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Molecular cloning and expression pattern of 14 kDa apolipoprotein in orange-spotted grouper, *Epinephelus coioides*

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Abstract

A novel fish-specific apolipoprotein (*apo-14 kDa*) has been recently cloned from eel and pufferfish. However, its expression pattern has not been elucidated. In this study, *EcApo-14* has been screened from hypothalamic cDNA library of male orange-spotted grouper, which shows 62.9%, 51%, 46.9%, 43.2%, and 31.9% identities to *Apo-14* of European flounder, pufferfish, Japanese eel, gibel carp, and grass carp, respectively. RT-PCR analysis reveals that this gene is first transcribed in neurula embryos and maintains a relatively stable expression level during the following embryogenesis. *EcApo-14* transcripts are at a very high level during embryonic and early larval development in the yolk syncytial layer (YSL), and decrease in YSL and form intense staining in liver at 3 days after hatching. In adult tissues, *EcApo-14* is predominantly expressed in liver and brain. The data suggested that EcApo-14 might play an important role in liver and brain morphogenesis and growth.

Keywords: Apolipoproteins; Grouper; Orange-spotted grouper; Apo-14; Embryogenesis; Yolk syncytial layer (YSL); Liver; Morphogenesis; Expression pattern; Whole-mount in situ hybridization

1. Introduction

Apolipoproteins, synthesized mainly in liver and intestine and bounded to lipids, play important roles in lipid transport and uptake through the circulation system (Havel, 1975). A great deal of attention had been focused on lipoproteins and apolipoproteins in humans ever since the relationship between specific lipoproteins and cardiovascular disease became apparent (Paolucci et al., 1998). However, little information has been dedicated to apolipoproteins in lower vertebrates (Paolucci et al., 1998). Since most fish utilize lipids as the most energy source in contrast to mammals which mainly use carbohydrates (Watanabe, 1982), lipid metabolism appears more important for homeostasis in fish than that in homeotherms (Kondo et al., 2005). Only several reports had been published on the structures of apolipoproteins from fish, such as ApoA-I isolated from zebrafish (Babin et al., 1997) and eel (Kondo et al., 2001), ApoC-II isolated from rainbow trout (Shen et al., 2000), ApoE isolated from zebrafish (Babin et al., 1997) and rainbow trout (Durliat et al., 2000). The expression pattern of apolipoproteins in embryogenesis was only investigated in zebrafish (Babin et al., 1997). Recently, a novel apolipoprotein (*Apo-14*) and its cDNA was isolated from eel (Kondo et al., 2001) and pufferfish (Kondo et al., 2005), respectively. While it has no homologous proteins with other vertebrates, the Apo-14 is specific to fish (Kondo et al., 2005). Its transcripts were mainly detected in liver and less abundantly in brain (Kondo et al., 2005). However, its expression pattern in embryogenesis has been unknown. The investigation on expression pattern of *Apo-14* throughout embryogenesis is able to obtain new insights about the apolipoprotein multigene family.

The orange-spotted grouper *Epinephelus coioides*, a protogynous hermaphroditic marine fish, is widely cultured in China and Southeast Asian countries. As a favorite marine food fish, it is commercially important. However, large-scale seed production is still encountering many difficulties. Grouper larvae are poor feeders. They are forced to shift to exogenous feeding at a small size because of their small endogenous energy reserves, and this may compromise their survival (Kohno, 1998). The study on *Apo-14* might help to understand the utilization of lipids in fish. Recently, we have constructed

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SMART cDNA plasmid libraries from the orange-spotted grouper pituitary and hypothalamus and initiated a series of molecular studies in order to reveal the regulative mechanisms of growth, development, reproduction, and sex inversion (Yao et al., 2003; Jia et al., 2004a,b; Wang et al., 2004; Li et al., 2005). By sequencing 352 clones from the hypothalamic cDNA library of the male grouper, a clone was screened to have high homology with pufferfish 14 kDa apolipoprotein (Kondo et al., 2005). Here, we report its molecular characterization, tissue distribution in adult, and expression pattern in embryogenesis.

2. Materials and methods

2.1. Isolation of cDNA clone and sequencing analysis

Hypothalamus was collected from a 7-year-old male orange-spotted grouper. Total RNAs were extracted using SV total RNA isolation system (Promega, USA). The quantity and quality of RNAs were measured at A260 nm and the ratio of A260:A280 nm by biophotometer (Eppendorf). Their cDNAs were synthesized from 50 ng of total RNAs according to the reports described previously (Yao et al., 2003; Wang et al., 2004; Li et al., 2005) using the Switching Mechanism At 5′ end of RNA Transcript (SMART) cDNA Library Construction Kit (Clontech). The cDNAs were ligated to pGEM-T vector (Promega) and the plasmids were used to transform *Escherichia coli* DH5á supercompetent cells. A clone was screened to have high homology with pufferfish 14 kDa apolipoprotein (Kondo et al., 2005).

Homology comparison was completed using ClustalW 1.8 program. The predications of signal peptide, transmembrane segments, glycosylation site and phosphorylation site were done by using SignalP V1.1 and YinOYang 1.2, NetOGlyc 2.0 and DictyOGlyc 1.1, and NetPhos 2.0, which were from the web site http://www.sxpasy.pku.edu.cn.

2.2. Total RNA isolation and semi-quantitative RT-PCR

Total RNAs of liver, kidney, spleen, fat, heart, muscle, brain and testis were isolated from the 7-year-old orange-spotted grouper, and RNAs of ovary were isolated from a 4-year-old orange-spotted grouper using SV Total RNA Isolation System according to the manufacturer's instructions (Promega). Total RNAs of eggs, embryos at different stages, such as morula, blastula, gastrula, neurula, optic vesicle, heart differentiation, prior to hatching, hatching, and 1-day-old fry were isolated. Total volume for each reaction was 25 μl containing 2 μl of the isolated RNAs, 5 mM of each dNTP, 0.5 μg primers, 200 units M-MLV RT, and 25 units of rRNasin® Ribonuclease Inhibitor with 1× M-MLV buffer (10 mM Tris–HCl, 25 mM KCl, pH 8.3, 0.6 mM $MgCl_2$ and 2 nM DTT). The reaction mixture was incubated at 37 °C for 1 h.

All of the resultant cDNAs were respectively diluted 1:10, and then used as templates for PCR with Taq DNA polymerase (MBI, Fermentas). One pair of primers (EcApo-14-F: 5′-ATGAATGCAAAATACGCCTTGG-3′, EcApo-14-R: 5′-TTACTCAGTGGGCATGAATTTG-3′) were synthesized (Sangon, Shanghai) according to the obtained nucleotide sequences of ORF and used to identify tissue distribution and expression level during embryogenesis. Amplification reactions were performed in volume of 25 μl containing 1 μl cDNA as template DNA, 0.2 μM each primer, 0.5 units Taq polymerase (MBI, Fermentas), 1 mM of each dNTP, 1× buffer for Taq polymerase (MBI, Fermentas). Each PCR cycle included denaturation at 95 °C for 40 s, annealing at 58 °C for 50 s, and extension at 72 °C for 50 s. 30 cycles were performed, followed by a final extension at 72 °C for 5 min.

As a positive control for the RT-PCR analysis, α-tubulin (tubulin-F: GTGCACTGGTCTTCAGGGGTT and tubulin-R: GGGAAGTGGATGCGTGGGTAT) was amplified to determine the template concentration and to provide a semi-quantitative external control for PCR reaction efficiency under

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    GACAGACAACTCGCTTGCAGAGAAGACTGTCACATCTTGGATCTGTACATCCAACACAGC  60

    AGCAGACATGAATGCAAAATACGCCTTGGCGCTGATCCTCGCTCTGCAGGTCTCTGTGAG 120
-18         M  N  A  K  Y  A  L  A  L  I  L  A  L  Q  V  S  V  S
    CCTGTATGAAGTTCCCGCACCATCGCAGGAGCTTGTTAACAAGTACGATGAATTGAAAGC 180
  1   L  Y  E  V  P  A  P  S  Q  E  L  V  N  K  Y  D  E  L  K  A
    TACGTTTTACAAGAGGCTGCTGACTGCTTACGGCAAGCTGCAGGCCGCTGCTGCTCCTAT 240
 21   T  F  Y  K  R  L  L  T  A  Y  G  K  L  Q  A  A  A  A  P  M
    GGTGGAGAAAGTCGGAGACAGTGCGCAGGGACAGACTGCTAAGGATTACATTGAGGAACT 300
 41   V  E  K  V  G  D  S  A  Q  G  Q  T  A  K  D  Y  I  E  E  L
    GCAGACTAAGCCTGAGCTCCAAGCCTTCGTCAAGGTTGCCACTGGCCTGGGCCAGGAGGC 360
 61   Q  T  K  P  E  L  Q  A  F  V  K  V  A  T  G  L  G  Q  E  A
    AGGTCCTCTGGTGGACAAGGCCCGTACTTCAGTGCTGGGTGCGTACGAATATTACCTGCG 420
 81   G  P  L  V  D  K  A  R  T  S  V  L  G  A  Y  E  Y  Y  L  R
    TCCCTACGTCGGCAACTTCCTGAGCGACAGCATTGATACCGCCAAGGTCTACCTGGACAA 480
101   P  Y  V  G  N  F  L  S  D  S  I  D  T  A  K  V  Y  L  D  K
    ATTCATGCCCACTGAGTAAAGAAGACCGACTGGAGGAAGCCATCTGTCAGCAGCCTGTAG 540
121   F  M  P  T  E STOP
    TAGATGATAGATCATAGAAACAAACACCAGTTTAGGCTGAGAAGAGCAGTGCACAATGCA 600

    ACAACCATCCTTTGCCTCTCATCAAATCAATATCTTTGCCACAACATGCAAAGTTAAAAG 660

    CAGGGCAAACAACACCTACGCAGTTTGTGCATGTACAGTATTTTGTGTTTGATACACTAA 720

    CCAAAGTCTCTGTGTGATTGTGTATGTATATGTGTGAGTGTGTTCAATAAAAAATGAACT 780

    GGAAACAAACAAAAAAAAAAAAAAAAAAAAAAAAAA                          817
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Fig. 1. Nucleotide and deduced amino acid sequences of *EcApo-14*. The signal peptide is underlined and the first amino acid of the mature peptide is numbered as +1. This sequence has been deposited in GenBank, and its accession number is DN552074.

the same reaction conditions as *EcApo-14*. The semi-quantitative RT-PCR was carried out as described previously (Wang et al., 2004). Briefly, 10 duplicate reactions were performed by alternate cycle numbers from 21 to 35 to ensure that the semi-quantitative RT-PCR products were in a linear range of accumulation. After the cycle number (30) was optimized, the expression analyses of *EcApo-14* were completed by semi-quantitative RT-PCR.

As another control, amplification was also performed on genome DNA of orange-spotted grouper. A negative control (C) lacking cDNA was performed.

2.3. Whole-mount in situ hybridization (WISH)

WISH was performed as previously described (Wilkinson, 1992) with minor modification. In brief, the embryos were fixed in 4% paraformaldehyde in PBS, dehydrated through a 25%, 50%, 75% methanol in PBS series and stored in 100% methanol at −20 °C. Rehydrated embryos were washed three times for 5 min in PBST and treated with proteinase K (10 μg/ml of final concentration) for 5 min at room temperature. After rinsing twice in PBST, embryos were refixed in 4% paraformaldehyde in PBS for 20 min at RT, washed twice with PBST and prehybridized for 5 min in hyb− (50% formamide, 5× SSC, 0.1% Tween-20) then 4 h in hyb+ (hyb−, 500 μg/ml yeast tRNA, 50 μg/ml heparin) at 58 °C. The fragment amplified by RT-PCR was cloned to the pGEM-T vector and linearized by *Eco*RI and *Xho*I, respectively. Antisense or sense digoxigenin-UTP labeled RNA probes were synthesized using T7 or Sp6 polymerase by in vitro transcription (DIG labeling kit; Roche Molecular Biochemicals). The probes were denatured at 70 °C for 10 min and added to fresh hyb+.

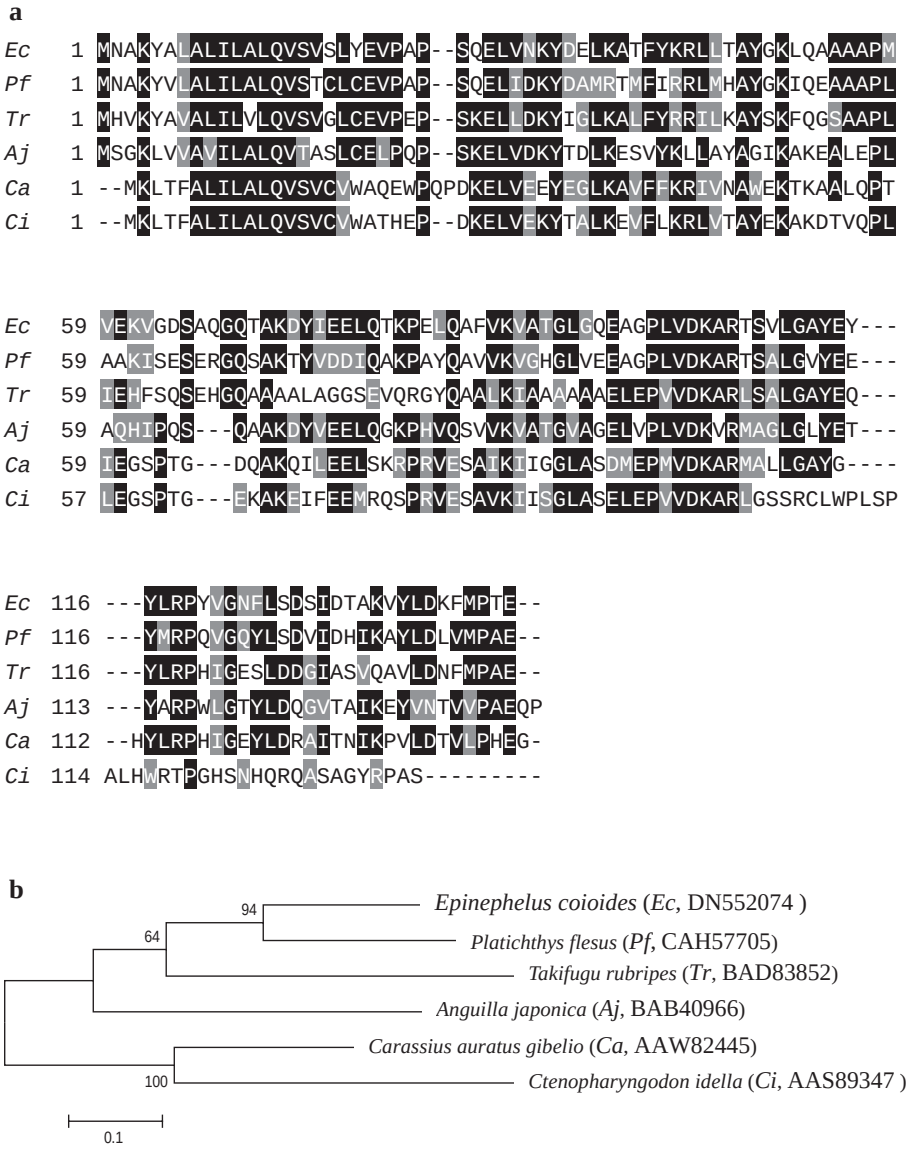

Fig. 2. Comparison of *EcApo-14* and other fish Apo-14. (a) Alignment of the amino acid sequences of *Apo-14* between orange-spotted grouper and other fishes. (b) Phylogenetic tree of *Apo-14* in fishes. Lengths of horizontal lines indicate the genetic distance. One hundred bootstrap repetitions were performed, and values are shown at the inner nodes.

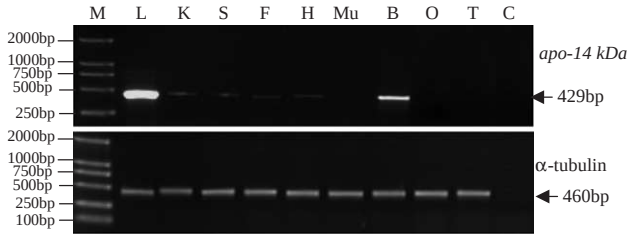

Fig. 3. RT-PCR detection of *EcApo-14* tissue distribution, such as liver (L), kidney (K), spleen (S), fat (F), heart (H), muscle (Mu), brain (B), ovary (O) and testis (T). α-Tubulin was used as control. A negative control (C) lacking cDNA did not generate any RT-PCR product. M is the 2 kb DNA ladder marker.

Hybridization was done at 58 °C overnight. Subsequent washes at 64 °C were as follow: twice for 30 min in 2× SSCT, 50% formamide, once for 15 min in 2× SSCT and twice for 30 min in 0.2× SSCT. These embryos were equilibrated for three times for 5 min in MABT, blocked for 1 h with buffer block (2% blocking reagent (Roche) and 10% fetal calf serum in MABT) at RT and then incubated at 4 °C overnight in anti-digoxigenin-alkaline phosphatase antibody (Roche Molecular Biochemicals) diluted to 1:5000 in fresh buffer block. Excess antibody was removed by washing five times for each 30 min in MABT at RT. Alkaline phosphatase activity was detected with NBT/BCIP (Sino–American Biotechnology Ins.). Color development was observed within 2 h.

3. Results

3.1. Full-length cDNA sequence and its characterization of EcApo-14

EcApo-14 cDNA is 817 bp long and has an open reading frame of 432 bp, starting with the first ATG codon at position 68 and ending with a stop TAA codon at position 499. A consensus polyadenylation signal AATAAA is located 25 bp upstream from the poly (A) tail. When a signal peptide of 18 amino acids is removed, the predicted mature *EcApo-14* consists of 123 amino acids, and starts with a Leu residue (Fig. 1). N-linked glycosylation site analysis by NetNGlyc 1.0 did not find any potential *N*-glycosylation site, but YinOYang 1.2 showed three potential *O*-glycosylation sites (Ser 16, Thr 107, and Thr 142). NetPhos 2.0 analysis revealed nine phosphorylation sites (Ser 18, Tyr 20, Thr 46, Ser 65, Thr 70, Thr 80, Ser 108, Tyr 116, and Thr 131).

Amino acid alignments and their identities of *Apo-14* were compared between the grouper and other fishes. As shown in Fig. 2a, *EcApo-14* has 62.9%, 51%, 46.9%, 43.2%, and 31.9% identities to the *Apo-14* of European flounder (*Platichthys flesus*), pufferfish (*Takifugu rubripes*), Japanese eel (*Anguilla japonica*), gibel carp (*Carassius auratus gibelio*) and grass carp (*Ctenopharyngodon idella*) respectively. Fig. 2b shows the phylogenetic tree of *Apo-14* among these fishes. Apparently, the phylogenetic tree is basically consistent with the known taxonomic relationships among these species. Three species that belong to percomorpha, such as *E. coioides*, *P. flesus* and *T. rubripes* are clustered together, and *A. japonica* is clustered together with the node of the three species of percomorphs. *C. auratus gibelio* and *C. idella*, belonging to the Cyprinidae, are clustered into another group.

3.2. Abundant expression of EcApo-14 in liver and brain

Tissue distribution of *EcApo-14* was analyzed by RT-PCR in adult orange-spotted grouper. As shown in Fig. 3, *EcApo-14* mRNA was detected abundantly in liver and brain, slightly in kidney, spleen, fat, heart, and no signals were detected in muscle, ovary and testis. The predominant expression of *EcApo-14* in liver and brain is basically similar to that in pufferfish, but the pufferfish *Apo-14* transcripts were observed only in liver and brain (Kondo et al., 2005). The subtle difference might be resulted from the detection methods, because the RT-PCR analysis used in this study is more sensitive than the Northern blot assay previously used in pufferfish.

3.3. Expression pattern of EcApo-14 during embryogenesis

According to the cDNA sequence, we employed RT-PCR and WISH to investigate spatial and temporal expression patterns of *EcApo-14* during embryogenesis. RT-PCR analysis revealed that this gene was first transcribed in neurula embryos and maintained a relatively stable expression level during the following embryogenesis (Fig. 4).

The expression pattern of *EcApo-14* determined by WISH revealed a very high level of transcripts in the yolk syncytial layer (YSL) during early embryonic development (Fig. 5a–c), which was similar to that of *ApoE* and *ApoA-I* in zebrafish embryogenesis. The YSL, an extraembryonic structure unique to teleosts (Kimmel et al., 1995), is formed during the blastula

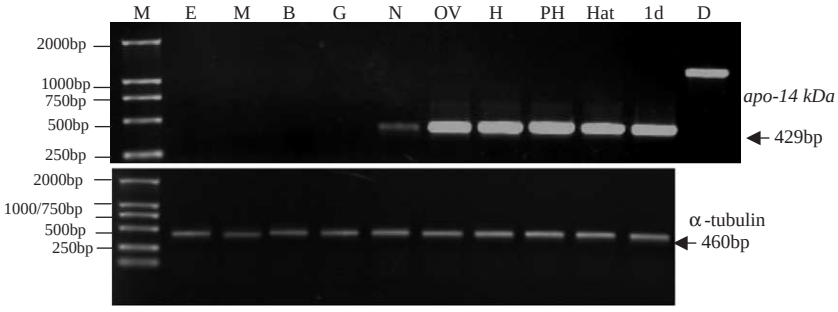

Fig. 4. RT-PCR detection of *EcApo-14* expression during embryogenesis. E: unfertilized egg, M: morula, B: blastula, G: gastrula, N: neurula, OV: optic vesicle stage, H: heartbeating stage, PH: prior to hatching, Hat: hatching, 1d: 1-day-old fry, D: genomic DNA control.

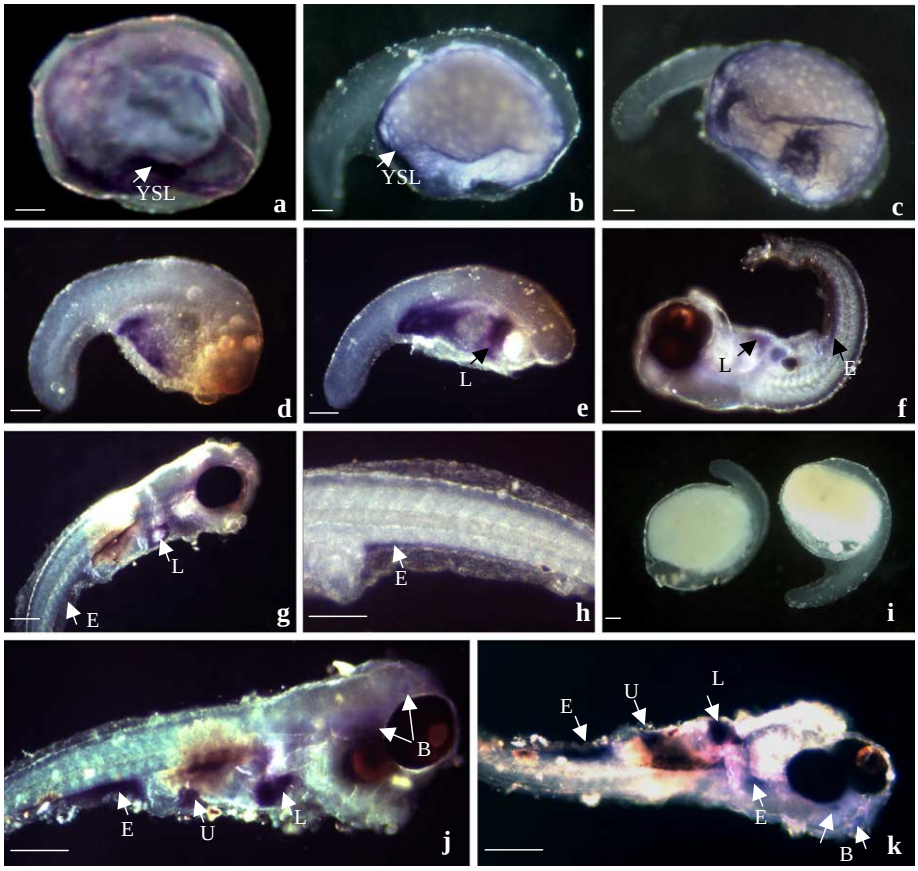

Fig. 5. Expression pattern of *EcApo-14* revealed by WISH during embryogenesis. (a) Heartbeating stage, (b) at 18 hpf embryo, (c) at 20 hbf embryo, (d) at 24 hpf embryo, (e) 2-day-old fry, (f) 3-day-old fry, (g) 4-day-old fry, (h) higher magnification of tail, (i) negative control (at 18 hpf embryos), (j) 7-day-old fry in lateral view, (k) 7-day-old fry in ventrolateral view. a–e, under 100× microscope, i, under 50 microscope, h, j and k, under 200× microscope. Scale bars are 75 μm. YSL—yolk syncytial layer; L—liver; U—urogenital opening; E—endoderm; B—brain.

stage and is responsible for yolk degradation and transfer to the embryo and early larva (Babin et al., 1997). It is most likely that *ApoA-I* and *ApoE* gene expression in the YSL is associated with lipoprotein synthesis and secretion (Babin et al., 1997). As shown in Fig. 5, the grouper embryos begin to hatch at 18 h after fertilization, and a very high level expression of *EcApo-14* is observed in YSL throughout the hatching stage (Fig. 5b, c). After hatching, the *EcApo-14* transcripts begin to restrict to a limited area in YSL (Fig. 5d). By the second day after hatching, the *EcApo-14* transcripts are obviously divided into anterior and posterior portions in YSL (Fig. 5e). After 3 days of hatching, the anterior *EcApo-14* transcripts are predominantly located in liver (Fig. 5f, g), and the posterior transcripts are concentrated to urogenital opening and endoderm region between trunk and abdomen (Fig. 5f–h, j, k). By the 7th day after hatching, the *EcApo-14* transcripts are predominantly expressed in liver, urogenital opening and the endoderm region (Fig. 5j, k). In addition, *EcApo-14* transcripts are also observed in brain, which is similar to *ApoE* expression in zebrafish (Babin et al., 1997).

4. Discussion

Apo-14 is a novel apolipoprotein specific to fish (Kondo et al., 2005). In the current study, *EcApo-14* has been screened from hypothalamic cDNA library of male orange-spotted grouper, and its expression patterns have been elucidated in adult tissues and during embryogenesis by RT-PCR and WISH analysis. It is the first time to reveal Apo-14 expression patterns. *EcApo-14* gene is first transcribed in neurula embryos and maintains a relatively stable expression level during the following embryogenesis. Its transcripts are at a very high level during embryonic and early larval development in the yolk syncytial layer (YSL). After 3 days of hatching, its transcripts are reduced in YSL, and concentrated on liver. In adult tissues, *EcApo-14* is predominantly expressed in liver and brain. The data suggested that EcApo-14 might play an important role in liver and brain morphogenesis and growth.

Liver morphogenesis in fish has been recently investigated by introducing a novel zebrafish transgenic line, the gutGFP line, which expresses GFP throughout the liver development (Field et al., 2003), and several gene expression patterns, such as *prox1*(Glasgow and Tomarev, 1998), *hnf4* (Kudoh et al., 2001), apan-endodermal markers (*foxA1*, *foxA2*, and *foxA3*) (Odenthal and NÜsslein-Volhard, 1998), liver-specific multi-copper oxidase gene (*ceruloplasmin*, *cp*) (Korzh et al., 2001), *selenoprotein Pb* (*sePb*) (Kryukov and Gladyshev, 2000; Kudoh et al., 2001), *sox17* (Alexander and Stainier, 1999), have been analyzed in the observed system. In this study, we find that *EcApo-14* transcripts are at a very high level during

embryonic and early larval development in the yolk syncytial layer (YSL), and are concentrated on liver after 3 days of hatching. The data suggested that EcApo-14 might play an essential role not only in the transport of yolk nutrients to the developing embryo, but also in liver morphogenesis and growth. On the other hand, *Ecapo-14* will be able to use as a molecular marker to reveal liver morphogenesis in teleost fish.

Predominant expression of *Ecapo-14* in liver and brain also implicates high lipid metabolic activity and synthetic activity in fish liver and brain. An important role of high-density lipoproteins (HDL) in the transport of excess cholesterol from peripheral tissues to liver has been well known in mammals (Jackson et al., 1976; Tailleux et al., 2002). However, previous reports also revealed unique lipid metabolism in fish. For example, Ando and Mori (1993) observed that pufferfish HDL transported lipids from liver to peripheral tissues as mammalian very low-density lipoproteins (VLDL) did. De Smet et al. (1998) reported that fish HDL transported free fatty acids instead of albumin in mammalian plasma. It awaits further investigation whether Apo-14 has functions different from other apolipoproteins or not.

Unlike other apolipoproteins which are expressed primarily in the intestine and liver, *EcApo-14* is similar to *ApoE*, which has the second highest level of expression abundance in brain (Mahley, 1988). To our knowledge, there exists high energy expenditure in brain (Erecinska and Silver, 1989). As suggested for ApoE (Babin et al., 1997), Apo-14 might also play a certain role in neuronal growth and repair.

Acknowledgements

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