development larvae by semi-quantitative RT-PCR assay. It was observed that IGF-IR mRNA expression was greater in the brain than in peripheral tissues. The level of IGF-IR mRNA expression was much higher in retina, gonad, skeletal muscle and gill tissues than in liver, heart and thymus tissues. The expression of IGF-IR can be visualized as a ubiquitous signal in unfertilized eggs, embryos and early development larvae. The distribution pattern of IGF-IR mRNA in grouper development suggests that IGF-IR plays an important role in the embryo and early larval development stages.

Key words orange-spotted grouper (*Epinephelus coioides*); insulin-like growth factor type 1 receptor; cDNA cloning; mRNA expression

The insulin-like growth factor (IGF) system, which consists of two ligands (IGF-I and IGF-II), two receptors (IGF-IR and IGF-IIR) and six binding proteins (IGFBP1–6), is well known for promoting proliferation and differentiation in many vertebrates. The biological effects of the IGF system are believed to be mediated mainly by the IGF-I receptor (IGF-IR) and modulated through multiple binding proteins. Recent experiments have shown that the normal postnatal growth and development may be a result of normal free IGF-I levels, although the role of autocrine/paracrine IGF-I has not been determined yet [1]. The IGF-IR belongs to the tyrosine kinase (TK) receptor family and is structurally similar to the insulin receptor [2]. It comprises two α-subunits and two β-subunits linked by disulphide bonds, forming α2β2 heterodimers. The α-subunits contain an extracellular ligand-binding domain.

Received: January 6, 2005 Accepted: March 7, 2005

This work was supported by the grants from the National High Technology Research and Development Program of China (No. 2003AA603410, No. 2003AA603011), the Major State Basic Research Development Program of China (2004CB117402) and the Natural Science Foundation of Guangdong Province (No. 20023002)

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DOI: 10.1111/j.1745-7270.2005.00045.x

The β-subunits are composed of a single transmembrane domain and highly conserved intracellular TK domains [3].

Over the past decade, there has been an accumulation of knowledge related to the IGF system in teleost fish. There is evidence to suggest that the major components of the system in teleosts are structurally and functionally similar to those in mammals [4]. IGF-IR cDNAs have been isolated from several vertebrates, including a number of teleost fishes. Partial cDNAs have been isolated from coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) [5,6]. It has been suggested that two separate genes encoding the IGF-IR exist in the rainbow trout. The full-length IGF-IR cDNA has been cloned from the turbot (*Psetta maxima*) and the gene has also been shown to be expressed at all stages of development and in all of the tissues examined [7]. Recently, two distinct IGF-IR genes named *igf-1ra* and *igf-1rb* were found in zebrafish (*Danio rerio*) [4]. Structural and phylogenetic analyses indicated that both genes are orthologous to the human *igf-1r* gene [4]. Almost at the same time, two different IGF-IR cDNA clones from the Japanese flounder (*Paralichthys olivaceus*) were reported [8]. RNase protection assays showed that the flounder *igf-1r* transcripts are present in a wide range of tissues [8].

The orange-spotted grouper, *Epinephelus coioides*, is a marine fish widely cultured in southern and eastern China, and it has a very high economic value. It is a protogynous hermaphroditic fish. The female fish reach sexual maturity at about 5 years of age. For the purpose of investigating the role of IGF-IR in the grouper, we cloned and characterized the complete cDNA for IGF-IR and determined the relative mRNA levels in various tissues and during early larval development.

Materials and Methods

Animals and tissues

Two-year-old orange-spotted groupers as well as larvae aged 1–50 days post-hatching were obtained from Daya Bay Fishery Development Center (Guangdong, China). The fish were decapitated and the tissues were dissected, frozen immediately in liquid nitrogen and stored at –70 °C until RNA extraction. The larvae were frozen in liquid nitrogen directly.

Collection of embryos

Newly fertilized eggs and embryos were collected from Daya Bay Fishery Development Center in May 2002. The embryos were maintained in seawater at about 25 °C and harvested at different developmental stages post-fertilization. The samples were frozen immediately in liquid nitrogen and stored at –70 °C.

RNA extraction

Total RNA was extracted from frozen tissues, embryos and larvae using Trizol (Invitrogen, USA). The concentration of RNA was determined by measuring the absorbance at 260 nm. The integrity of the RNA was examined by electrophoresis on 1% agarose gel.

Oligonucleotide design

All oligonucleotides were synthesized by BioAsia Limited (Shanghai, China). The oligonucleotides used as PCR primers are listed in **Table 1**. Oligonucleotides P1703, P2935 and P3100 were degenerate primers designed to amplify the *igf-1r* partial fragment. Oligonucleotides 5R336 and 5R104n were used to clone the 5′ region of *igf-1r* cDNA. Oligonucleotides 3R707, 3R1109n and 3RYR1 were primers used to amplify the TK domain coding sequence of *igf-1r*. 3R3700, 3R3500 and 3R6000 were used to clone the 3′ region of *igf-1r*. ProFor and ProRev, designed as the primers for RT-PCR of tissue distribution, were also the primers in PCR of probe preparation for Southern blot. 18SU and 18SD were used to amplify the 18S ribosome RNA as a positive control in the RT-PCR.

cDNA cloning

After a partial 1250 bp cDNA fragment of *igf-1r* was generated from the hypothalamus by standard molecular cloning procedures, the 5′ and 3′ regions of *igf-1r* were further synthesized by the rapid amplification of cDNA ends (Invitrogen). PCRs were performed at 94 °C (0.5 min), 45–65 °C (0.5 min) and 72 °C (1–2 min) for 30 cycles using the TGradient thermal cycler (Biometra, Germany). The amplified cDNA fragments were cloned into the pGEM-T easy vector (Promega, USA) and sequenced by the dideoxy chain-termination method.

RT-PCR analysis of IGF-IR mRNA expression

The total RNA (2 μg) from adult tissues, embryos and larvae were reverse transcribed into single-stranded DNA using the oligo(dT) primer and SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). Each RNA sample was treated with RNase-free DNase I (Invitrogen) to remove any genomic DNA contamination. The primers ProFor and ProRev were used to amplify a 300 bp *igf-1r* fragment for use as a probe. The integrity of all RNA

Table 1 Oligonucleotide sequences

Fragment	Oligonucleotide	Sequence
Partial fragment of *igf-1r*	P1703 (+)	5′-T**K**AA**R**CC**Y**TGGAC**H**CA**R**TA**Y**GC-3′
	P2935 (–)	5′-CACTGAA**R**TA**Y**TC**N**GG**R**TT**V**AC-3′
	P3100 (–)	5′-TC**R**TT**N**AC**H**GTCTT**D**ATGGC-3′
5′ region of *igf-1r*	5R336 (–)	5′-CCTCTTCCTGGTCTCCCACACCTATG-3′
	5R104n (–)	5′-GGTGCGGATGTAGACCACTTTGC-3′
TK domain of *igf-1r*	3R707 (+)	5′-GCCGTTCACAGTTTACCGCATCG-3′
	3R1109n (+)	5′-CGTCCTCTACGCTATGATCTTCG-3′
	3RYR1 (–)	5′-GGCTTGTT**S**TC**MK**C**R**CTGTAG-3′
3′ region of *igf-1r*	3R3700 (+)	5′-GTGTTGGCAGTACAATCCTAAG-3′
	3R3500 (+)	5′-AGGGTTTGCTTCCTGTCCGATG-3′
	3R6000 (–)	5′-CCCACTGTGACATCAGGTTC-3′
Probe of *igf-1r*	ProFor (+)	5′-AGGGATCCCATGCCAAGAGCAAAGTG-3′
	ProRev (–)	5′-GGAATTCCGGGATCTGGCTTAGTG-3′
18S ribosome RNA	18SU	5′-CCTGAGAAACGGCTACCACATCC-3′
	18SD	5′-AGCAACTTTAGTATACGCTATTGGAG-3′

Codes for degenerate primers (in bold): R=A+G; K=G+T; V=A+G+C; Y=C+T; N=A+G+C+T; H=A+C+T; D=A+G+T; M=A+C. (+) and (–) indicate sense and antisense primers, respectively.

samples was verified by the successful amplification of 18S ribosome RNA. The primers 18SU and 18SD generated a PCR product of 250 bp. The cycling conditions were as follows: 95 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. The RT-PCR products were separated on 1.8% agarose gel and transferred onto a nylon membrane by the capillary method. The detection of the *igf-1r* transcript was performed using a digoxigenin (DIG)-labeled product amplified with the PCR-DIG probe synthesis kit (Roche). Hybridization, washing and autoradiography were carried out according to the manufacturer's instructions.

Results

Cloning and characterization of the IGF-IR cDNA

The orange-spotted grouper IGF-IR cDNAs were isolated from the hypothalamus using RT-PCR and RACE method. The length of the complete IGF-IR cDNA sequence is 4908 bp, which includes a 595 bp 5′-untranslated region (UTR), an open reading frame (ORF) of 4242 bp and a 71 bp 3′-UTR (**Fig. 1**). The ORF encodes a putative protein of 1413 amino acid residues. The deduced amino acid sequence is shown in **Fig. 2**. The receptor can be

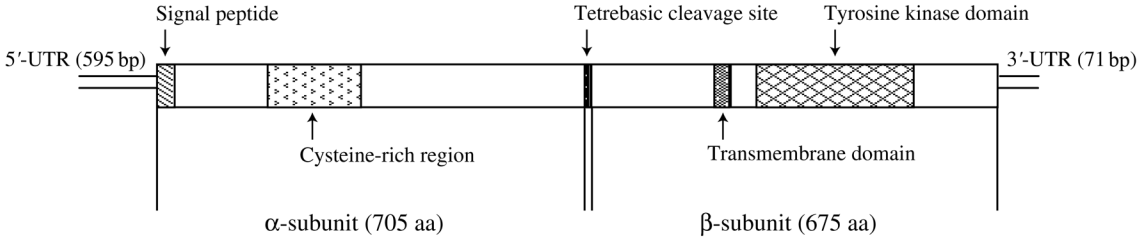

Fig. 1 Primary structure of IGF-IR protein

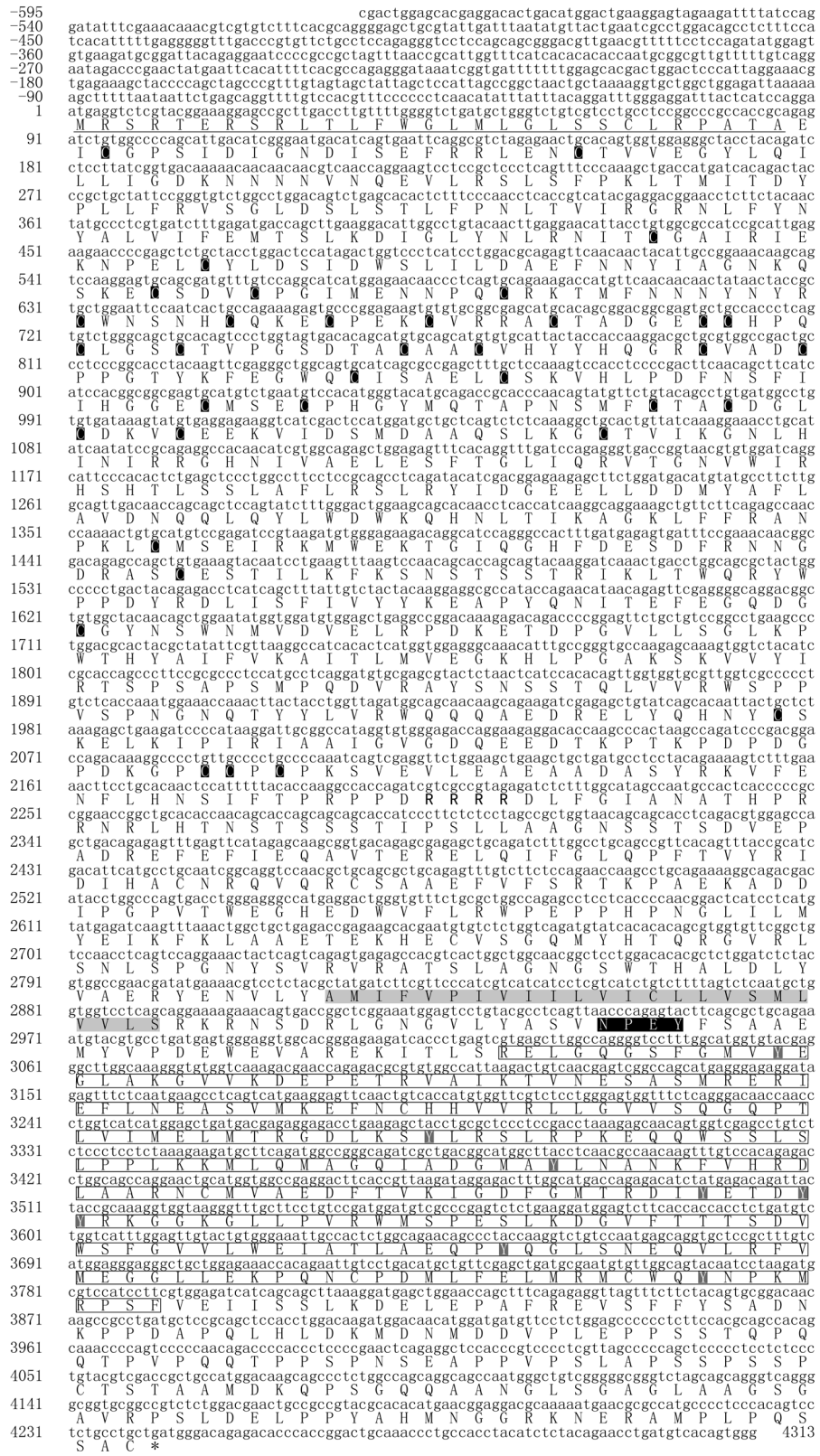

Fig. 2 Nucleotide sequence and amino acid sequence of IGF-IR

The signal sequence is underlined; cysteine residues are shown by black boxes; the proteolytic cleavaging sequence is shown by bold characters; the transmembrane domain is shaded; and the tyrosine kinase domain is shown by the boxed areas in which tyrosine residues have a black background. The potential IRS-I binding site (NPEY) is indicated by a black box. The stop code is followed by an asterisk.

Table 2 Phylogenetic distance alignment of IGF-IR sequence between the grouper and other species

Species	1	2	3	4	5	6	7	8	9	10	11	12
1	–	66.5	66.1	67.0	84.9	83.9	80.4	85.1	65.5	78.6	69.5	65.9
2	43.3	–	67.8	68.6	64.6	63.8	64.9	64.8	67.4	64.0	75.2	69.6
3	43.2	40.0	–	90.2	64.6	63.1	62.6	64.4	90.5	63.0	71.6	70.5
4	41.7	38.5	10.0	–	65.2	64.1	63.8	64.8	89.6	63.5	71.9	72.0
5	16.9	46.7	46.5	45.0	–	96.0	76.2	98.4	63.7	75.1	67.2	63.6
6	18.2	47.4	47.4	46.1	4.0	–	75.1	95.9	63.0	74.4	65.9	61.7
7	22.2	46.6	49.3	47.2	27.5	29.1	–	76.5	61.8	75.3	67.9	63.3
8	16.6	46.3	46.8	45.3	1.6	4.1	27.2	–	63.6	75.1	67.0	62.9
9	44.3	41.2	9.9	10.4	47.4	48.1	50.1	47.5	–	62.7	71.1	70.1
10	24.7	48.0	49.4	48.2	29.4	30.6	29.1	29.4	50.0	–	66.6	62.9
11	37.2	29.8	35.4	34.0	41.5	42.9	40.9	41.9	36.1	43.2	–	73.6
12	41.3	36.6	35.0	33.3	45.0	46.0	45.0	45.0	35.6	46.4	30.8	–

A multiple comparison was performed with Lasergene megalign with ClustalW method. 1, chicken; 2, flounder-a; 3, flounder-b; 4, grouper; 5, human; 6, mouse; 7, newt; 8, porcine; 9, turbot; 10, *Xenopus*; 11, zebrafish-a; 12, zebrafish-b.

subdivided into several major domains, including a cysteine-rich region, a tetrebasic cleavage site, a transmembrane domain, a TK domain and a carboxyl-terminal region (**Fig. 1**).

Using the ClustalW method, we aligned the amino acid sequence of the grouper IGF-IR with those of several other species, including human (X04434), mouse (AF056187), chicken (AJ223164), porcine (AB003362), newt (AB050625), *Xenopus* (AF055980), zebrafish (AF400275), turbot (AJ224993) and flounder (AB065098) (**Table 2**). The grouper IGF-IR was found to be 90.2%, 89.6%, 71.9% and 72% homologous to the IGF-IR of the Japanese flounder, turbot, zebrafish-a and zebrafish-b, respectively. Similarities with mammalian and avian IGF-IRs were all found to be below 70%. A potential proteolytic cleavaging sequence R-X-R-R was conserved in the species compared. The cysteine-rich domain in the α-subunit of the grouper IGF-IR contains 24 cysteine residues, which was observed in mammalian IGF-IR. In the β-subunit, an IRS-I binding site (NPEY), a potential ATP binding site (G-X-G-X-X-G-21-X-K) and an autophosphorylation site (YETDYY) were highly conserved in all species. The least conserved region was the carboxyl-terminal domain. Large insertions were observed in the teleost IGF-IR compared with those of mammals.

A phylogenetic tree of the IGF-IRs in the orange-spotted grouper and other vertebrates (**Fig. 3**) was constructed by the maximum parsimony method using Phylip software. The IGF-IRs in teleosts were arranged into two distinct groups; namely the a-subtype and b-subtype receptor branches. The orange-spotted grouper IGF-IR

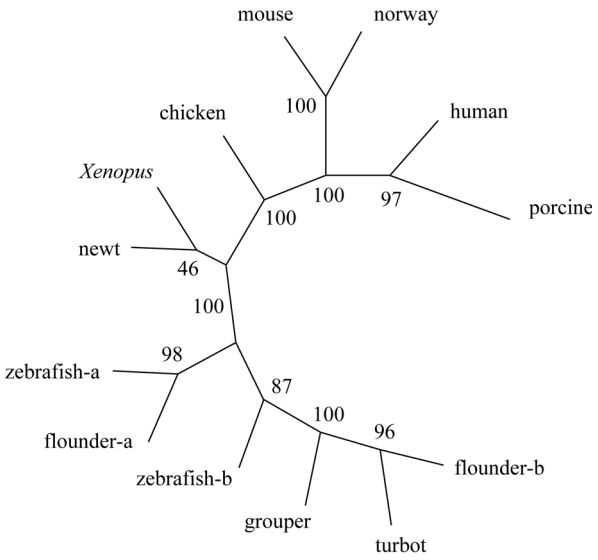

Fig. 3 Phylogenetic tree of IGF-IR amino acid sequences of the orange-spotted grouper and other species constructed using Phylip software

Sequences were obtained from the following data libraries: human IGF-IR (X04434); porcine IGF-IR (AB003362); norway IGF-IR (NM_052807); mouse IGF-IR (AF056187); chicken IGF-IR (AJ223164); *Xenopus* IGF-IR (AF055980); newt IGF-IR (AB050625); zebrafish-a IGF-IRa (AF400275); zebrafish-b IGF-IRb (AF400276); flounder-a IGF-IRa (AB065098); flounder-b IGF-IRb (AB065099); turbot IGF-IR (AJ224993).

belonged to the b-subtype branch. Phylogenetic analysis showed that the evolutionary position of the orange-spotted grouper IGF-IR was close to that of the flounder, turbot, zebrafish-a and especially zebrafish-b.

Tissue distribution of IGF-IR mRNA

The expression of IGF-IR mRNA in different adult tissues was detected using semi-quantitative RT-PCR followed by Southern blot, and the results are shown in **Figs. 4** and **5**. High levels of IGF-IR mRNA expression were observed in the forebrain, hypothalamus, cerebellum, retina, gonad and skeletal muscle tissue. The IGF-IR mRNA expression detected in the liver and thymus was at low levels. Weak signs of expression were detected in the head kidney, kidney and heart after Southern blot analysis. Other tissues showed intermediate levels of expression.

Expression of IGF-IR mRNA during embryonic and larval development

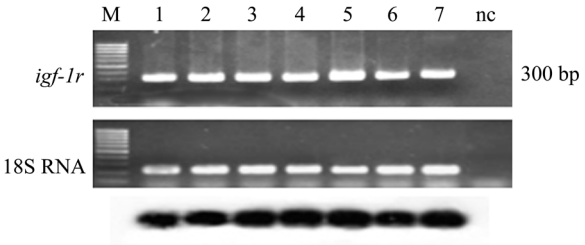

Fig. 4 mRNA expression of IGF-IR and 18S ribosome RNA in each domain of the brain and IGF-IR Southern blot

1, olfactory bulb; 2, end-brain; 3, epithalamus; 4, hypothalamus; 5, cerebellum; 6, medulla oblongata; 7, pituitary; M, 100 bp DNA ladder; nc, negative control.

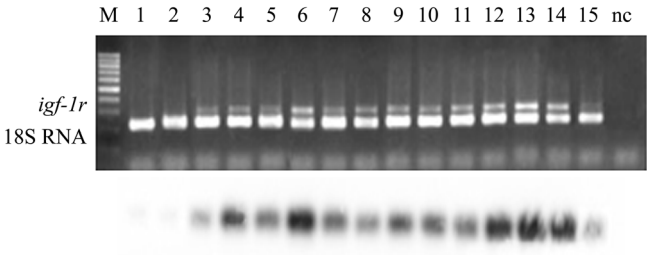

Fig. 5 mRNA expression of IGF-IR and 18S ribosome RNA in other tissues and IGF-IR Southern blot

1, head kidney; 2, heart; 3, liver; 4, spleen; 5, kidney; 6, gonad; 7, stomach; 8, foreintestine; 9, midintestine; 10, posterior intestine; 11, fat; 12, gill filament; 13, retina; 14, muscle; 15, thymus; M, 100 bp DNA ladder; nc, negative control.

The temporal expression patterns of IGF-IR during early development were detected using semi-quantitative RT-PCR followed by Southern blot, and the results are shown in **Figs. 6** and **7**. Analysis indicated that IGF-IR expression was detected at all early developmental stages of the orange-spotted grouper. IGF-IR mRNA was expressed in unfertilized eggs and the signal began to increase after fertilization and during the whole embryo development stage until the hatching stage, when it began to decrease. The transcript in larvae showed a similar expression pattern to that in embryos. The IGF-IR mRNA expression in larvae increased gradually and was maintained at high levels up to 30 days post-hatching, and then appeared to drop at 35–50 days post-hatching.

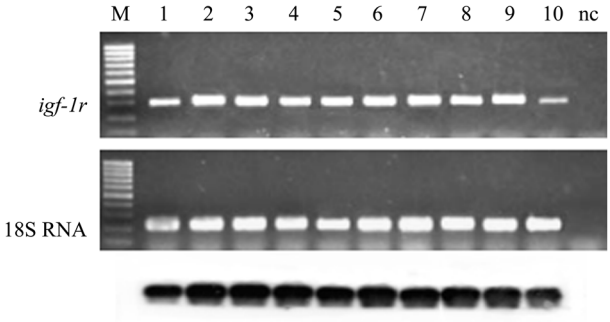

Fig. 6 mRNA expression of IGF-IR and 18S ribosome RNA during embryogenesis and IGF-IR Southern blot

1, unfertilized eggs; 2, fertilized eggs; 3, 16-cell stage; 4 and 5, neurula stage; 6, gastrula stage; 7, vesicle stage; 8, crystal stage; 9, hatch prophase; 10, hatching; M, 100 bp DNA ladder; nc, negative control.

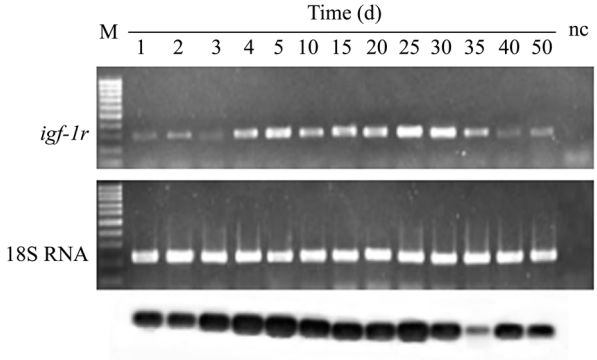

Fig. 7 mRNA expression of IGF-IR and 18S ribosome RNA during early larval development and IGF-IR Southern blot

M, marker; nc, negative control.

Discussion

In this study, we cloned the full-length IGF-IR cDNA encoding a protein containing 1413 amino acids from the orange-spotted grouper hypothalamus. The alignment of the orange-spotted grouper IGF-IR amino acid sequence with those of other vertebrates indicates the high degree of conservation of this receptor during vertebrate evolution. In particular, most of the conserved regions are known to be critical for IGF-IR biological activity, such as the ligand-binding motif, TK domain, ATP-binding site, autophosphorylation site and IRS-I binding site. In contrast, the carboxyl-terminus of the receptors is the most divergent region. In humans, this C-terminal region exhibits only limited homology between insulin receptor and IGF-IR [9]. There is evidence to show that the carboxyl-terminus is important for cell signaling specificity [10]. Teleosts share insertions in the carboxyl-terminus and this suggests that the role played by this region may differ between mammalian and more ancient receptors.

Two isoforms of IGF-IR in teleost fish have been reported. Two partial cDNAs have been identified in coho salmon and rainbow trout, and it has been suggested that the IGF-IRs are encoded by two genes in these species [5,6]. It has also been reported that two full-length IGF-IR cDNAs exist in the zebrafish and Japanese flounder [4,8]. These subtypes were found to be coded by distinct genes orthologous to the human gene. Sequence comparison and phylogenetic analysis revealed that the IGF-IR cDNA obtained in this study belongs to the b-subtype gene. We can not rule out the possibility that a second gene coding this receptor exists in the orange-spotted grouper, given that the likely genome duplication event has occurred in other teleosts [11].

The IGF-IR mRNA is found in a wide variety of tissues in the adult orange-spotted grouper. RT-PCR revealed high levels of IGF-IR mRNA in brain, retina, gonad, skeletal muscle and gill tissue. The tissue distribution in the grouper is almost consistent with what has been reported in other teleosts. The high level expression of the receptor in the brain suggests IGF's involvement in neural development [12]. Fish IGF-IR has been shown to stimulate DNA synthesis and cell proliferation [13]. The presence of high levels of IGF-IR mRNA in gonad, skeletal muscle and gill tissue is consistent with the biological activity mediated by the receptor. IGF-IR is increasingly recognized as a potentially important regulator in germ cell proliferation and differentiation, including steroidogenic and gonadotrophic functions in fish [14]. It has also been proven to stimulate oocyte maturation, hormone synthesis and secretion [15].

IGF-IR has been reported to stimulate glucose uptake, metabolism and protein synthesis in mammalian skeletal muscles [16]. The present study found a high level of IGF-IR mRNA in orange-spotted grouper skeletal muscle. However, in the rainbow trout, turbot and flounder, low levels of IGF-IR mRNA expression were observed in the skeletal muscle [6–8]. It has been reported that the glucose uptake and metabolism in rainbow trout are higher in the heart than in skeletal muscles. This suggests that discrepant expression exists in the same gene of different species even if they are highly similar.

It has been hypothesized that IGF-IR is involved in osmoregulation cartilage sulfation in the gills of fishes [17, 18]. IGF-IR stimulates sulfate uptake by isolated gill cartilage and enhances seawater adaptability. High levels of IGF-IR mRNA found in gill tissue confirm the biological function modulated by the receptor. Moreover, the intestine participates in osmoregulation, especially in marine fishes. The presence of IGF-IR in orange-spotted grouper intestine is consistent with its osmotic role.

We also examined the transcription of IGF-IR in the orange-spotted grouper at the different stages: unfertilized eggs, embryos and larvae. IGF-IR appears to be the product of both maternal and embryonic genomes. The IGF-IR mRNA was detected in eggs just after fertilization. Its concentration increases gradually as the embryo grows and is maintained at high levels in two critical stages after fertilization and hatching (4 days and 35 days post-hatching). The expression of IGF-IR mRNA in orange-spotted grouper embryos and larvae indicates that the growth factor may play a key role and is important during early embryonic development. Unlike mammalian embryos, fish embryos develop outside the maternal body and rely on growth factors stored maternally. Indeed, these maternal mRNA synthesized during oogenesis are stored in a translationally inactive form until they are activated mainly by polyA elongation during oocyte maturation, fertilization and early embryonic development [19].

In summary, we have demonstrated the existence in the orange-spotted grouper of an IGF-IR that is a b-subtype receptor and contains the main features of its counterpart in other vertebrate species. The ubiquitous expression of IGF-IR in adult tissues suggests that the IGF influence is exerted locally either at its site of synthesis (autocrine mode) or on nearby cells (paracrine mode). The widespread presence of IGF-IR at the early developmental stage of the orange-spotted grouper suggests the role of IGFs as a potential regulator during

embryogenesis. This initial study therefore provides a basis for the further investigation of IGF-IR functions, such as its physiological functions.

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Edited by
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