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Identification and Characterization of Immunoglobulin T Heavy Chain in Large Yellow Croaker (*Larimichthys crocea*)

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Abstract: Three immunoglobulin (Ig) isotypes have been identified in teleosts, IgM, IgD, and IgT or IgZ. IgT, a new teleost Ig isotype, plays a vital role in mucosal immunity. However, information on molecular and functional characteristics of fish IgT is still limited. In this study, an IgT heavy chain (*Lc*IgT) gene was cloned and characterized in large yellow croaker (*Larimichthys crocea*). Complete cDNA of *Lc*IgT was 1930 bp in length, encoding a protein of 554 amino acids. The deduced *Lc*IgT contains a V_H region and only three C_H regions (C_H1, C_H2, C_H4), but no transmembrane region was predicted. Phylogenetic analysis showed that IgT heavy chain sequences from all fish species are grouped together. Homology comparison showed that *Lc*IgT shares the highest amino acid identity of 58.73% with IgT heavy chain in *Scophthalmus maximus*. The V_H domain of *Lc*IgT has the highest identity of 72.50% with that of *Scophthalmus maximus* IgT. Relatively, each constant domain of *Lc*IgT exhibits the highest amino acid identity with that of IgT in *Oreochromis niloticus* (67.61% identity for C_H1, 61.11% identity for C_H2, and 63.74% identity for C_H4). *Lc*IgT was constitutively expressed in various tissues tested, with the highest levels in mucosa-associated tissues such as gills and skin. After *Cryptocaryon irritans* infection, the mRNA levels of *Lc*IgT were significantly up-regulated in the spleen (3.27-fold) at 4 d, in the head kidney (3.98-fold) and skin (2.11-fold) at 7 d, and in gills (4.45-fold) at 14 d. The protein levels in these detected tissues were all significantly up-regulated; the peak of its up-regulation was 6.33-fold at 28d in gills, 3.44-fold at 7d in skin, and 3.72-fold at 14d in spleen. These results showed that IgT response could be simultaneously induced in both systemic and mucosal tissues after parasitic infection and that IgT may be involved in systemic immunity and mucosal immunity against parasitic infection.

Keywords: immunoglobulin T; large yellow croaker (*Larimichthys crocea*); expression modulation; parasitic infection

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1. Introduction

Immunoglobulins (Igs) consist of two light (L) chains and two heavy (H) chains [1], which have a critical role in the vertebrate adaptive immune system as an important class of immune effector molecules. Five Igs isotypes have been identified in mammals: IgM, IgG, IgA, IgD, and IgE, based on the difference of heavy chain [2]. In teleosts, only three Igs isotypes, IgM, IgD, and IgT or IgZ are discovered as yet [3–7]. IgM is the most predominant and stable Ig in systemic immunity, while IgT or IgZ is considered an Ig specialized in mucosal immunity in teleosts [8]. IgD has an unknown function in teleosts, but studies have suggested that IgD might be involved in surveillance and immune regulation as an antigen-binding receptor [9].

IgT or IgZ has been identified in several teleost fish, including rainbow trout (*Oncorhynchus mykiss*) [6], zebrafish (*Danio rerio*) [10], fugu (*Takifugu rubripes*) [11], common carp (*Cyprinus carpio*) [12], Japanese flounder (*Paralichthys olivaceus*) [13], turbot (*Scophthalmus maximus*) [14], European seabass (*Dicentrarchus labrax* L.) [15], three-spined stickleback (*Gasterosteus aculeatus*) [15], Pacific bluefin tuna (*Thunnus orientalis*) [16], and Atlantic salmon (*Salmo salar*) [17]. Interestingly, some teleost species possess multiple IgT subclasses, such as rainbow trout with three subclasses, termed IgT1, IgT2, and IgT3 [18], zebrafish with two functional molecules, IgZ and IgZ-2 [7], common carp with IgZ1 and IgZ2 [12], and Atlantic salmon with IGH-A and IGH-B [17]. Nevertheless, no IgT ortholog has been found in channel catfish (*Ictalurus punctatus*) [19] and medaka (*Oryzias latipes*) [20] until now.

Two forms of IgT, membrane-bound IgT (mIgT) and secreted IgT (sIgT), exist in teleosts. The heavy chain of both IgT forms contains a variable region (V_H) and 2–4 constant regions (C_H), while the mIgT possesses additional single- or double-spinning transmembrane regions (TMs). The C_H number of IgT varies among different species; for example, four C_H domains are found in most teleosts, whereas only two exist in fugu [11] and three in stickleback [21] and emerald rock cod [22]. IgH gene locus in teleosts has multiple variables (V), diversity (D), joining (J), and constant (C) segments in the following order: (Vn-Dn-Jn-Cn) [10,16]. RAG-1 and RAG-2 assemble the V, D, and J segments by recognizing the recombination signal sequences at the borders of the V, D, and J segments [23]. IgT heavy chain (τ) exons are located either in the 5′ regions of the D and J gene segments of IgM and IgD heavy chains in zebrafish [10] and stickleback [24] or inserted within the V gene segments of IgM and IgD heavy chains in rainbow trout [6] and Atlantic salmon [25], which has resulted in the evolution of a distinct B cell lineage. The structure and feature of the IgT heavy chain genes in teleosts are diversified with the evolution of species, which might cause differential functional characteristics of IgT. Thus, the cloning of IgT genes from more fish species could increase the understanding of the molecular structure of fish IgT.

Tissue expression of fish *IgT* genes has been widely studied, and patterns were variable among different species. The mucosa-associated tissues such as gills and gut were found to be the primary tissues that *IgT* was expressed in several species [6,17,21,22]. The zebrafish *IgZ1* was detected only in the kidney, whereas *IgZ-2* was in the kidney and skin [7]. The high *IgT* expression levels in common carp were found in gills, head kidney, muscle, and brain [26]. The flounder *IgT* was highly expressed in the spleen, liver, and gills [13]. After *Edwardsiella tarda* infection, the flounder *IgT* expression was strongly induced in gills, skin, spleen, and head kidney [13]. The turbot *IgT* was up-regulated after infection with *Vibrio anguillarum* in gills, skin, kidney, spleen, liver, and gut [14]. The trout *IgT* expression was significantly increased when infected with parasites or bacteria, mainly in mucosal tissues including gut [27], skin [28], gill [29], and nasal [30]. These data suggested that teleost *IgT* may participate in both systemic and mucosal immunity against pathogens, especially in mucosal immunity. Therefore, expression analyses of IgT molecules based on various pathogen infections will provide valuable clues to fish IgT function in-depth knowledge.

Large yellow croaker, belonging to the Perciformes, is an important mariculture fish in China, with the highest annual production among marine species [31]. In recent years, the outbreak of infectious diseases caused by various pathogens, including bacteria, viruses, and parasites, has severely affected the development of large yellow croaker farming [32–34]. *Cryptocaryon irritans*, a ciliate ectoparasite, can infect large yellow croakers, leading to high mortality and huge economic losses [35]. Understanding the function of the immune system of *L. crocea* will contribute to the prevention and control of the infectious diseases that occur in this species. Igs are an important class of immune effector molecules in the vertebrate adaptive immune system. A previous study elucidated the molecular characteristics of IgM heavy and light chain genes in large yellow croakers, and their expression patterns upon immune stimuli were also analyzed [36,37]. Here, we describe the cloning and molecular characterization of a secretory IgT heavy chain (*Lc*IgT) in large yellow croaker. The expression pattern of *Lc*IgT in several tissues was analyzed. After a challenge with *C. irritans*, the expression changes of *Lc*IgT mRNA and protein were

determined by real-time PCR (RT-PCR) and western blotting. These results will contribute to the understanding of the molecular and functional characteristics of fish IgT.

2. Materials and Methods

2.1. Experimental Infection and Tissue Preparation

Healthy large yellow croakers (body weight: 20 ± 3.6 g; length: 9 ± 1.5 cm) were obtained from a mariculture farm at Fujian, China. The fish were maintained in a 3000-L aerated water tank with a flow-through seawater supply at 25 °C. The individuals were acclimatized for 7 days prior to being used for the challenge experiments. Then, fish were divided into two groups and cultured in separate 3000-L closed tanks. Thirty fish in the experimental group were infected with 3000 theronts of *C. irritans* per fish by bath for 4 h. The infection was performed in a 100-L fiberglass aquarium with about 60-L water containing a proper number of theronts of *C. irritans* and then transferred into 3000-L aerated water tanks with fresh seawater. Another thirty fish were treated in the same way but without *C. irritans*, and designated as the control group. The fish were euthanized with eugenol, and various tissues including spleen, head kidney, skin, and gills were sampled from five fish of each group at different time points (4 d, 7 d, 14 d, 21 d, 28 d, 35 d) after infection. These isolated tissues were frozen immediately in liquid nitrogen and stored at −80 °C before RNA extraction. The Committee on the Ethics of Animal Experiments of the Chinese Academy of Sciences approved all experiments on animals.

2.2. Complete cDNA Cloning and Sequence Analysis of LcIgT

To obtain the full-length cDNA of large yellow croaker IgT heavy chain (*Lc*IgT) gene, 5′ and 3′ RACE-PCR were performed using the 5′ and 3′-Full RACE Kit (TaKaRa, China). Primers (Table S1) for the 5′ and 3′ RACE were designed based on IgT heavy chain gene sequences obtained from the genome database of large yellow croaker (JRPU00000000) [38]. Both 5′ and 3′ RACE-PCR were performed according to the manufacturer's instructions. The resulting PCR product was cloned into the pMD19-T simple vector (TaKaRa, Beijing, China) and sequenced. All sequences were assembled to obtain the full-length cDNA of the IgT heavy chain. The deduced amino acid sequence of IgT heavy chain was analyzed for a signal peptide using SignalP software and N-glycosylation sites with the NetNGlyc 1.0 Server. Sequence similarity analysis was performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 12 May 2021). Protein domains were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/, accessed on 17 May 2021). The isoelectric point (pI) and the molecular weight of the deduced IgT protein were calculated by the ExPASy-Compute pI/Mw tool (https://web.expasy.org/compute_pi/, accessed on 23 June 2021). Multiple sequence alignment was performed using the ClustalW 2.0. A phylogenetic tree was conducted by the neighbor-joining (NJ) method using the MEGA 6 program [39]. The bootstrap method with 1000 replicates was used to examine the veracity of these trees and the statistical significance of each branch. The amino acid similarity among each C_H region of IgT was also analyzed using protein Blastp at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 July 2021), and the corresponding phylogenetic trees were constructed as performed above.

2.3. Tissue Distribution of LcIgT by RT-PCR

To determine the tissue distribution of *Lc*IgT, eleven tissues, including liver, heart, head kidney, gills, brain, spleen, stomach, intestine, skin, muscle, and blood, were collected from at least five healthy juveniles. Total RNA was extracted from these tissues using Eastep® Super Total RNA Extraction Kit (Promega, Shanghai, China), referring to the manufacturer's protocol. Subsequently, Oligo dT-Adaptor primer (Promega, Shanghai, China) was used to reverse-transcribe 1 μg total RNA into the first-strand cDNA. The cDNA was diluted with RNase-free water and stored at −20 °C before RT-PCR. The special primers (Supplementary Table S1) were used to determine the transcriptional levels of

*Lc*IgT in all tissues with a QuantStudioTM 1 (Applied biosystems, Thermo, Waltham, MA, USA) equipped with Design & Analysis Software v1.5.1. PCR was performed in a reaction volume of 20 µL. PCR amplification was performed under the following conditions: one cycle of 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. In addition, *β-actin* was amplified as the internal control for RT-PCR. The expression levels of *Lc*IgT were normalized by *β-actin* using the $2^{-\Delta\Delta CT}$ method and expressed as the ratio of *Lc*IgT expression levels in the blood [40]. The collected tissues were examined in triplicate.

2.4. LcIgT Expression Analysis after Infection with C. irritans

To further understand the modulation of *Lc*IgT expression upon *C. irritans* infection, four tissues (spleen, head kidney, gills, and skin) were isolated at 4 d, 7 d, 14 d, 21 d, 28 d, and 35 d post-infection as above. Total RNA extraction and first-strand cDNA synthesis were performed as the previous protocol. Then these templates were used for RT-PCR with respective primer sets of *Lc*IgT and *β-actin* genes. RT-PCR conditions were the same as described above. The relative expression levels of *Lc*IgT were normalized by *β-actin* and expressed as fold changes by comparing the normalized gene expression levels of *C. irritans*-infected fish with those of the non-infected fish (defined as 1) at the same time point. The samples were detected in triplicate.

2.5. Western Blotting Analysis of LcIgT Expression

To detect the modulation of *Lc*IgT protein levels, each tissue was homogenized in a homogenizer IKA T10 (Truelab, Shanghai, China) with RIPA lysis buffer (Beyotime, Shanghai, China) containing 2% protease inhibitor, and instantly centrifuged at 4 °C (13,000× *g* for 5 min). Under the reducing conditions, the supernatants of lysate were separated by 12% SDS-PAGE, and then the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GE, Boston, MA, USA). The membranes were immersed in blocking buffer, consisting of TBST buffer containing 0.2% Tween-20 (Sangon Biotech, Shanghai, China) and 5% nonfat dried milk for 1 h at room temperature with shaking to block completely. Then the membranes were incubated in blocking buffer with primary antibody (1.2 µg/mL mouse anti-IgT mAb, 1:1000; prepared in our laboratory [41]) overnight at 4 °C. The membranes were rinsed twice with TBST for 10 min each time, following incubation with diluted secondary antibody, HRP-labelled Goat Anti-Mouse IgM (1:5000, Thermo Fisher Scientific, Waltham, MA, USA), shaken for 1 h at ambient temperature. After three extra washes with TBST as described above, the membranes were covered up with detection solution, a mixture of an equal amount of detection solution A and B, and placed in a gel imaging system (Azure Biosystems, Dublin, CA, USA) to detect. Alternatively, β-tubulin was selected as the internal control to achieve protein quantification. The samples were detected in triplicate. Image J v1.8.0 software was used to analyze the protein gray values of IgT.

2.6. Statistical Analysis

All data of each repeated experiment were analyzed by GraphPad Prism 8 software and denoted as the mean ± standard error of the mean. Using IBM SPSS Statistics 19.0, the analysis of independent samples t-test and one-way ANOVA was applied to the data of each group, and the p-value < 0.05 represents statistical difference.

3. Results

3.1. Identification and Characterization of LcIgT cDNA

The cloned *Lc*IgT cDNA sequence (GenBank accession number: MW450786) was 1930 bp long, containing an open reading frame (ORF) of 1665 bp, a 5′-untranslated region (5′-UTR) of 31 bp, and a 3′-UTR of 234 bp with a putative atypical polyadenylation signal sequence AATAAA (Supplement Figure S1). The ORF of *Lc*IgT encodes a protein of 554-amino acids (aa), containing a putative 29-aa signal peptide, a 117-aa variable region (V_H, H^{47}-E^{163}), and three constant domains (C_H1, D^{164}-G^{236}; C_H2, T^{259}-T^{331}; C_H4, M^{453}-N^{542}), but no transmembrane

region was predicted, indicating that *Lc*IgT cDNA sequence cloned here encodes a secreted form of IgT heavy chain. The *Lc*IgT sequence contains twelve conserved cysteine residues (Cys^{51}, Cys^{123}, Cys^{158}, Cys^{170}, Cys^{227}, Cys^{264}, Cys^{321}, Cys^{361}, Cys^{422}, Cys^{478}, Cys^{528}, and Cys^{553}) and two potential N-glycosylation sites (N^{398} and N^{499}). Additionally, *Lc*IgT has a predicted molecular weight of 58.69 kDa and an isoelectric point of 7.18.

3.2. Multiple Sequence Alignment and Phylogenetic Analysis of LcIgT

The multiple alignments of amino acid sequences of *Lc*IgT and other known teleost IgT heavy chains showed the conservation of typical Ig structural domains (Figure 1), with a V_H and two-four C_H domains. *Lc*IgT contains three C_H domains, C_H1, C_H2, and C_H4, which were different from other fish IgT heavy chains at C_H number and arrangement. Homology comparison showed that *Lc*IgT shares the highest amino acid identity of 58.73% to *Scophthalmus maximus* IgT heavy chain (Table 1). Then, the V_H and C_H domains of *Lc*IgT were compared with the corresponding V_H and C_H domains in other teleosts IgT. As shown in Table 2, the V_H domain of *Lc*IgT has the highest identity of 72.50% to that of *Scophthalmus maximus* IgT. Nevertheless, the C_H1, C_H2, and C_H4 domains of *Lc*IgT all share the highest identity to those of *Oreochromis niloticus* IgT with 67.61%, 61.11%, and 63.74% identities, respectively.

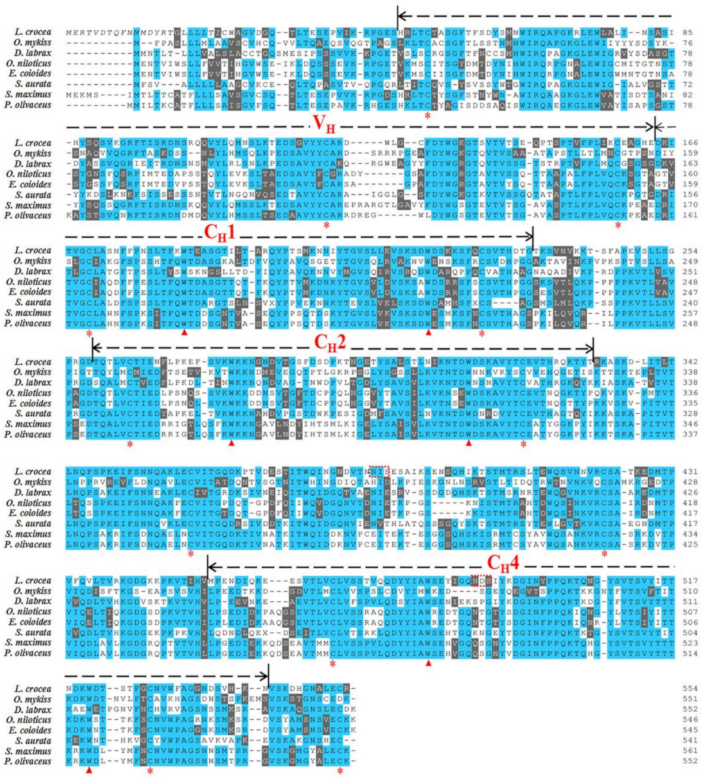

Figure 1. Alignment of the predicted large yellow croaker IgT heavy chain amino acid sequence with other known IgT heavy chain molecules. The conserved amino acids are indicated in blue fill. The predicted signal peptide sequence of large yellow croaker IgT heavy chain is indicated in italics. The C_H domains are divided into three domains (C_H1, C_H2, and C_H4). The conserved cysteines and tryptophan are marked with red asterisk and triangle. The first cysteine corresponds to the cysteine required for binding to the light chain. Potential glycosylation sites (NIS and NDS) are shown with a red dotted box.

Table 1. The IgT heavy chain protein sequences used for phylogenetic analysis.

Species	IgT Heavy Chain (Identity, %)	NCBI Accession Number
	Entire Sequence	
Larimichthys crocea	100	MW450786
Scophthalmus maximus	58.73	AMQ49170.1
Sparus aurata	56.10	ASK39430.1
Paralichthys olivaceus	55.88	ANS12795.1
Dicentrarchus labrax	55.04	AKK32388.1
Epinephelus coioides	53.96	ACZ54909.1
Oncorhynchus mykiss	44.14	AAW66978.1
Oreochromis niloticus	36.84	AUV64181.1
Danio rerio	32.14	ABF19723.1
Ctenopharyngodon idella	28.24	AAT67444.1

Table 2. The amino acid identity analysis of the V_H and each C_H domain of IgT heavy chain between *L. crocea* and other fish species.

Species	IgT Heavy Chain (Identity, %)			
	V_H	C_H1	C_H2	C_H4
Larimichthys crocea	100	100	100	100
Scophthalmus maximus	72.50	61.29	52.05	63.33
Oreochromis niloticus	41.05	67.61	61.11	63.74
Paralichthys olivaceus	56.10	61.29	52.05	63.33
Epinephelus coioides	43.97	66.20	56.94	63.74
Sparus aurata	47.47	62.90	61.11	60.24
Dicentrarchus labrax	51.90	47.89	56.94	58.43
Oncorhynchus mykiss	44.21	50.00	40.85	47.73
Ctenopharyngodon idella	45.30	35.14	35.14	25.97

To understand the relationship between the IgT heavy chain of large yellow croaker and other teleosts, a phylogenetic tree was constructed with the protein sequences from various species. As shown in Figure 2, the IgT, IgM, and IgD of all species were clustered into three respective groups. Additionally, the constructed phylogenetic tree also showed that the corresponding C_H1, C_H2, C_H3, and C_H4 domains of the IgT heavy chain of the teleosts were clustered together, although *Lc*IgT lacks C_H3 domain (Figure 3).

3.3. Tissue Distribution of LcIgT by RT-PCR

Tissue expression analysis showed that *Lc*IgT mRNA was expressed in all tested tissues of large yellow croaker, including liver, heart, head kidney, gills, brain, spleen, stomach, intestine, skin, muscle, and blood (Figure 4). The highest expression levels of *Lc*IgT were detected in gills, while the lowest levels were in blood. These results indicated that *Lc*IgT transcripts were constitutively expressed in all tissues tested, with the highest expression levels in gills.

3.4. Modulation of LcIgT Gene Expression after C. irritans Infection

We further analyzed the modulation of *Lc*IgT gene expression after *C. irritans* infection. As shown in Figure 5, expression levels of *Lc*IgT in gills, skin, spleen, and head kidney were significantly up-regulated after *C. irritans* infection at several time points. *Lc*IgT transcripts were increased in the spleen at 4 d (3.27-fold increase) and in head kidney and skin at 7 d (3.98- and 2.11-fold increases, respectively). In comparison, its expression levels in the gills were substantially up-regulated at 14 d post-infection (4.45-fold increase). Thus, *Lc*IgT expression was differentially modulated in different tissues after *C. irritans* infection.

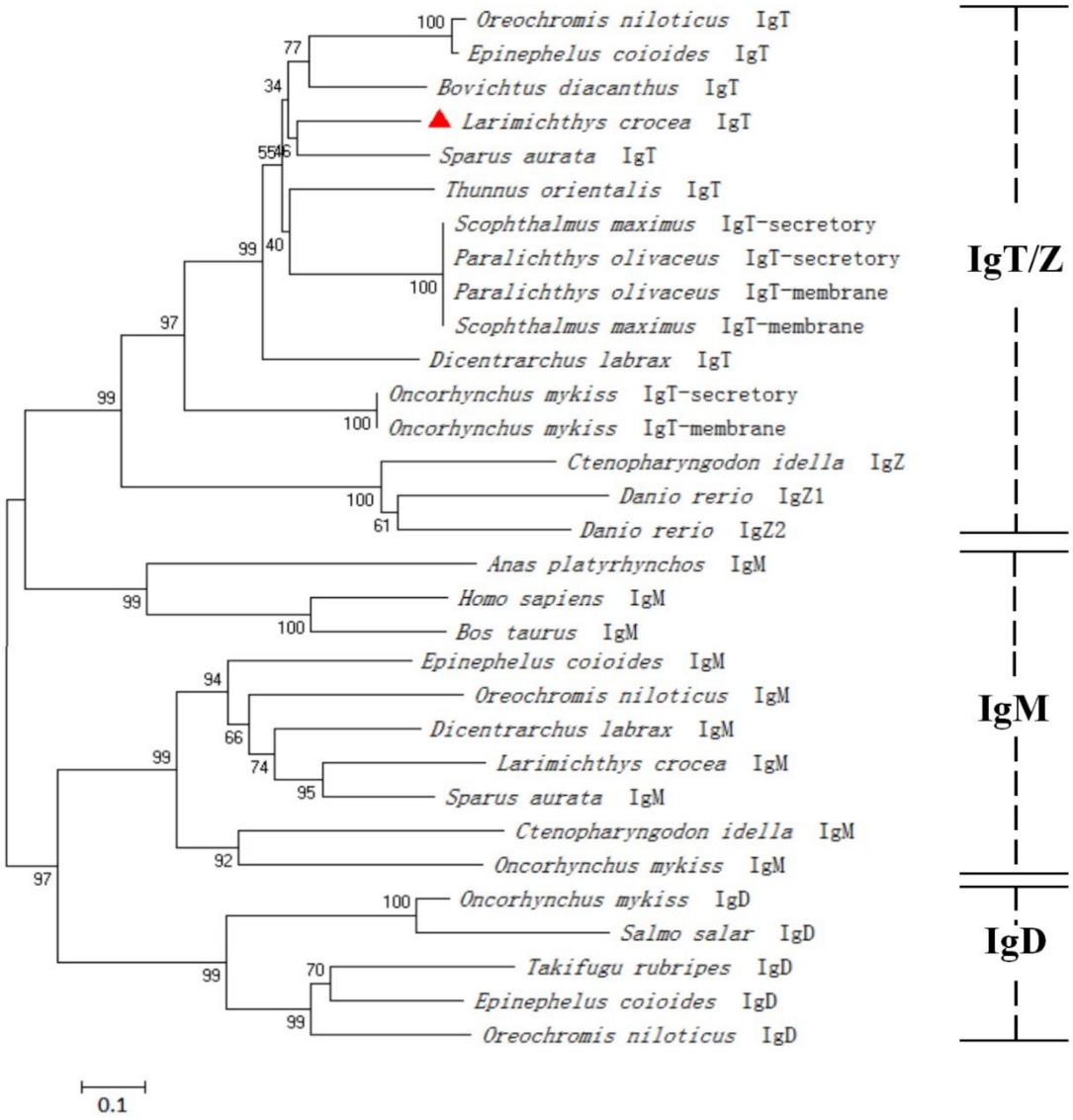

Figure 2. Phylogenetic tree of IgT heavy chain molecules from large yellow croaker and other fish species. A polygenetic tree was constructed to show the genetic relationship between large yellow croaker IgT and other fish species IgT. IgM, IgD heavy chain molecules from fish and vertebrates, were used as an outgroup. The tree was constructed by MEGA software using the Neighbor Joining (NJ) method, and the numbers on nodes represent the frequency with which this node is recovered per 100 bootstrap replications in a total of 1000. Large yellow croaker IgT was marked with a solid red triangle. GenBank accession numbers for IgT heavy chain sequences are as follows: MW450786 *Larimichthys crocea* (large yellow croaker) IgT heavy chain, AKA09828.1 *Bovichtus diacanthus* (Bovichtus) IgT heavy chain, AUV64181.1 *Oreochromis niloticus* (Nile tilapia) IgT, ACZ54909.1 *Epinephelus coioides* (juvenile grouper) IgZ heavy chain, ASK39430.1 *Sparus aurata* (juvenile seabream) IgT heavy chain secretory form, ANS12794.1 *Paralichthys olivaceus* (Japanese flounder) IgT membrane form, ANS12795.1 Japanese flounder IgT secretory form, AMQ49170.1 *Scophthalmus maximus* (turbot) IgT secretory form, AHC31432.1 *Thunnus orientalis* (Pacific bluefin tuna), AKK32388.1 *Dicentrarchus labrax* (European seabass), AAW66980.1 *Oncorhynchus mykiss* (rainbow trout) IgT membrane form, AAW66978.1 rainbow trout IgT secretory form, ANS12795.1 *Paralichthys olivaceus* (Japanese flounder), ABF19723.1 *Ctenopharyngodon idella* (grass carp), AAT67444.1 *Danio rerio* (zebrafish) IgZ1, ACH92959.1 zebrafish IgZ2. GenBank accession numbers for IgM heavy chain sequences are as follows: AAK69167.1 zebrafish IgM secretory form, ABD76396.1 grass carp IgM heavy chain, AAB27359.2 rainbow trout IgM heavy chain, ACM24795.1 large yellow croaker IgM heavy chain, ACH87158.1 bovichtus IgM secretory

form, AAX78211.1 juvenile grouper IgM heavy chain, AAA49774.1 *Xenopus laevis* (African clawed frog) IgM heavy chain, CAC43280.1 *Anas platyrhyncho* (duck) IgM heavy chain, NP_001192115.1 *Bos taurus* (cattle) IgM heavy chain, and AAS01770.1 *Homo sapiens* (human) IgM heavy chain. GenBank accession numbers for IgD heavy chain sequences are: AAW66977.1 rainbow trout IgD membrane form, AHY86392.1 Nile tilapia IgD membrane form, BAD34541.1 *Takifugu rubripes* (fugu) IgD, AFI33218.1 juvenile grouper IgD heavy chain, ADD59896.1 IgD heavy chain constant region.

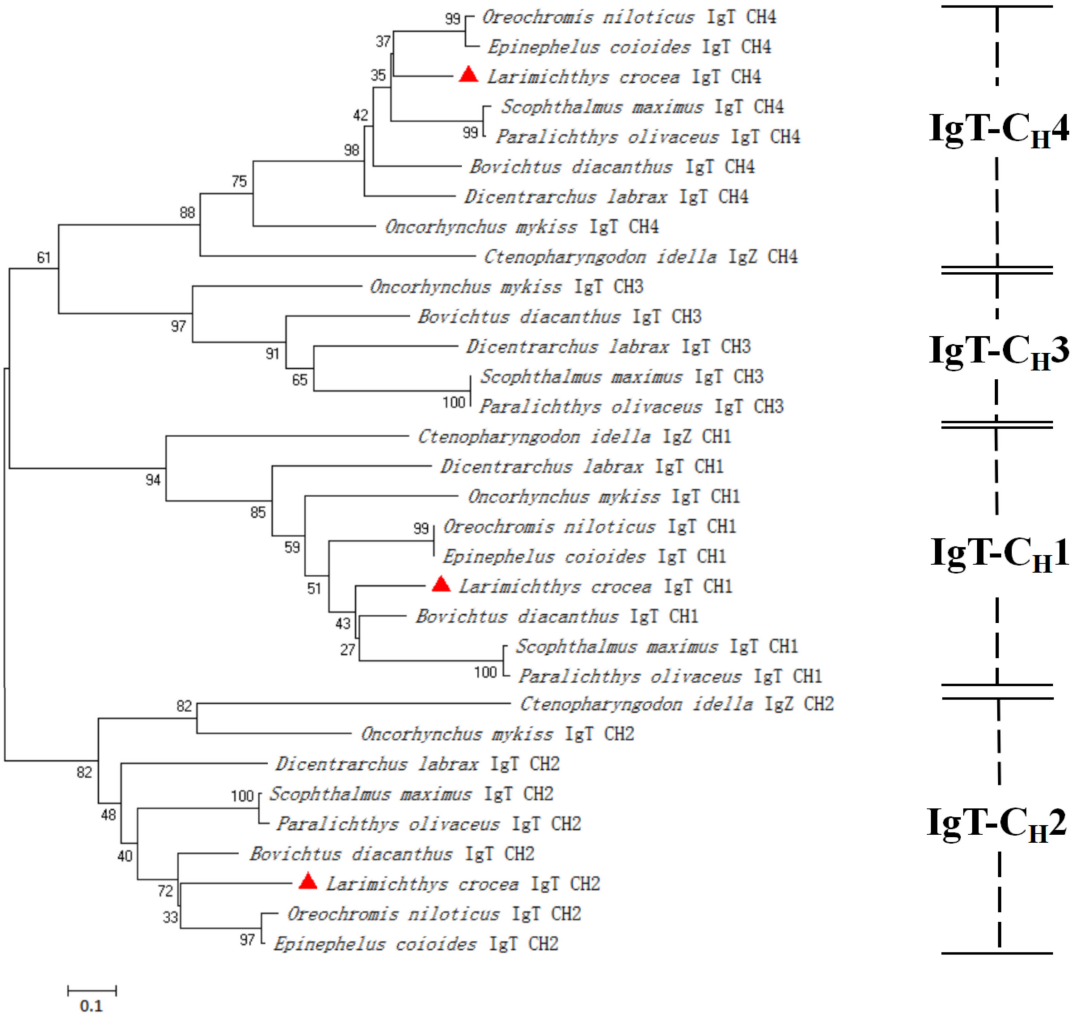

Figure 3. Phylogenetic tree of each constant domain of fish IgT heavy chains. A polygenetic tree was constructed to show the genetic relationship of each constant domain of IgT heavy chains from large yellow croakers and other fish species. The tree was constructed by MEGA software using the Neighbor Joining (NJ) method, and the numbers on nodes represent the frequency with which this node is recovered per 100 bootstrap replications in a total of 1000. The single constant domain of large yellow croaker IgT was marked with a solid red triangle.

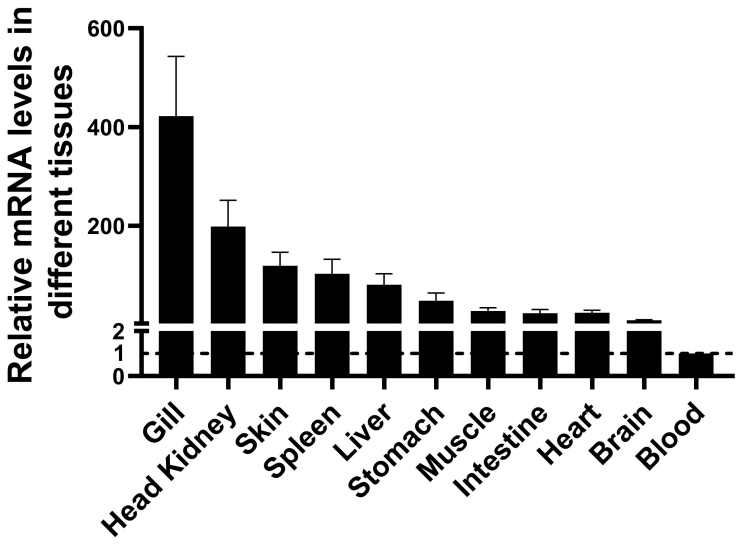

Figure 4. Tissue expression profile of *Lc*IgT. The expression levels of the *Lc*IgT gene in eleven tissues of healthy large yellow croaker (liver, heart, head kidney, gills, brain, spleen, stomach, intestine, skin, muscle, and blood) were detected by real time-PCR. Total RNA from various tissues of five fish was isolated and then transcribed into the first cDNA. Error bars represent the mean ± standard error of the mean (mean ± SEM).

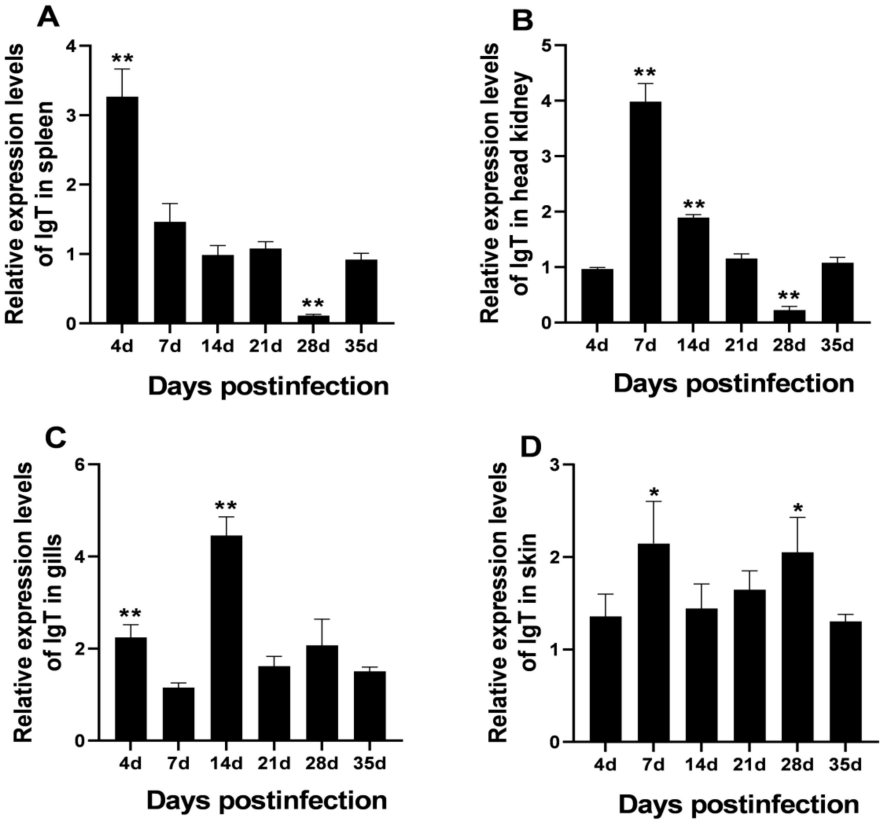

Figure 5. Expression modulation of *Lc*IgT in large yellow croakers infected by *C. irritans*. Expression change of *Lc*IgT mRNA upon *C. irritans* infection was analyzed by real-time PCR. Four tissues, spleen (**A**), head kidney (**B**), gills (**C**), and skin (**D**), were collected from five *C. irritans*-infected fish or non-infected

fish and used for total RNA extraction. The relative expression levels of *Lc*IgT expressed as fold changes by comparing the normalized gene expression levels of *C. irritans*-infected fish with those of non-infected fish at the same time point. Error bars represent the mean ±SEM. Statistically significant differences are indicated with asterisks where * $p < 0.05$, ** $p < 0.01$.

3.5. Expression Change at Protein Levels of LcIgT after C. irritans Infection

Finally, we investigated the expression change at the protein levels of *Lc*IgT after *C. irritans* infection. The protein expression of *Lc*IgT was significantly increased in gills, skin, and spleen upon *C. irritans* infection. The results of gray values analysis showed that the protein levels of *Lc*IgT in the *C. irritans*-infected group were commonly higher than those in the control group, with a 3.43-fold increase in the skin at 7 d, 3.72-fold increase in the spleen at 14 d, and 6.33-fold in gills at 28 d (Figure 6).

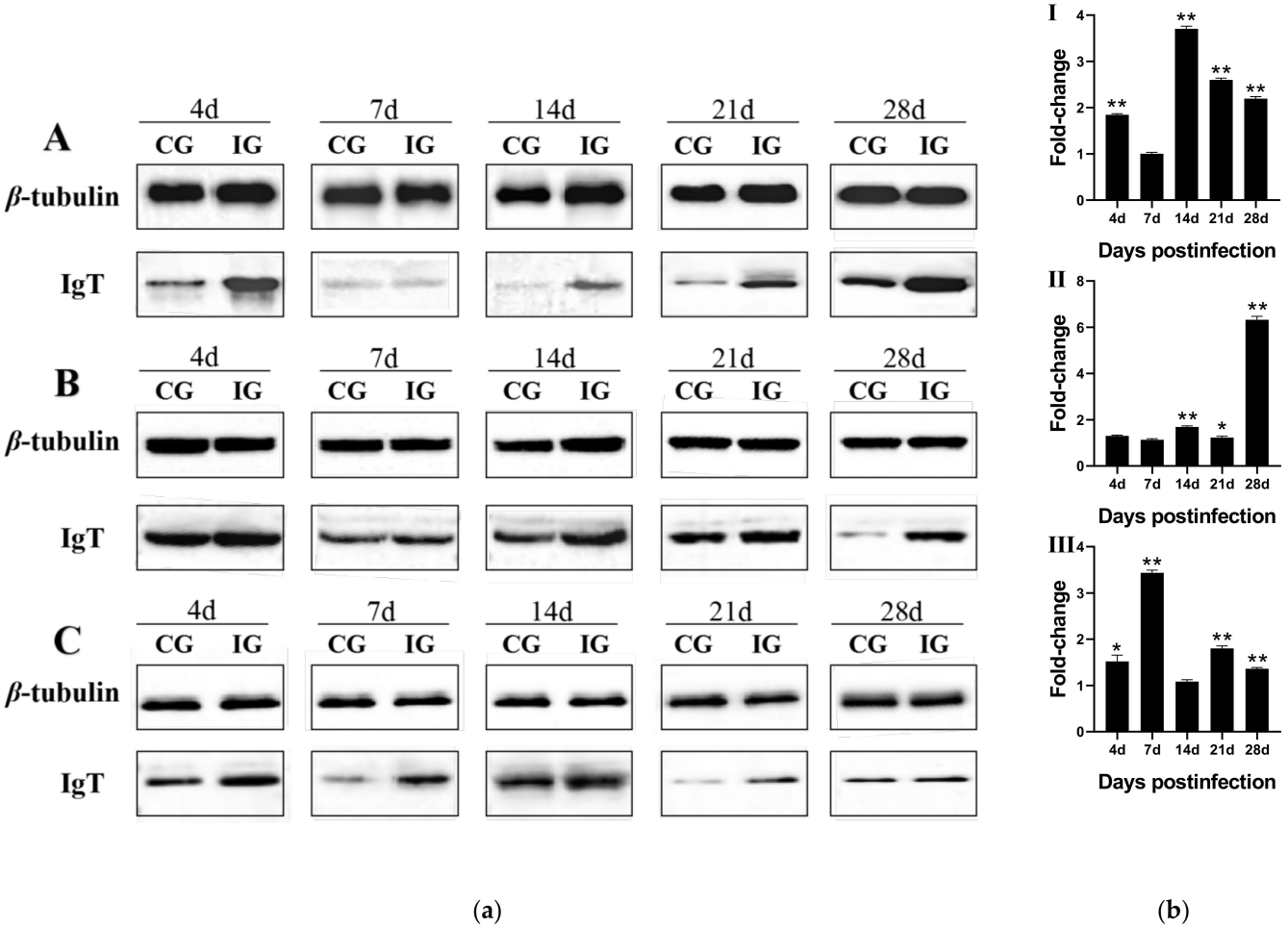

Figure 6. Western blotting analysis of *Lc*IgT expression in large yellow croaker infected by *C. irritans*. Western blotting analyzed the expression of LcIgT protein upon *C. irritans* infection. (**a**) Three tissues, spleen (**A**), gills (**B**), and skin (**C**), were collected from five *C. irritans*-infected fish or non-infected fish at 4 d, 7 d, 14 d, 21 d, and 28 d post-infection. CG: control group; IG: infection group. (**b**) The fold change values of spleen, gills, and skin, marked with (I), (II), and (III), respectively, were calculated by normalizing to the control group. Statistically significant differences are indicated with asterisks where * $p < 0.05$ and ** $p < 0.01$.

4. Discussion

The present study characterized a secretory IgT heavy chain (*Lc*IgT) in large yellow croaker. The deduced *Lc*IgT has the conserved Ig structural characteristics, including

a V_H and three C_H domains (C_H1, C_H2, and C_H4), lacking the C_H3 domain (Figure 2). IgT heavy chain in most known teleosts possesses four C_H domains, but stickleback and emerald rock cod IgT heavy chains contain C_H1, C_H3, and C_H4 domains (with a lack of C_H2 domain) [21,22], and fugu IgT heavy chain contain only two C_H domains, C_H1 and C_H4 [11]. These data indicated that the C_H domains of IgT heavy chain might vary in C_H number and arrangement among different teleosts. The cysteine and tryptophan residues, likely to contribute to the formation of disulfide bonds and the maintenance of the spatial structure of immunoglobulin [13,14], are well conserved in *Lc*IgT (Figure 2; Cys^{170}, Trp^{184}, Trp^{219}, Cys^{227}, Cys^{264}, Trp^{241}, Trp^{313}, Cys^{321}, Cys^{478}, Trp^{492}, $Trp^{521,}$ and Cys^{528}). The third conserved cysteine residue, involved in forming an interchain disulfide bond, and the two cysteine residues in each C_H domain, indispensable for intrachain disulfide bridge in the tertiary structure of IgT [14], are also conserved in large yellow croaker. The neighbor-joining phylogeny revealed that *Lc*IgT formed a well-supported clade with IgT molecules from other fish species. Single domain comparison showed that each C_H domain of *Lc*IgT shared a high amino acid identity with the corresponding domain of IgT in other species, with the highest identity with those of *Oreochromis niloticus* IgT. This further supported that the equivalent evolution extent may occur in each C_H domain in teleosts.

Expression analyses showed that *Lc*IgT was constitutively expressed in all tissues tested, with the highest levels in gills (Figure 5), which was consistent with the results observed in trout [30], flounder [13], European seabass [15], blunt snout bream [42], and emerald rock cod [43]. In addition, *IgT* was also highly expressed in the main mucosal tissues such as skin and intestine in large yellow croaker and other teleosts [13,14]. Thus, IgT plays a role in the mucosal immunity of fish. After bacterial infection, the flounder and turbot *IgT* expressions were up-regulated in several tissues, especially in gills and skin [13,14]. In trout, the *IgT* mRNA was significantly increased in gills, skin, nasopharynx, and gut after infection with *Ichthyophthirius multifiliis* or *Ceratomyxa shasta* [28,30,44]. Besides, significant increases of IgT-specific titers and IgT^+ B-cells were detected in the gill, skin, and nasal mucus of *I. multifiliis*-infected trout [45]. In our study, the expression levels of both *Lc*IgT mRNA and protein were found to be up-regulated in gills, skin, and spleen after *C. irritans* infection (Figure 6), suggesting that *Lc*IgT may be involved in the systemic immunity and mucosal immunity against parasitic infection. In the previous study, *Lc*IgT protein was expressed at the highest levels in the skin [41]. This may be the reason that the fold change of *Lc*IgT protein in the skin (3.43-fold increase) was not very remarkable after *C. irritans* infection. All these data indicated that IgT plays a vital role in the immune defense against pathogenic infections in fish.

5. Conclusions

In conclusion, we cloned and characterized a secretory IgT heavy chain (*Lc*IgT) from large yellow croaker. *Lc*IgT was constitutively expressed in all tissues tested, with the highest levels in mucosa-associated tissues such as gills and skin. After *C. irritans* infection, both mRNA and protein levels of *Lc*IgT were significantly up-regulated in systemic and mucosal immune tissues, suggesting that *Lc*IgT may play a role in the systemic immunity and mucosal immunity against parasitic infection. However, further investigations are required to clarify the exact roles in immunity against parasitic infections of IgT in large yellow croakers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7010029/s1, Figure S1: Nucleotide and deduced amino acid sequences of *Lc*IgT gene (GenBank accession number: MW450786.1), Table S1: Primers and their sequences used in this study.

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