

Observation of the middle intestinal tight junction structure, cloning and studying tissue distribution of the four Claudin genes of the grass carp (*Ctenopharyngodon idellus*)

Fan Xu · Yuan-Tu Ye · Chun-Fang Cai · Ping Wu · Lin Song · Meng Liu ·
Lin-Jie Yao · Jiao-Jiao Dong · Yu-Wei Huang · Zhi Gong · Jie Qin ·
Liang Song

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Abstract To confirm the existence of the tight junction (TJ) in middle intestine and obtain the genetic information of Claudin-3, Claudin-15a, Claudinb and Claudinc of grass carp, we observed the physical structure of TJ by transmission electron microscopy and cloned the partial cDNAs of the four Claudins using reverse transcriptase PCR technique. The four partial cDNAs consist of 1,261, 490, 776 and 662 bp encoded 131, 150, 195 and 171 amino acids, respectively. Homology analysis showed that the grass carp Claudin shared high homology with other teleost species, especially with *Danio rerio* and *Carassius auratus*. Multi-alignments of the four Claudin amino acid sequences have seen the two conserved cysteines existing in the first extracellular loop of Claudin-15a, Claudinb and Claudinc, and the sequence diversity of the four Claudins mainly lies within the C-terminal tails, which usually end with the -Y-V motif, except the -F-V motif in Claudinb. Tissue distributions of the four Claudins were measured by applying quantitative real-time PCR technique. Results showed that Claudin-3 was mainly expressed in liver and middle intestine and Claudinb was ubiquitously expressed with a higher expression in middle intestine while Claudin-15a and

Claudinc were mainly expressed in middle intestine. Our study revealed the existence of the TJ in the middle intestinal and obtained the genetic information of Claudin-3, Claudin-15a, Claudinb and Claudinc of grass carp, aiming to found the molecular biology basis for the further study of the intestinal barrier function of grass carp.

Keywords *Ctenopharyngodon idellus* · Intestine · TJs · Claudin-3 · Claudin-15a · Claudinb · Claudinc

Introduction

Feed oxidative lipids have had great side effects on the health and growth of farmed fish including reduced feed intake, low survival, impaired growth, depletion of tissue vitamins E storage, hemolysis, liver degeneration and skeletal deformation (Hamre et al. 2001; Lewis-McCrea and Lall 2007). In this regard, side effect of the feed oxidative lipids on the health and growth of farmed fish is a serious problem in need to be researched. As feed digestion and absorption firstly occur in fish intestine, the feed oil oxides and their main components would firstly damage the epithelial mucosa in intestine, which consists of a dense mucous layer containing secretory IgA and antimicrobial peptides as well as dynamic junctional complexes that regulate permeability between cells (Ohland and Macnaughton 2010). If

F. Xu · Y.-T. Ye (✉) · C.-F. Cai · P. Wu ·
L. Song · M. Liu · L.-J. Yao · J.-J. Dong ·
Y.-W. Huang · Z. Gong · J. Qin · L. Song
Basic Medical and Biological Science College, Soochow
University, 199 Renai Road, Suzhou 215123, China
e-mail: yeyt@suda.edu.cn

the structure and function of the intestinal epithelial mucosa barrier are normally maintained, damage from feed oxidative oils is only inner-intestinal damage; otherwise, bacteria and anatoxin would be transferred into the systemic circulation through the damaged barrier, which may have further effects on the remote substantive organ, liver for example. Thus, the epithelial mucosa barrier is vital to fish intestine.

As tight junction (TJ) is the most important structure of the intestinal epithelial mucosa barrier, it has been attracting great attention from researchers for years. The TJ complex is composed of transmembrane and cytosolic TJ proteins (Yuriko et al. 2003), the latter of which provides structural support to the complex by linking transmembrane TJ proteins to the actin cytoskeleton of an epithelial cell (Mariscal et al. 2003). However, it is the transmembrane TJ proteins, such as occludin and members of the Claudin superfamily, who bridge the intercellular space between epithelial cells and largely influence TJ complex permeability (Mariscal et al. 2003; Van Itallie and Anderson 2006). Research showed that mammals express about 24 Claudins while *Fugu rubripes* express about 56 Claudins (Loh et al. 2004). Their tissue-specific combination results in tissue-specific barrier characteristics (Shen et al. 2008), which is vital to the TJ barrier function. So far, existence of the TJ in the middle intestine and genetic information of Claudins genes of the grass carp are still unknown, and hence, our experiments have focused on the observation of the TJ structure in the middle intestine of grass carp and cloning and tissue distribution analysis of the Claudins genes in order to (1) confirm the existence of the TJ structure in the middle intestine of grass carp; (2) obtain genetic information of the four Claudins (Claudin-3, Claudin-15a, Claudinb and Claudinc) chosen from our laboratory's intestinal transcriptome sequencing results of grass carp; and (3) investigate their tissue distribution in liver, spleen and middle intestine of grass carp. The work was done in order to found the molecular biology basis for the further study of the intestinal barrier function.

Materials and methods

Sample source

Healthy grass carp were purchased from a local fish pond (Changzhou, Jiangsu Province) and acclimatized

Table 1 Primers for cloning

Primers	Sequence (5'–3')
Claudin-3-F	CTGTCTGCCACCACCAATAG
Claudin-3-R	CGGTCATCGCCATCATCTT
Claudinb-F	GGTGTCTGCTGGGCTTCA
Claudinb-R	TTGTCCTTGTGTCTTTGTGC
Claudin 15a-F	GGATGATTTTGGAGCCTGTG
Claudin 15a-R	GGGTAGAGCGGGTTGAAGA
Claudin c-F	GAGTGACAATGGACACCGAA
Claudin c-R	GGAGGGAATCTGGATGAGC

in indoor tank (Soochow University, Jiangsu Province) with filtered water (25.0 ± 3.0 °C). The body weight of the fish ranged from 20 to 40 g. Three individual middle intestines (the middle part of the middle intestine, a length of 1 cm) were sampled from randomly selected grass carps, and pieces (about 6 mm × 6 mm) were fixed overnight in 2.5 % glutaraldehyde for the transmission electron microscopy (TEM) observation. Tissues (spleen, liver, middle intestine) and middle intestinal mucosa were immediately frozen in liquid N₂ and stored at –80 °C until RNA extraction.

The first-strand cDNA synthesis

Total RNA was extracted from frozen tissues using RNAiso Plus (TaKaRa, Japan) according to the manufacturer's instructions. The isolated RNA samples were dissolved in DEPC water and then analyzed for its integrity on agarose gel. The concentration and the purity were determined by UV spectroscopy at 260 and 280 nm. The first-strand cDNA was synthesized from total RNA (1 µg) in a total volume of 10 µL using M-MLV reverse transcriptase (Takara Bio., China).

Molecular cloning of TJ genes cDNA

According to the intestinal transcriptome sequencing results of the grass carp in our laboratory, primers (Table 1) were designed with Primer Premier 5.0 software (Premier, Canada) to enable cloning the four Claudin genes including Claudin-3, Claudin-15a, Claudin b and Claudin c. All the amplified fragments of the expected sizes were purified with Tiangen gel

extraction kit (Tiangen, China) and ligated into pMD19-T vector (Takara Bio., China), then transformed into *Escherichia coli* DH5 α for amplification. The sequences were performed by Genewiz Biotech Ltd. (Suzhou, China).

Sequence analysis

The deduced amino acid sequences, multiple sequence alignments and transmembrane domain prediction were performed with DNAMAN software (<http://www.shinegene.org.cn/q2.html>). Homology analysis of the amino acid sequences was performed with the online BLASTP software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic trees were constructed using the bootstrap neighbor-joining method of MEGA 5.0. The bootstrap values were replicated 1,000 times to obtain the confidence value for the analysis.

Q-RT-PCR detection

Q-RT-PCR was applied to determine tissue distribution in spleen, liver and middle intestine. It was conducted on a ABI 7,300 Real-Time PCR system (ABI, USA) using SYBR[®] Premix Ex TaqTMII (Perfect Real Time) kit (TaKaRa Code: DRR081A). Each sample was triplicated with sterile ddH₂O as template in the blank control. Amplifications were performed in a finally 20 μ L solution, which contained 2 μ L cDNA, 10 μ L SYBR Premix Ex TaqTM (Takara Bio., China), 1 μ L of each primer (4 μ M) and 6 μ L sterile ddH₂O. PCR conditions were as follows: an initial denaturing at 95 $^{\circ}$ C for 1 min, followed by 45 cycles of 15 s at 95 $^{\circ}$ C, 31 s at 60 $^{\circ}$ C (Primers in the Table 2); then, a dissociation step at 95 $^{\circ}$ C for 15 s was performed to determine the target specificity. The comparative threshold (CT) cycle method was used to calculate the relative concentrations. This method involves obtaining CT values for the target genes, normalizing to β -action (Δ Ct) and comparing the relative expression level of the target gene in different tissues according to Δ Ct values. Data from all experiments were expressed as the mean \pm SD and analyzed by one-way ANOVA method to determine significant differences between samples using SPSS 17.0. The differences between values were considered statistically significant when $P < 0.05$.

Table 2 Primers for Q-RT-PCR

Primers	Sequence (5'–3')
Claudin-15a-F	ACAGATTCTCGTACAGGGTTGA
Claudin-15a-R	CGGTTGTTGAAGTCGTTGC
Claudin-3-F	TCGGTGGATTGTACTTCTTCTC
Claudin-3-R	CCAAATCACTCGGGACTTCTA
Claudinc-F	GTGGTGGGCTGAGTAGGCT
Claudinc-R	GCTGCTGCGGCTCTGTT
Claudinb-F	ACAGACTTCGTGGGAGGGA
Claudinb-R	CGATGGCAATGATGGTGAGA
β -Actin-F	CGTGACATCAAGGAGAAG
β -Actin-R	GAGTTGAAGGTGGTCTCAT

Results

Transmission electron microscopy (TEM) observation

Tight junction structure in middle intestine of grass carp was observed by TEM. The complete intestinal villi and IECs layer were shown in Fig. 1a, b. The black connecting lines of high electron density were shown at the sides of adjacent IECs. When under higher magnification (Fig. 1c), black spots along the connecting lines can be clearly seen.

Molecular cloning and characterization of four Claudin cDNA

From our established intestinal transcriptome laboratory of the grass carp, we obtained four partial cDNA sequences of Claudin using RT-PCR strategy. The nucleotide and deduced amino acid sequences of the grass carp Claudin-3, Claudin-15a, Claudinb and Claudinc cDNA were shown in Fig. 2, respectively.

The Claudin-3 cDNA was 1,261 bp (GenBank Accession No. KF193858), which encoded 131 amino acids from 1 to 393; after termination codon TAA, a sequence of 865 bp was included in the 3' untranslated region without AATAAA tailing signal, indicating that the Claudin-3 cDNA we have obtained was partial. After domain analysis, three transmembrane domains, one intracellular loop, one extracellular loop and one C-terminal tail were completely identified. According to the four transmembrane domains' position in the Claudin amino acid sequence, from 4

to 22 (19aa) was the second transmembrane domain, from 39 to 65 (27aa) was the third and from 80 to 108 (29aa) was the fourth while the intracellular loop was from 23 to 38(16aa), the extracellular loop was from 66 to 79 (14aa) and the C-terminal tail was from 109 to 131(23aa) (Fig. 2a), which ends with a -Y-V motif, a known PDZ binding motif interacting with the cytoplasmic and scaffolding proteins such as ZO-1, ZO-2 and ZO-3.

The Claudin-15a cDNA was 490 bp (GenBank Accession No. KF193857), which encoded 150 amino acids from 36 to 489; there was no termination codon TAA, indicating that the Claudin-15a cDNA we have obtained was partial. After domain analysis, three transmembrane domains, one extracellular loop and one intracellular loop were completely identified. According to the four transmembrane domains' position in the Claudin amino acid sequence, from 1 to 24 (24aa) was the first transmembrane domain, from 72 to 100 (29aa) was the second and from 113 to 140 (28aa) was the third while the extracellular loop was from 25 to 71 (46aa) and the intracellular loop was from 101 to 112 (12aa) (Fig. 2b).

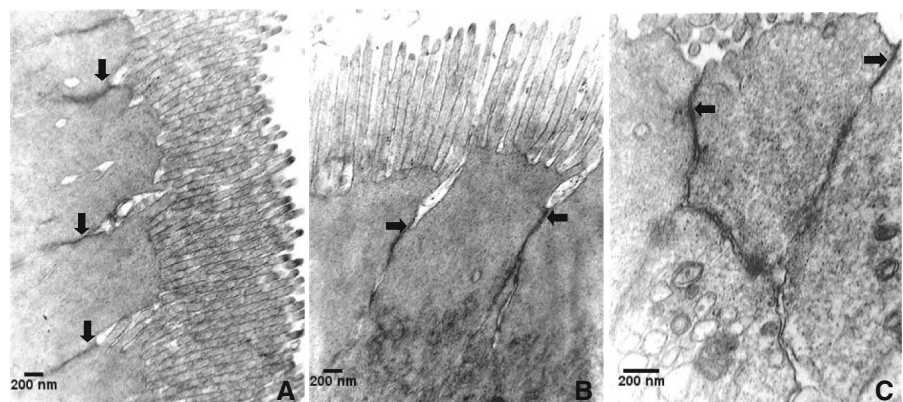
The Claudinb cDNA was 776 bp (GenBank Accession No. KF193860), which encoded 195 amino acids from 1 to 585; after termination codon TAA, a sequence of 188 bp was included in the 3' untranslated region without AATAAA tailing signal, indicating that the Claudinb cDNA we have obtained was partial. After domain analysis, four transmembrane domains, two extracellular loops, one intracellular loop and one C-terminal tail were completely identified. According to the four transmembrane domains' position in the Claudin amino acid sequence, from 4 to 21(18aa) was

Fig. 2 Partial cDNA sequence and the deduced amino acid sequence of grass carp Claudin-3 (a), Claudin-15a (b), Claudinb (c) and Claudinc (d). Sequences indicating the transmembrane domains are *straightly underlined*. The -Y-V and -F-V motifs are *boxed*

the first transmembrane domain, from 55 to 83(29aa) was the second, from 104 to 131 (28aa) was the third and from 141 to 164 (24aa) was the fourth while the first extracellular loop was from 22 to 54(33aa), the second extracellular loop was from 132 to 140(9aa), the intracellular loop was from 84 to 103 (20aa) and the C-terminal tail was from 165 to 195(31aa) (Fig. 2c), which ends with a -F-V motif, not the normally known -Y-V motif.

The Claudinc cDNA was 662 bp (GenBank Accession No. KF193859), which encoded 171 amino acids from 2 to 519; after termination codon TAA, a sequence of 140 bp was included in the 3' untranslated region without AATAAA tailing signal, indicating that the Claudinc cDNA we have obtained was partial. After domain analysis, three transmembrane domains, one intracellular loop, one extracellular loop and one C-terminal tail were completely identified. According to the four transmembrane domains' position in the Claudin amino acid sequence, from 28 to 56 (29aa) was the second transmembrane domain, from 69 to 95 (27aa) was the third and from 113 to 136 (24aa) was the fourth, while the intracellular loop was from 57 to 68(12aa), the extracellular loop was from 96 to 112 (17aa) and the C-terminal tail was from 137 to 171(35aa) (Fig. 2d), which ends with a -Y-V motif, a known PDZ binding motif interacting with the cytoplasmic and scaffolding proteins such as ZO-1, ZO-2 and ZO-3.

Fig. 1 TEM micrographs of the junctional structures in the middle intestine of grass carp seen in **a**, **b** (TEM \times 20,000) and **c** (TEM \times 40,000). TJs are marked with *arrows*



Homology analysis and phylogenetic analysis

Homology analysis of the amino acid sequences was performed with the online BLASTP software. The amino acid sequence of the grass carp Claudin-3 showed 88, 69, 69, 64 and 67 % identity with *Danio rerio* Claudin-3, *Takifugu rubripes* Claudin-3, *Oryzias latipes* Claudin-3, *Salmo salar* Claudin-3 and *Epinephelus coioides* Claudin-3, respectively. The amino acid sequence of the grass carp Claudin-15a showed 92, 78 and 79 % identity with *D. rerio* Claudin-15a, *T. rubripes* Claudin-15a and *S. salar* Claudin-15a, respectively. The amino acid sequence of the grass carp Claudinb showed 84 and 89 % identity with *D. rerio* Claudinb and *Carassius auratus* Claudinb, respectively. The amino acid sequence of the grass carp Claudinc showed 95 and 91 % identity with *D. rerio* Claudinc and *C. auratus* Claudinc, respectively. Comparison of the Claudin amino acid sequences revealed that the grass carp Claudin shared high homology with other teleost species, especially with the *D. rerio* and *C. auratus*.

Multi-alignments between grass carp and other teleost species of the four Claudin amino acid sequences were performed with DNAMAN software. It is easy to find that there were always two conserved cysteines in the first extracellular loop of Claudin proteins such as Claudin-15a (Fig. 3b), Claudinb (Fig. 3c) and Claudinc (Fig. 3d). The C-terminal tails showed relatively high-sequence diversity compared with other domains, especially that of Claudin-3 (Fig. 3a). C-terminal tails of both Claudin-3 and Claudinc among different teleost species end with the -Y-V motifs while that of Claudinb was the -F-V motifs (Fig. 3).

Phylogenetic analysis of Claudin-3 amino acid sequences indicated that there were two major classes of Claudin-3 in vertebrates, teleost species Claudin-3 and tetrapod Claudin-3. The grass carp Claudin-3 together with the Claudin-3 from *D. rerio* was clustered in the same branch, showing the highest similarity. To Claudin-15a amino acid sequences, the two major classes were teleost species Claudin-15a and mammalian Claudin-15a. The grass carp Claudin-15a shared the high similarity with all other teleost species, also highest with the *D. rerio* Claudin-15a (Fig. 4).

Tissue distribution

Tissue distribution of the four Claudin of the grass carp was analyzed by Q-RT-PCR (Fig. 5). According

Fig. 3 Multi-alignments of the Claudin-3 (a), Claudin-15a (b), Claudinb (c) and Claudinc (d) amino acid sequences between grass carp with other teleost species. Sequences indicating the first extracellular loops are straightly underlined. The two conserved cysteines in the first extracellular loops are boxed. The C-terminal tails are marked with wavy lines. The -Y-V and -F-V motifs are marked with shadows

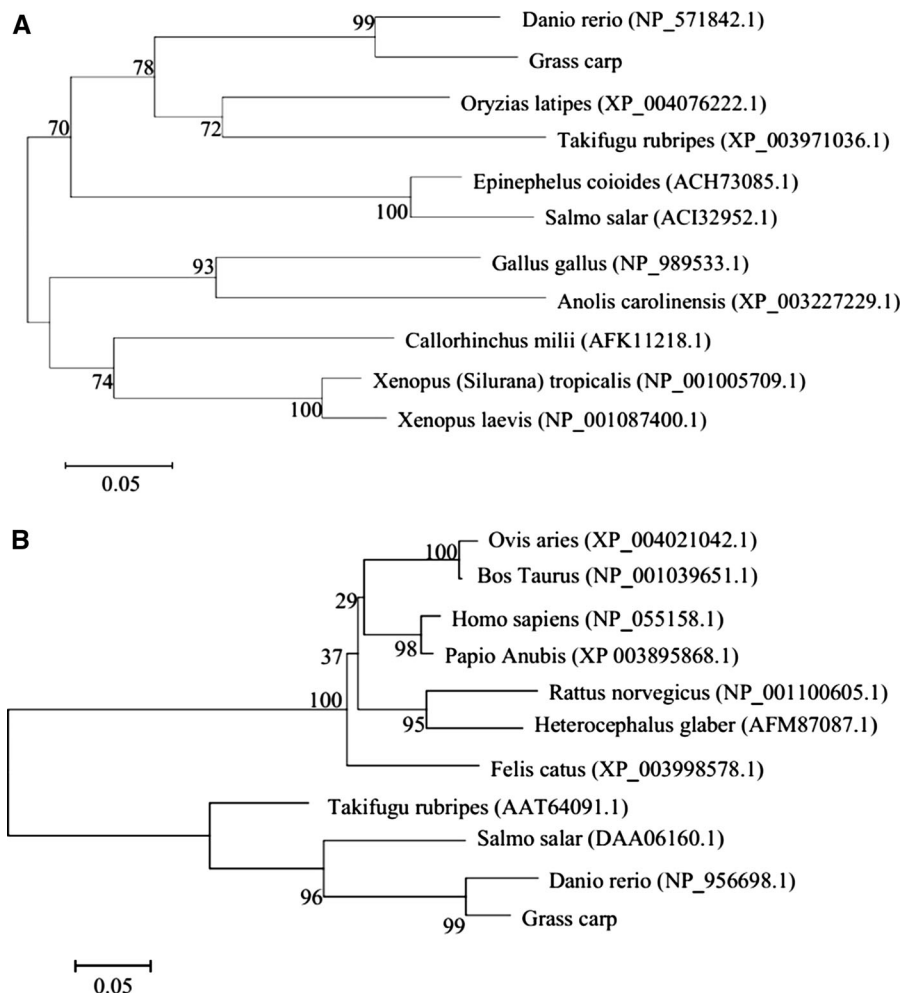
to relative quantification analyses, Claudin mRNA expressions were detected in liver, spleen and middle intestine. Claudin-3 (Fig. 5a) was abundantly expressed in liver, lowly expressed in middle intestine and slightly expressed in spleen. Claudinb (Fig. 5c) was highly expressed in middle intestine, lowly expressed in liver and spleen. Claudin-15a (Fig. 5b) and Claudinc (Fig. 5d) were both abundantly expressed in the middle intestine, but slightly expressed in liver and spleen.

Discussions

In TEM micrographs of the middle intestine of grass carp (see Fig. 1), complete intestinal villi, IECs layer and the black connecting lines of high electron density at the sides of adjacent IECs were observed. TJs observed by TEM usually appear at sites where the intercellular space between neighboring cells (Farquhar and Palade 1963). It is always obliterated and the adjoining membranes appear to fuse. In the TEM micrographs of the European sea bass, the junction structures between enterocytes in the posterior intestinal region appeared as a set of black particle strands, which begin from the base of villi, through the side of the IECs, to the cell basement (Silvia et al. 2013). The top area near the villi is the real TJ. These descriptions are very similar to what was showed in our micrographs; thus, we confirmed the existence of TJs in the middle intestine of grass carp, and the top area of the black particle strands between the IECs is the right TJ. Later study also revealed the existence of middle intestinal Claudin proteins, one member of the TJ proteins. After totally considered from the two aspects of structure and functional proteins, we confirmed the existence of TJ in the middle intestine of grass carp.

As known to all, Claudin proteins are characterized by several characteristic domains, including four transmembrane domains, one intracellular loop, two extracellular loops, two cytoplasmic tails (C-terminal, N-terminal) and two conserved cysteines in the first

Fig. 4 Phylogenetic trees of Claudin-3 (a) and Claudin-15a (b) amino acid sequences based on neighbor-joining (NJ) method. The bootstrap confidence values shown at the nodes of the tree are based on a 1,000 bootstrap procedure, and the scale for the branch length is shown below the tree

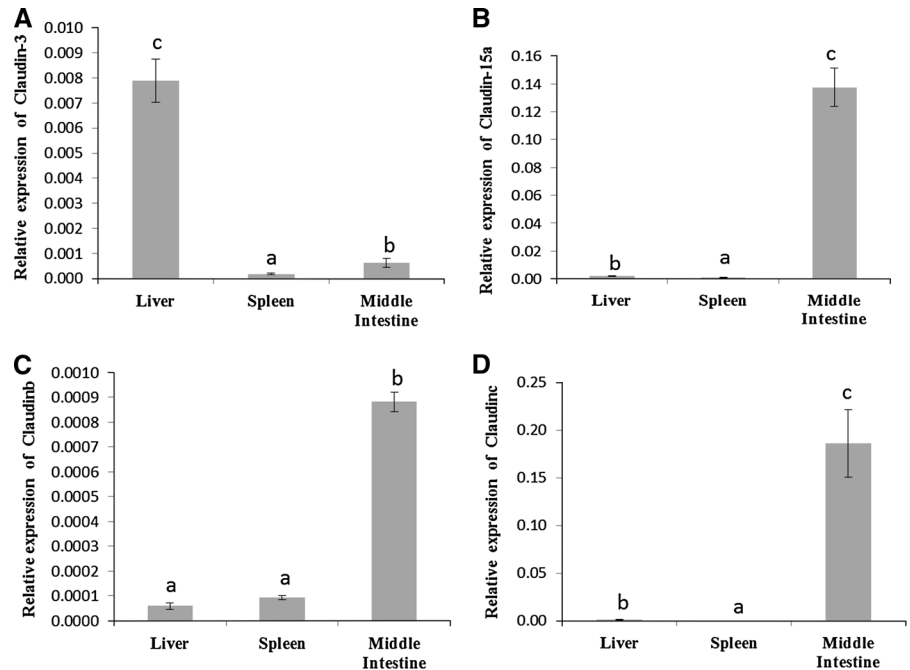


extracellular (Lapierre, 2000). Our study revealed the existence of the two conserved cysteines in the first extracellular part of all the four Claudins among the teleost species, which, in some extent, also revealed the accuracy of our obtained Claudin sequences in turn. The sequence diversity of the Claudins was reported to lie within the third and fourth transmembrane domains and within the C-terminal tail (Morita et al. 1999a, b, c), which was consistent with our results. From our multi-alignment results of the C-terminal tails of Claudin-3, Claudinb and Claudinc, we found that the C-terminal tails showed relatively high-sequence diversity compared with other domains, especially in Claudin-3, almost reaching a diversity of above 50 % among the teleost species. The -Y-V motif is a known PDZ binding motif and indicated to be interacting with the cytoplasmic and scaffolding proteins such as ZO-1, ZO-2 and ZO-3

(Itoh et al. 1999). Usually, among the classic Claudins (Claudin-2, -8, -10, -14, -17, -20 and so on), the -Y-V motif in the C-terminal positions -1 and 0 exhibits 100 % conservation, in contrast to a greater variety in nonclassic Claudins (H/S/Y/D/E/R-V/L) (Krause et al. 2008). In our study, all Claudin proteins showed the conserved -Y-V motif in the C-terminal, except that of Claudinb. It was a -F-V motif, which maybe a new kind motif found in the C-terminal positions of grass carp.

Homology analysis revealed that the grass carp Claudin-3, Claudin-15a, Claudinb and Claudinc are highly homologous with other teleost species, especially with *D. rerio* and *C. auratus*. Phylogenetic analysis showed that the similarity between each species of the Claudin-3 and Claudin-15a amino acid sequences was in conformity with the traditionally morphological and biochemical characteristics of

Fig. 5 Relative mRNA expression of Claudin-3 (a), Claudin-15a (b), Claudinb (c) and Claudinc (d) gene in spleen, liver and middle intestine of the grass carp. Data (mean \pm SE) with different letters among different tissues are significantly different ($P < 0.05$)



species classification status. To be specific, grass carp has a close relationship with the *cyprinidae* fish such as crucian carp and zebra fish, a relatively far relationship with the *salmonidae*, *sparidae* and *cyprinodontidae*, and the furthest relationship with mammals such as *Bos taurus*, *Sus scrofa*, *Mus musculus* and *Homo sapiens*, which conforms to the species molecular evolution.

To mention the tissue distribution, some Claudins are expressed only in specific tissues while some are widely expressed in different tissues (Morita et al. 1999a, b, c). For example, in the heart, liver, gut, kidney, gill, eye, brain and muscle of zebrafish, Claudinc was mainly expressed in gut; Claudind and ClaudinI were mainly expressed in gill, while, some isoforms such as Claudin-2, -7 and -12, appeared to be expressed ubiquitously (Kumai et al. 2011). In our study, tissue distribution of Claudin-3, Claudin-15a, Claudinb and Claudinc of grass carp was analyzed using the Q-RT-PCR technique. Results showed that Claudin-3 was mainly expressed in liver, Claudin-15a and Claudinc were both mainly expressed in the middle intestine and Claudinb was highly expressed in middle intestine and lowly expressed in liver and spleen. Our research was consistent with the results in rat, Atlantic salmon and zebra fish; Claudin-3 was

expressed in liver, pancreas and intestine of rat, among which liver showed the highest expression (Christoph et al. 2001). Claudin-15 was abundantly expressed in intestine of Atlantic salmon (Tipsmark et al. 2010). Claudinc was mainly expressed in gut of zebrafish; Claudinb had no significantly tissue specificity with ubiquitously expressed in heart, liver, gut, kidney, gill, eye and muscle (Kumai et al. 2011). Focusing on the middle intestine of grass carp, we can find that three among the total four Claudins were abundantly expressed. This phenomenon may be closely related to the intestine demand for the TJ barrier function, because the barrier properties of some particular tissues were closely related to the types and amount of corresponding TJ protein compounds (Förster 2008). As middle intestine is the main place where fish digestion occurs with quite a large amount of material exchange and absorption, which strongly demands for the epithelial barrier function as well, the Claudin compounds there should be certainly varied. Thus, we speculated that these demands resulted in the high expression of Claudin15a, Claudinb and Claudinc in the middle intestine. Future work that focuses on the vital function of the Claudins in different tissues of the grass carp, especially in the intestine, is still needed.

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