Molecular characterization of a new IgZ3 subclass in common carp (Cyprinus carpio) and comparative expression analysis of IgH transcripts during larvae development

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Abstract

Background: Immunoglobulins (Igs) distributed among systemic immune tissues and mucosal immune tissues play important roles in protecting teleosts from infections in the pathogen-rich aquatic environment. Teleost IgZ/IgT subclasses with different tissue expression patterns may have different immune functions.

Results: In the present study, a novel secreted IgZ heavy chain gene was cloned and characterized in common carp (Cyprinus carpio). This gene exhibited a different tissue-specific expression profile than the reported genes IgZ1 and IgZ2. The obtained IgZ-like subclass gene designated CcIgZ3, had a complete open reading frame contained 1650 bp encoding a protein of 549 amino acid residues. Phylogenetic analysis revealed that CcIgZ3 was grouped with carp IgZ2 and was in the same branch as IgZ/IgT genes of other teleosts. Basal expression detection of the immunoglobulin heavy chain (IgH) in healthy adult common carp showed that CcIgZ3 transcripts were widely expressed in systemic immune tissues and mucosal-associated lymphoid tissues. CcIgZ3 was expressed at the highest levels in the head kidneys, gills, and gonads, followed by the spleen, hindgut, oral epithelium, liver, brain, muscle, foregut, and blood; it was expressed at a very low level in the skin. The transcript expression of CcIgZ3 in leukocytes isolated from peripheral blood cells was significantly higher than that in leukocytes isolated from the spleen. Different groups of common carp were infected with Aeromonas hydrophila via intraperitoneal injection or immersion. RT-qPCR analysis demonstrated that significant differences in CcIgZ3 mRNA levels existed between the immersion and injection groups in all the examined tissues, including the head kidney, spleen, liver, and hindgut; in particular, the CcIgZ3 mRNA level in the hindgut was higher in the immersion group than in the injection group. The different routes of A. hydrophila exposure in common carp had milder effects on the IgM response than on the CcIgZ3 response. Further study of the relative expression of the IgH gene during the development of common carp showed that the tissue-specific expression profile of CcIgZ3 was very different from those of other genes. RT-qPCR (Continued on next page)
analysis demonstrated that the CclgZ3 mRNA level increased gradually in common carp during the early larval development stage from 1 day post fertilization (dpf) to 31 dpf with a dynamic tendency similar to those of IgZ1 and IgZ2, and IgM was the dominant Ig with obviously elevated abundance. Analyses of the tissue-specific expression of IgHs in common carp at 65 dpf showed that CclgZ3 was expressed at mucosal sites, including both the hindgut and gill; in contrast, IgZ1 was preferentially expressed in the hindgut, and IgZ2 was preferentially expressed in the gill. In addition to RT-qPCR analysis, in situ hybridization was performed to detect CclgZ3-expressing cells and IgM-expressing cells. The results showed that CclgZ3 and IgM transcripts were detectable in the spleens, gills, and hindguts of common carp at 65 dpf.

**Conclusions:** These results reveal that CclgZ3 gene transcripts are expressed in common carp during developmental stage not only in systemic tissues but also in mucosal tissues. CclgZ3 expression can be induced in immune tissues by *A. hydrophila* challenge via immersion and intraperitoneal injection with significantly different expression profiles, which indicates that CclgZ3 is involved in the antimicrobial immune response and might play an important role in gut mucosal immunity.

**Keywords:** Common carp, Immunoglobulin, IgZ, Ontogeny

**Background**

Fish, like other vertebrates, possess an extensive defence system that enables each individual to survive and maintain its integrity in a hostile environment. The humoral immune system responds to a variety of pathogens by producing specific antibodies. Antibodies produced by B lymphocytes exist in vertebrates from mammals to cartilaginous fish [1]. There are five types of heavy (H) chains in mammals, including µ, δ, γ, ε, and α, which differ in their constant regions. The antibodies that contain these different H chains are considered different isotypes and are named immunoglobulin (Ig) M, IgD, IgG, IgE, and IgA, respectively. Each isotype has distinct physical and biological properties and effector functions [2]. Fish are the most primitive group of vertebrates that possess an adaptive immune system capable of generating antibodies in response to pathogenic challenges. Aside from IgM and IgD, a novel isotype, IgZ (ζ) or IgT (τ), has been identified in many teleost fish species [3]. This isotype has also been found to have varied subclasses with different gene sequences and different tissue expression patterns, such as IgZ1 and IgZ2 in zebrafish (*Danio rerio*) [4, 5]; IgT1, IgT2, IgT3, IgT4, and IgT5 in rainbow trout (*Oncorhynchus mykiss*) [6]; IgZ and a chimeric IgZ (IgZ2) in grass carp (*Ctenopharyngodon idella*) [7]; IgT1, IgT2, IgT3 and IgT4 in stickleback (*Gasterosteus aculeatus*) [8]; five IgT-A and three IgT-B in Atlantic salmon (*Salmo salar*) [9]; and IgZ1 and IgM-IgZ (IgZ2) in common carp (*C. carpio*) [10]. Most of the IgZ/T molecules have four CH domains that are encoded by the Cc/Ct genes, but others have two CH domains or three CH domains; for example, there are two constant domains in common carp IgZ2 [11] and torafugu (*Takifugu rubripes*) IgH [12] and three constant domains in stickleback IgT [8] and sea bass (*Dicentrarchus labrax*) IgT [13]. In addition to exhibiting diversity in domain numbers, the IgZ/T subclasses display varied expression patterns and functions among fish species. The reported IgZ1 of common carp is expressed mainly in blood and has activity against blood pathogens, while the IgZ2 chimera is preferentially expressed in the mucosal compartment to respond to mucosal infections [10]. The rainbow trout Ig1 is expressed mainly in both systemic and mucosal lymphoid tissues, while Ig2 is expressed largely in systemic lymphoid organs. After poly (I:C) treatment, the Ig1 and Ig2 genes exhibit different expression profiles, and Ig1 transcript levels peak at 7 days in the spleen and 14 days in the gut. However, Ig2 levels increase slightly, peaking at 7 days in both the spleen and gut [6]. Thus, previous research on this novel immunoglobulin heavy chain has indicated that teleost fish IgZ/T is more diverse than previously thought.

The teleost IgZ/T considered a primitive Ig class specialized in mucosal immunity, is equivalent to the IgA in mammals and plays an important role in the mucosal immune response [3, 14]. IgZ/T is a comparatively new teleost Ig class, and its presence, expression, and tissue distribution during the early developmental stage are not very clear. Many studies have investigated the ontogeny of IgM-positive cells and IgM-secreting cells. Previous research has indicated that the first B cells are most likely generated in the head kidneys and that B cells populate gut-associated lymphoid tissue (GALT) much later than the spleen or kidneys [15]. In channel catfish, a specific IgM can be detected in eggs that provides an immune barrier at the surface of the egg as well as protection for the developing fry [15]. In one study on carp, surface IgM+ cells were first detected in the head kidneys at 2 weeks post fertilization (wpf) using WCI12 and WCI4 (monoclonal antibodies against the IgM H chain), and carp injected with a T cell-independent antigen...
(lipopolysaccharide, LPS) developed antibody responses and memory from 4 wpf, while they responded to a T cell-dependent antigen (human gamma globulin, HGG) from 8 wpf [16]. RT-qPCR using primers specific for the IgH constant sequence has been performed to detect the presence of IgH during the early developmental stages of some teleost fish species. In zebrafish, all Ig isotypes effectively responded to LPS challenge from 21 dpf onwards, while IgZ1 responds to LPS challenge faster and more strongly than IgM and IgD at 28 dpf. IgZ-2 transcripts can be detected at 14 dpf [17]. In common carp, whole embryos show constitutive expression of all three Igs (IgM, IgZ1, and IgZ2) as early as 4 dpf with IgM being the predominant form. IgZ1 and IgZ2 expression increases rapidly to peak at 12 dpf whereas IgM peaks at 30 dpf [9]. The appearance of immunoglobulin during the early developmental stage varies considerably among different teleost species due to important differences in developmental status at hatch and the aquatic environment. The presence of Ig molecules in fish embryos and larvae suggests that these molecules are important for defence against pathogens. The carp is one of the most popular cultured fish in China, and diseases caused by A. hydrophila can cause great harm to the carp aquaculture industry. A. hydrophila exhibits antibiotics resistance which is attributed to the indiscriminate use of antibiotics in aquaculture. Vaccines based on antibody-mediated immune responses can enable defence against bacterial infection without the use of antibiotics [18]. In the present study, we cloned a new gene, CcIgZ3 from common carp and detected its expression patterns in adult fish and during embryonic development compare to those of the other known IgHs of this species. The immune responses of CcIgZ3 and IgM after challenge with A. hydrophila by immersion and injection were also compared. Our results provide additional experimental evidence regarding IgZ/T ontogeny and immune function in teleost fish.

**Results**

**Molecular cloning and analysis of common carp CcIgZ3**

Identification of the constant region of CcIgZ3 cDNA in common carp

The obtained full-length CcIgZ3 cDNA sequence was 2144 bp with a 5'UTR of 112 bp, a 3'UTR of 382 bp and a putative typical polyadenylation signal sequence (AATAAA) located upstream of the poly(A) tail. The deduced CcIgZ3 amino acid sequence contained 549 aa and spanned the V domain, four constant domains and a secreted tail (Fig. 1). Analysis of the IgT sequence showed the presence of a putative 20 aa signal peptide. IMGT unique numbering was used for the V domain of the CcIgZ3 sequence. The entire C-domain could be divided into four CH domains.

**Multiple amino acid sequence alignment**

Alignment of common carp CcIgZ3 with zebrafish IgZ1 and IgZ2, common carp IgZ1 and IgZ2, and grass carp IgZ and IgZ2 showed that CcIgZ3 was composed of four Ig-like constant domains (CH1, CH2, CH3, CH4) and a secretory tail. Conserved cysteine residues for disulfide bond formation and tryptophan residues for folding of the IgSF domain were present in each CH domain of CcIgZ3 (Fig. 2). Five N-linked glycosylation sites were predicted to be present in CcIgZ3 (in CH2, CH3 and CH4) (Fig. 1). The number and distribution of putative N-glycosylation sites, including the sequon NXS or NXT for each domain, varied in different teleost species. The CH1 domain possessed none zero to three putative N-glycosylation sites. There was no N-linked glycosylation site in the CcIgZ3 CH1 domain, unlike in the grass carp sequence CH1 domain(three N-linked glycosylation sites) and the zebrafish sequence CH1 domain (two N-linked glycosylation sites). The first N-linked glycosylation site of CcIgZ3 existed in the CH2 domain and was at the very beginning of the CH2 domain. CcIgZ3 presented two N-linked glycosylation sites in the CH3 and CH4 domains, similar to the grass carp sequence [5].

The percent identity values between the amino acid sequences of all the IgZ subclasses of common carp and those of grass carp, zebrafish and rainbow trout were calculated considering every single domain. The results showed that the identity values among all three IgZ subclasses of common carp ranged from 37.5 to 74.76 %, those between the three IgZ subclasses of common carp and grass carp IgZ1/IgZ2 ranged from 11.59 to 70.21 %, those between the three IgZ subclasses of common carp and zebrafish IgZ1/IgZ2 ranged from 24.18 to 64.13 %, and those between the three IgZ subclasses of common carp and rainbow trout IgT1 ~ IgT3 ranged from 21 to 40.82 %. Interestingly, the identity between the CcIgZ3 CH1 domain and grass carp IgZ CH2 domain (71.26 %) was higher than that between the CcIgZ3 CH1 domain and grass carp IgZ CH1 domain (11.69 %), and the other two IgZ of common carp also showed this characteristic (41.67 % vs. 13.1 in IgZ1, 66.67 % vs. 17.07 % in IgZ2).

**Phylogenetic analysis**

Phylogenetic analysis was carried out for the deduced amino acid sequences of the constant domains of CcIgZ3 with their counterparts in other vertebrates. The results indicated that CcIgZ3 was grouped with common carp mlgZ2/slgZ2 and grass carp mlgZ2/mlgZ3. The IgT/ IgZ genes from teleost species formed a distinct cluster separate from those of other IgM and IgD genes identified from fish and other vertebrates (Fig. 3).
Comparison of the basal expression of CcIgZ3 with that of other Ig molecules in common carp

To investigate tissue-specific expression patterns, we performed RT-qPCR analysis using gene-specific primers for all three IgZ and IgM genes of common carp in normal adult tissues, including the liver, spleen, head kidneys, gills, skin, gonads, brain, muscle, blood, foregut, hindgut and oral epithelium. CcIgZ3, IgZ1, IgZ2 and IgM were expressed in all the tested tissues, and IgM was the most abundant Ig in all tissues. The expression of CcIgZ3 was found to be highest in the head kidneys, gills, and gonads, followed by the spleen, hindgut, oral epithelium, liver, brain, muscle, foregut, and blood; CcIgZ3 was expressed at a very low level in the skin. IgZ1 and IgZ2 demonstrated expression patterns similar to that of CcIgZ3 in most of the detected tissues except that the lowest transcript levels of IgZ1 and IgZ2 were found in blood (Fig. 4). To compare the expression of the four IgH transcripts in leukocytes, we performed RT-qPCR analysis using gene-specific primers for all three IgZ subclasses and IgM of common carp on isolated leukocytes from peripheral blood cells and spleen tissues. The results showed that a significant difference in CcIgZ3 expression existed between peripheral blood lymphocytes (PBLs) and leukocytes of the spleen. In contrast, no significant differences in IgM, IgZ1 and IgZ2 expression existed between PBLs and leukocytes of the spleen (Fig. 5).

Fig. 1 Nucleotide and deduced amino acid sequences for secreted form of common carp (C. carpio) IgZ3 (CcIgZ3, GenBank Accession No. MN1707441). a In the nucleotide sequence, the start codon (atg) and the stop codon (taa) are indicated in red. The typical polyadenylation signal (AATAAA) located upstream of the poly(A) tail is indicated in red. b In the amino acid sequence, the signal peptide (SP) is marked in green, the framework (FR) is shown in blue and the complementarity determining regions (CDR) are shown in red. c The immunoglobulin domains CH1, CH2, CH3, and CH4 and the secreted tail are indicated with slashes above the nucleotide sequence. d Potential N-glycosylation sites are underlined.
Comparison of the basal expression of CcIgZ3 with that of other Ig molecules in common carp during different developmental stages

Constitutive expression of the four Ig genes during the early developmental stages of common carp (from 1 to 65 days post fertilization, dpf) was assessed by RT-qPCR (Figs. 6 and 7). Expression of CcIgZ3 and the other three Ig genes was detected beginning at 6 dpf. IgM was expressed at the highest level and was the dominant Ig isotype during the early developmental stages tested (Fig. 6). To investigate the tissue expression patterns of IgH transcripts, the expression levels of CcIgZ3, IgM, IgZ1, and IgZ2 were evaluated in gill, spleen, hindgut and liver tissues from common carp at 65 dpf. The expression of IgM was still higher than that of the other three subclasses, and IgM was the only isotype detectable in all the tested tissues. The genes CcIgZ3, IgZ1 and IgZ2 exhibited varied tissue expression patterns. CcIgZ3 was expressed in the spleen, hindgut and gills but not in the liver, IgZ1 expression was elevated in the liver but undetectable in the gills. IgZ2 expression was detected primarily in the gills and spleen but was hardly detected in the liver and hindgut (Fig. 7).

Tissue localization of CcIgZ3 and IgM mRNA-expressing cells in common carp at early developmental stages

In situ hybridization (ISH) analysis of spleen, gills and hindgut sections showed the presence of IgM-expressing cells and CcIgZ3-expressing cells in common carp at the early developmental stage (65 dpf). IgM-expressing cells were detected in both the spleen and gills with strong positive signals. In the spleen, IgM-positive cells were scattered throughout the haematopoietic tissues and in clusters close to the splenic sinus (Fig. 8f). In the gills, IgM-expressing cells were apparently distributed along gill filaments (Fig. 8j). In the hindgut, IgM-expressing cells were detected in the lamina propria, but no positive cells were found in the lamina muscularis (Fig. 8b). No signals were revealed using an IgM mRNA sense probe (Fig. 8a, e, i). CcIgZ3-expressing cells were detected in the liver and hindgut (Fig. 7).
Fig. 3 Phylogenetic analysis of vertebrate IgH based on CH sequences. The phylogenetic tree was obtained from a Clustal W alignment and MEGA X by the neighbor-joining method with pairwise gap deletions. The scale indicated the genetic distance. Clusters of teleost Ig sequences are highlighted with different colours. The GenBank accession numbers are listed in Table 2.
the spleen, gills and hindgut with comparatively weak positive signals. In the spleen, some single positive cells were found close to the splenic sinus (Fig. 8h). In the gills, CcIgZ3-expressing cells were also detected along gill filaments (Fig. 8l). In the hindgut, CcIgZ3-expressing cells were detected in the lamina propria and epithelium (Fig. 8d). The use of CcIgZ3 mRNA sense probes did not result in any staining (Fig. 8c, g, k).

Organ-specific CcIgZ3 and IgM expression after A. hydrophila challenge by different routes

The RT-qPCR results showed that the mRNA expression of both CcIgZ3 and IgM was upregulated in all tested tissues in the injection and immersion groups compared to the blank control group from 3 days to 21 days, except for CcIgZ3 in the liver in the injection group (0.46-fold at 3 days) (Fig. 9f). Compared with those in the control group, the peak values of CcIgZ3 expression in the immersion group were 86.78-fold in the spleen (Fig. 9d), 31-fold in hindgut (Fig. 9h), 13.61-fold in head kidney (Fig. 9b), and 6.39-fold in the liver (Fig. 9f), while the peak values of CcIgZ3 in the injection group were 65.13-fold in the spleen (Fig. 9D), 10.96-fold in the hindgut (Fig. 9h), 26.91-fold in the head kidneys (Fig. 9b), and 1.21-fold in the liver (Fig. 9f). Compared to those in the injection group, the peak expression levels of CcIgZ3 in the immersion group were higher in the liver (5.28-fold), hindgut (2.83-fold), and spleen (1.33-fold) but...
lower in the head kidneys (0.51-fold). However, the peak IgM expression was 13.79-fold in the head kidneys (Fig. 9a), 0.57-fold in the spleen (Fig. 9c), 6.5-fold in the liver (Fig. 9e) and 4.07-fold in hindgut (Fig. 9g) in the immersion group compared with the control group; the peak IgM expression was 3.09-fold in the head kidneys (Fig. 9a), 5.27-fold in the spleen (Fig. 9c), 5.85-fold in the liver (Fig. 9e), and 5.8-fold in the hindgut (Fig. 9g) in the injection group compared with the control group. The peak expression of IgM was higher in the head kidneys (4.46-fold), spleen (1.25-fold), and liver (1.11-fold) but lower in the hindgut (0.7-fold) in the immersion group compared with the injection group. The peak expression of IgM was higher in the head kidneys (4.46-fold), spleen (1.25-fold), and liver (1.11-fold) but lower in the hindgut (0.7-fold) in the immersion group compared with the control group. The results displayed different tissue expression patterns and smaller fold changes for IgM than for CclgZ3.

Peak CclgZ3 and IgM expression occurred at 3–7 days in the spleen, liver, and hindgut in both the immersion group and injection group. However, peak IgM expression in the head kidneys occurred much later in the immersion group (14 days) than in the injection group (1 day). Peak CclgZ3 expression in the head kidneys also occurred later in the immersion group (14 days) than in the injection group (3 days). In the hindgut, peak CclgZ3 expression appeared later in the immersion group (3 days) than in the injection group (1 day). In these tissues, the immersion challenge resulted in a slower immune response mediated by CclgZ3 and IgM than the injection challenge (Fig. 9).

Regarding the response in the hindgut, the upregulation of CclgZ3 expression was significantly stronger in the immersion group than in the injection group from 3
days to 21 days post challenge, while the expression of IgM was not significantly different during this time period. Compared with that in the control group, CcIgZ3 expression in the hindgut in the immersion group was 1.73-fold at 1 day, 31-fold at 3 days, 11.53-fold at 7 days, 5.97-fold at 14 days, and 3.76-fold at 21 days, while CcIgZ3 expression in the hindgut in the injection group was 10.96-fold at 1 day, 1.69-fold at 3 days, 1.13-fold at 7 days, 1.06-fold at 14 days, and 1.09-fold at 21 days. Compared with that in the injection group, the expression of CcIgZ3 in the immersion group was 0.15-fold at 1 day, 18.34-fold at 3 days, 10.2-fold at 7 days, 15.63-fold at 14 days, and 3.45-fold at 21 days (Fig. 9h). Compared with that in the control group, the value of IgM expression in the hindgut in the immersion group was 0.75-fold at 1 day, 2.93-fold at 3 days, 4.07-fold at 7 days, 2.07-fold at 14 days, and 1.73-fold at 21 days, while the value of IgM expression in the hindgut in
the injection group was 2.94-fold at 1 day, 2.13-fold at 3 days, 5.80-fold at 7 days, 3-fold at 14 days, and 1.62-fold at 21 days. The IgM expression in the immersion group was 0.26-fold that in the injection group at 1 day, 1.38-fold that in the injection group at 3 days, 0.7-fold that in the injection group at 7 days, 0.69-fold that in the injection group at 14 days, and 1.07-fold that in the injection group at 21 days (Fig. 9g). The results showed that *A. hydrophila* challenge, especially by the immersion route, triggered a stronger *CcIgZ3* immune response than IgM in the hindgut.

**Discussion**

In teleosts, a new Ig isotype apart from IgM and IgD, IgZ/T, has been discovered, and more than one subclass of IgZ/T has been found in some fish species. Although alignment of sequences in these subclasses has indicated that the member of each IgZ/IgT subclass exhibit some common structural features, specific gene organization and conserved amino acid residues, their expression and distribution patterns in immune organs and tissues clearly vary, implying that there are functional differences among these IgZ/T molecules [19, 20].

To date, in common carp, two IgZ subclasses have been reported, IgZ1 and chimeric IgZ2, both of which have membrane-bound and secretory forms. In the current study, alignment of *CcIgZ3* with other IgZ sequences revealed the presence of conserved cysteine residues that participate in the formation of intrachain disulfide bonds in each CH domain of all three IgZ subclasses in common carp. In addition, the conserved cysteine residues for interchain disulfide bonds existed in all three IgZ CH1 domains. However, the conserved tryptophan residues for the folding of the IgSF domain were found only in the CH domains of *CcIgZ3* and IgZ2, and the positions of the tryptophan residues in the CH4 domains of *CcIgZ3* and IgZ2 in the common carp were different from those in rainbow trout and zebrafish. The sequence of the secretory tail was conserved among the three subclasses. Alignment of the CH sequences indicated that the CH4 domain was the most highly conserved domain and that the CH1 domain exhibited a lower level of sequence identity. Phylogenetic analysis showed that *CcIgZ3* was grouped with IgZ2, and the sequence identities for the two molecular CH1 and CH4 domains were 69.52 and 74.49%, respectively. In addition, the *CcIgZ3* CH1 domain was highly similar to the grass carp IgZ CH2 domain, while the CH1 domain sequence identity was very low (11.69%). These differences made it possible to discriminate the IgZ subclasses on the basis of their expression and localization.

In the current study, the results of constitutive expression analyses of different subclasses in normal tissues showed that the IgZ2 chimera was expressed at slightly higher levels in mucosal tissues, including the gills, skin, hindgut and oral epithelium, than IgZ1 and *CcIgZ3*; however, IgZ2 was expressed at a lower level in the blood than the other three subclasses, which may indicate that the IgZ2 chimera is preferentially expressed at mucosal sites. This result corroborates previous findings indicating that IgZ2 is expressed at higher levels in the gut and gills than in other tissues [9]. Unlike all three subclasses of IgZ, IgM was predominantly expressed in all organs, including both lymphoid and nonlymphoid organs and tissues. IgZ1 was expressed systemically, as indicated by the finding that it was expressed at higher levels than IgZ2 and *CcIgZ3* in blood. As the third member of the group of new Ig subclasses, *CcIgZ3* was expressed at higher levels in the gonads and liver than IgZ1 and IgZ2. To investigate whether *CcIgZ3* has immune function, an *A. hydrophila* challenge experiment was performed. We found that *CcIgZ3* expression was strongly induced in the hindgut during *A. hydrophila* challenge by immersion, although basal *CcIgZ3* expression was not very high in the hindgut. The teleost gut interacts with foreign pathogens from the water directly and is a main site for pathogen entry in teleost fish. Teleost Igs elicit the mucosal immune response in GALT via specific B lymphocyte proliferation and local antibody secretion within mucosa-associated lymphoid tissues (MALTs) [21, 22]. The posterior segment of the teleost intestine has been discovered to contain several immune cell types and has been shown to play a more important role in immune responses to pathogen invasion than the first segment and the second segment of the teleost intestine [13, 22]. The *CcIgZ3* immune response in the hindgut of common carp indicates that this molecule may play an important role in protecting the host from *A. hydrophila* infection, and the *CcIgZ3* immune response is even stronger than that of IgM.

Ontogeny studies have been conducted on Igs in several fish species, including IgZ1 and IgZ2 in common carp. In previous studies, IgM and IgZ have been found to be expressed during the same stage of development in fugu [11] and trout [23], but IgZ was the first detected antibody in zebrafish [5]. In common carp, IgZ1 and IgZ2 can be detected as early as 4 dpf, and tissue-specific expression is first observed in the head and trunk kidneys, which exhibit the highest expression [9, 10]. To compare the different subclasses, we first detected IgM, *CcIgZ3*, IgZ1, and IgZ2 expression during the early developmental stage from 1 to 31 dpf by RT-qPCR. In the current study, IgM and *CcIgZ3* were expressed at all time points beginning at the same stage (6 dpf) and their expression increased gradually. However, IgZ1 and IgZ2 were expressed beginning at 16 dpf, and their expression levels at a greater rate than those of *CcIgZ3* to exceed those of *CcIgZ3* at 31 dpf. In
addition, analysis of tissue-specific expression at 65 dpf showed that IgM was the only detectable antibody in the four tissues and it was comparatively abundant in the spleen, hindgut and gills. CcIgZ3 existed primarily in the spleen, followed by the gills and hindgut. The IgZ1 gene was expressed at relatively higher levels in the liver, and then in the hindgut and spleen, and the IgZ2 gene was expressed in the gills and spleen but not in the hindgut and liver. These observations may indicate the existence of functional differences among these subclasses. Furthermore, the production of these three subclasses of IgZ might be attributable to the differentiation and maturation of B lymphocytes residing within systemic lymphoid tissues and MALTs. The subpopulations of IgZ-positive B lymphocytes need further investigation.

Although considerable IgZ/T characterization has been conducted, little information is available on the in situ detection of IgZ-expressing cells in fish, especially during development [11, 24, 25]. Based on the results of RT-qPCR analysis of Ig expression during development, ISH was employed to investigate the CcIgZ3-expressing cells and IgM-expressing cells in the tissues of carp at the developmental stage of 65 dpf. The ISH results clearly showed that the localization of CcIgZ3-expressing cells in the spleen, gills and hindgut was similar to that of IgM-expressing cells; however, the transcripts levels of the two antibodies were different, as their positive reactivity varied. Our findings imply that ISH might reveal the distribution and localization of CcIgZ3-expressing cells even in the early developmental stage in common carp, and our results might help to clarify the production of CcIgZ3 and the maturation of CcIgZ3-positive B lymphocytes.

Conclusions
In conclusion, a third IgH member in common carp, CcIgZ3, was cloned and characterized and the expression and localization of CcIgZ3 compared with those of IgM, IgZ1 and IgZ2 during larval development were investigated. CcIgZ3 and IgM in systemic immune tissues and GALTs of common carp challenged with A. hydrophila via injection and immersion were compared. We hope that our findings help to expand the information on adaptive immunity in common carp and other teleost fish. Further research is required to understand the roles of the different IgZ/T subclasses and their corresponding B lymphocytes.

Methods
Ethics statement
All experiments on live animals were carried out in accordance with relevant guidelines and regulations. The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSDNU2017004). All efforts were made to minimize suffering. The study was carried out in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Experimental animals
Common carp (C. carpio) weighing between 150 and 200 g were obtained from the Fresh Water Fishery Research Institute of Shandong Province (China) and maintained in tanks with aerated freshwater at 22–25°C. The fish were acclimated to the aquaria for at least 2 weeks before being used in experiments. The fish were anaesthetized by immersion in a solution of tricaine methane sulfonate (MS222, Sigma-Aldrich) and killed. Blood, head kidney, spleen, liver, gill, foregut, midgut, hindgut, oral epithelium, skin, gonad, and muscle tissue samples were collected immediately, frozen in liquid nitrogen, and stored at -80°C until use [26, 27]. All animal experiments were approved by the Committee on the Ethics of Animal Experiments of Shandong Normal University.

Molecular cloning and analysis of common carp CcIgZ3
Total RNA extraction and first-strand cDNA synthesis
Each frozen sample was ground in a mortar with liquid nitrogen, and then total RNA was isolated using the TRIzol universal reagent (Tiangen, China). The quality and concentration of all total RNA samples were assessed using a NanoDrop Spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized from 2 µg total RNA with a FastQuant RT Kit (with gDNase) (Tiangen, China) according to the manufacturer’s instructions [28, 29]. Total RNA from the collected samples was extracted following the procedure above, and then the cDNA was stored at -80°C until use for real-time quantitative PCR (RT-qPCR).

Molecular cloning and sequencing of common carp CcIgZ3
A common carp CcIgZ3 cDNA fragment was first amplified by PCR with primers IgZ3 F1 and IgZ3 R1, which were designed based on known IgZ sequences from teleost fish (GenBank Accession No.: D. rerio AY643750, EU732710.1, AY643750, C. idella DQ478943, GQ201421, C. carpio AB004105, AB598367, AB598368, AB598369). cDNA from common carp head kidneys was used as the template. The reactions steps were as follows: 3 min of initial denaturation at 94°C; 35 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 68°C; and 5 min of final extension at 68°C. Ex Taq HS (Takara) was used for PCR, and the PCR products were loaded on a 1% agarose gel and visualized by staining the gel in 0.1 mg/mL-ethidium bromide. The DNA amplified in each reaction system by
PCR was purified using a Gel Extraction Kit (Tiangen), inserted into the pMD19-T vector (Takara) and transformed into competent TOP 10 E. coli cells for sequencing. Subsequently, rapid amplification of cDNA ends (RACE) was performed using a 3'-Full RACE Core Set (Takara) and a SMARTer RACE cDNA Amplification Kit (Takara) to obtain the full-length CcIgZ3 cDNA sequences with specific primers that were designed based on the obtained partial sequence. The 3'-Full RACE Core Set (Takara) was utilized to obtain 3'-unknown regions with the specific forward primers and the adaptor primers listed in Table 1. The first round of PCR was performed using the primer pair IgZ3 race-3'outer primer/3'RACE outer primer, under the following conditions: one cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 2 min; and a final extension step at 72°C for 10 min. The resultant product was diluted and reamplified in a second round of PCR using the primer pair IgZ3 race-3'inner primer/3'RACE inner primer under the same reaction conditions. All PCR products were purified using a Gel Extraction Kit (Tiangen) and then were cloned into the pMD19-T vector (Takara) for sequencing. For the 5’ RACE reaction, first strand cDNA was synthesized from 1 µg of total spleen lymphocyte RNA after adding the 5’ RACE adapter to RNA following the manufacturer’s instructions. PCR was performed with the 5’ RACE Ready cDNA samples using an Advantage 2 PCR Kit (Clontech) according to the manufacturer’s specifications and the specific primers shown in Table 1. The reaction steps were as follows: one cycle of 95°C for 1 min, 30 cycles of 95°C for 30 s and 68°C for 3 min, and a final extension step at 68°C for 3 min. All PCR products were purified using a NucleoSpin Gel and PCR Clean-Up Kit (Takara) and then cloned into the pRACE vector (Takara) for sequencing.

**Bioinformatic analysis of common carp CcIgZ3**

The full-length sequence of CcIgZ3 was confirmed by PCR using sequence-specific primers IgZ3-1817F2/R2 and IgZ3-1817F3/R3. The open reading frames (ORFs)

| Name                  | Sequence(5’-3’)                          | Application                  |
|-----------------------|------------------------------------------|------------------------------|
| IgZ3-F1               | TCGTTTGTGACACTGTGGGAG                   | ccIgZ3 gene cloning          |
| IgZ3-R1               | GCTTGTTGACACTGTGGGAG                   | ccIgZ3 gene cloning          |
| IgZ3race-3’ outer primer | CTCCTGATTACCTGACCCTGGGAG            | ccIgZ3 race gene specific primer |
| IgZ3race-3’ inner primer | ATTACTCCAGGTCCAGAAGAATCCCA          | ccIgZ3 race gene specific primer |
| IgZ3race-5’ outer primer | GGATGGGGACACGGTTGCGGCCCTCT         | ccIgZ3 race specific primer  |
| IgZ3-1817F2           | AGGGCAGCAGATGCTGGCCCTGG              | ccIgZ3 gene cloning          |
| IgZ3-1817R2           | GCAACAGGACAAAGACACTGA                 | ccIgZ3 gene cloning          |
| IgZ3-1803F3           | CAACGCAGAGATCATGGGGGA                | ccIgZ3 gene cloning          |
| IgZ3-1803R3           | GAGCAACAGGACAAAGACACTG               | ccIgZ3 gene cloning          |
| S11qF                 | CCGTGGGTGACATCGTTACA                 | gene expression analysis     |
| S11qR                 | TACACCCATGACACCTGACCTGCT            | gene expression analysis     |
| IgZ3-qF3              | GGCTGACTCGAGCTTGA                    | gene expression analysis     |
| IgZ3-qR3              | GACAGGACAAAGACTGACGGG               | gene expression analysis     |
| IgM-qF1               | GGTGTTGTTGCGCTGGCTGGTCT         | gene expression analysis     |
| IgM-qR1               | CGTCCAGCTGGAATATACACTG             | gene expression analysis     |
| IgZ1-qF               | GAGAATTTCTACCCCAAGG                | gene expression analysis     |
| IgZ1-qR               | GACCTTCAGATATCCTGCT                 | gene expression analysis     |
| IgZ2-qF1              | GCTGAGGATAGATGCGTCC                | gene expression analysis     |
| IgZ2-qR1              | TGGAGAGACCACGATCGCTGAAT            | gene expression analysis     |
| IgZ2-qF2              | AATCTTGAAGGACACCTGACTAGA          | gene expression analysis     |
| IgZ2-qR2              | CACACACATGAGACCCCGAT               | gene expression analysis     |
| IgZ3_situ-F4          | TTCAGCCACACATGGGACATAGAAA         | ccIgZ3 in situ hybridization |
| IgZ3_situ-R4          | TGGTGGGCTGAGCTGAAGT                | ccIgZ3 in situ hybridization |
| IgM_situ-F2           | ATGACCGCCGCTGTTGAGTGGAG           | IgM in situ hybridization   |
| IgM_situ-R2           | CTCAAAGAAGCAGAAGGCGGCAA          | IgM in situ hybridization   |
and deduced protein sequences of $C_{c}IgZ3$ were predicted using the ORF Finder program and by blasting genomic stretches against protein databases at NCBI (blastx) [30]. The locations of Ig domains were predicted using the InterProScan program, the PROSITE Database and the NCBI Conserved Domain Databases. Posttranslational modifications were predicted with the NetNGlyc 1.0 program. The theoretical isoelectric point and the molecular weight of the amino acid sequence were calculated using the ExPASy Compute pi/Mw program. The theoretical molecular weight of the amino acid sequence were calculated using the ExPASy Compute pi/Mw program. Molecular weight of the amino acid sequence were calculated using the ExPASy Compute pi/Mw program. Multiple sequence alignment was conducted using Clustal X version 2.1 with the default parameters, and the resulting alignment was adjusted manually [12]. Based on the alignment, a phylogenetic tree was generated from the deduced amino acid sequence using the neighbour-joining method with MEGA X. All the sequences used for the phylogenetic analysis are listed in Table 2.

**Gene expression studies of common carp igs**

**Basal expression of ig isotypes in organs of common carp**

For tissue expression analysis, total RNA was isolated from the head kidneys, spleens, livers, blood, skin, gills, foreguts, midguts, hindguts, oral epithelia, gonads and brains of normal common carp and then reverse-transcribed into first-strand cDNA as described above. PCR was conducted with specific primers as indicated in Table 1, with SuperReal PreMix Plus (SYBR Green, Tiangen, China). The RT-quantitative PCR analysis was performed using the primers S11F/S11R and the amplification reactions for IgM, IgZ1, IgZ2, and $C_{c}IgZ3$ consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s using the primers (Table 1). The reactions for the standards and target genes were conducted in parallel tubes. The relative expression of each Ig gene was calculated and normalized to the expression of S11.

**Basal expression of ig isotypes in leukocytes isolated from different tissues of adult common carp**

Whole blood was collected from the caudal vein for isolation of PBLs (peripheral blood lymphocytes) with a heparinized syringe and centrifuged at 4°C and 500×g for 10 min, the serum was then removed. The cells were diluted 5-fold in the same volume of RPMI-1640 medium as the original volume of blood at room temperature and then placed on ice. The spleen was dissected from each anaesthetized fish and placed in a sterile plastic culture dish containing 5 mL of RPMI-1640 with 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma-Aldrich, USA). A single-cell suspension was first obtained from the spleen by teasing apart the tissue with sterile dissecting scissors, repeatedly aspirating it and then passing it through a 100 μm nylon mesh with RPMI-1640 medium. A total volume of 10 mL of single-cell suspension was gradually layered upon the same volume of Histopaque 1077 (Sigma-Aldrich, USA) in a 50 mL centrifuge tube, and the tube was centrifuged 500×g for 40 min at 4°C. Leukocytes were collected from the interface layer and washed three times with medium [31, 32]. The cell quantity and viability were determined with 0.4% trypan blue (Sigma-Aldrich, USA), and cells were collected for RT-qPCR analysis [33]. The expression of Ig genes was calculated and normalized to that of S11.

**Relative expression of ig isotypes during the development of common carp**

For expression analysis of Igs during different developmental stages, fertilized eggs (n=100) were obtained, and total RNA was extracted from embryos or larvae 1, 6,10,16 or 31 dpf using a FastQuant RT Kit (with gDNase) (Tiangen) following the steps described above. Ig expression in tissues from common carp at 65 dpf, including gill, spleen, hindgut and liver tissues was detected by RT-qPCR following the same procedure.

**ISH**

**Synthesis of RNA probes**

Common carp IgM and $C_{c}IgZ3$ cDNAs sequences were amplified with primers (Table 1,) and subcloned into the pSPT18 vector (Roche). The primers IgZ3_situ_F4 and IgZ3_situ_R4 were designed to amplify a 244 bp product corresponding to the constant region of the $C_{c}IgZ3$ CH4 domain. The primers IgM_situ_F2 and IgM_situ_R2 were designed to amplify a 435 bp product corresponding to the constant region of common carp IgM CH4 domain. The cycling protocol was as follows: denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, final extension step at 72°C for 10 min. The PCR products were visualized on 1% agarose gels containing ethidium bromide. The fragments were purified using a TIANgel Midi Purification Kit (Tiangen), inserted into the pSPT18 vector (Roche) and transfected into competent E. coli DH5α cells. Plasmid DNA from four clones was purified and sequenced. Sequence analysis was performed to confirm the sequence identity and insert orientation.

To generate RNA probes, the clones were digested with EcoR-I or Hind-III, and the fragments were purified on an agarose gel and used for in vitro transcription reactions with a DIG RNA Labelling Kit (Roche). Transcription was performed with SP6 RNA polymerase and T7 RNA polymerase according to the protocol to generate antisense and sense RNA probes.
Spleen, gill and hindgut tissues were aseptically extracted from fish and fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS)-H₂O DEPC for at least 4 h. The tissues were then immersed in a 15 % sucrose solution for 8 h and transferred to a 30 % sucrose solution for soaking overnight. Tissue embedded in optimum cutting temperature (OCT) compound was sectioned at a thickness of 4 μm and mounted onto poly-L-lysine-coated slides. The frozen slides were removed from the freezer, fixed in paraformaldehyde (4 % in PBS, pH 7.4) for 20 min, washed three times with DEPC-treated buffer (pH 7.4) and permeabilized with protein K (5 μg/mL) buffer at 37℃ with gentle rocking. After washing in PBS-glycine buffer, the sections were washed twice with PBS-H₂O DEPC. Prehybridization was performed by incubating the sections with prehybridization buffer (Servicebio) for 60 min at 37℃. DIG-labelled antisense RNA probes (1 µg/ml) were applied with hybridization solution to the tissues, and the tissues were incubated at 55℃ overnight in a moistened chamber. To remove the hybridization solution, the sections were washed sequentially with 5× SSC, 1×SSC, 0.5×SSC and 20 % formamide (50 min). The tissue sections were blocked with 5 % serum blocking reagent for 30 min at room temperature. The anti-DIG-AP antibody used for detection was diluted 1:500 in blocking buffer solution containing 5 % serum. The sections were washed twice with PBS; subsequently, BCIP/NBT reagent (Roche) was applied according to the protocol. The

Table 2  Sequences of Igs used for phylogenetic tree construction and multiple sequence alignment

| Protein            | species                      | Accession number | Protein            | species       | Accession number |
|--------------------|------------------------------|------------------|-------------------|---------------|------------------|
| chicken IgA        | Gallus gallus                | AAB22614.2       | human IgE         | Homo sapiens  | AAB59424         |
| chicken IgM        | Gallus gallus                | CAA25762.1       | human IgG1        | Homo sapiens  | CAA75032         |
| chicken IgY        | Gallus gallus                | CA30161.1        | human IgM         | Homo sapiens  | CAB37838         |
| pacific cod        | Gadus macrocephalus          | AKL81191         | human IgM         | Homo sapiens  | CAC20458         |
| atlantic cod       | Gadus morhua                 | CA41680.1        | channel catfish IgD | Tetraodon nigroviridis | AAD60133 |
| Chinese soft-shelled turtle | Peladiscus sinensis IgM | ACU45376         | channel catfish IgD | Tetraodon nigroviridis | ADF56020 |
| Chinese soft-shelled turtle | Peladiscus sinensis IgY | ACU45374         | channel catfish IgH | Tetraodon nigroviridis | AAA9003 |
| mandarin fish IgD  | Siniperca chuatsi            | ACO88906         | channel catfish IgM | Tetraodon nigroviridis | A45804 |
| mandarin fish IgM  | Siniperca chuatsi            | AAQ14862         | little skate IgM  | Leucoraja erinacea | AAB04671.1 |
| mandarin fish IgT  | Siniperca chuatsi            | AAY42141         | little skate IgW  | Leucoraja erinacea | AAA9546 |
| cattle IgM         | Bos taurus                    | AAC71048         | mouse IgA         | Mus musculus  | AAI10324         |
| common carp IgM    | cyprinus carpio              | BAA34718         | mouse IgG         | Mus musculus  | AAB59658         |
| common carp IgZ1   | cyprinus carpio              | BAJ41037         | mouse IgM         | Mus musculus  | AAB59650         |
| common carp mlgZ2  | cyprinus carpio              | BAJ41038         | rainbow trout IgD | Oncorhynchus mykiss | AAW66976 |
| common carp slgZ2  | cyprinus carpio              | BAJ41039         | rainbow trout IgM | Oncorhynchus mykiss | AAB27359 |
| common carp IgZ3   | cyprinus carpio              | MN170744         | rainbow trout IgM | Oncorhynchus mykiss | AAW66972 |
| zebrafish IgM      | Danio rerio                  | AAK96442         | rainbow trout IgT1 | Oncorhynchus mykiss | AAW66978 |
| zebrafish IgM      | Danio rerio                  | AAT67444         | rainbow trout IgT2 | Oncorhynchus mykiss | AAB48553 |
| zebrafish mlgZ     | Danio rerio                  | AAT67444         | human IgA1        | Homo sapiens  | BAC87456.1       |
| zebrafish mlgZ2    | Danio rerio                  | ACH92959         | human IgD         | Homo sapiens  | EAW81936         |
| zebrafish slgZ     | Danio rerio                  | AAT67446         | sheep mlgM        | Ovis aries     | AAA51379         |
| Fugu rubripes IgM  | Takifugu rubripes            | BAD26619         | African clawed frog IgM | Xenopus laevis | AAA49774 |
| Fugu rubripes IgT  | Takifugu rubripes            | BAD69712         | African clawed frog IgX | Xenopus laevis | CAA32027 |
| Fugu rubripes IgD  | Takifugu rubripes            | BAD34541         | Atlantic salmon IgM | Salmo salar  | AAB24064         |
| Fugu rubripes IgH  | Takifugu rubripes            | BAD89297         | Atlantic salmon IgT2 | Salmo salar  | ADD59873         |
| Atlantic salmon IgD| Salmo salar                  | ADD59896         | Atlantic salmon mlgM | Salmo salar  | ACN10415         |
| grass carp IgD     | Ctenopharyngodon idella      | ADK66818         | African clawed frog IgY | Xenopus laevis | CAA33212 |
| grass carp IgM     | Ctenopharyngodon idella      | ABD76396         | tropical clawed frog IgF | Xenopus tropicalis | AAH87793 |
| grass carp slgZ    | Ctenopharyngodon idella      | ADD82655         | rainbow trout IgT3 | Oncorhynchus mykiss | ANW11927 |
| grass carp IgZ2    | Ctenopharyngodon idella      | ABF19723         | Japanese flounder IgD | Paralichthys olivaceus | BAB41204.1 |
reaction was visualized and documented using a bright-field microscope.

**Organ-specific IgM and CctgZ3 expression after A. hydrophila challenge through different routes**

Fifty common carp were divided into two groups for immune stimulation and challenged with *A. hydrophila via* intraperitoneal (i.p.) injection or immersion as previously described [28]. Briefly, the *A. hydrophila* used in the study was obtained from the China Center for Type Culture Collection and incubated in LB medium at 28°C overnight under continuous shaking. For the injection challenge, *A. hydrophila* was inactivated in 0.5% formalin at 4°C overnight and then suspended in sterile 0.1 M PBS. Each fish was challenged by i.p. injection with 5 x 10⁷ CFU per fish. For the immersion challenge, cultured *A. hydrophila* was added to the aquarium to a concentration of 1 x 10⁸ CFU/ml. After being treated for 40 min, the carp were removed and transferred to a tank containing fresh water. On days 0, 1, 7, 14, 21 and 28 after stimulation, three fish from each group were anaesthetized with MS-222. Tissue samples, including spleen and hindgut samples, were taken, frozen in liquid nitrogen, and used for total RNA extraction and subsequent RT-qPCR analysis following the same procedure described above. The relative mRNA expression was determined via real-time quantitative PCR with the comparative cycle threshold (Ct) \(2^{(-\Delta\Delta Ct)}\) method; the level of target mRNA was normalized with respect to S11, an internal reference gene, and the results are expressed relative to the levels in the unchallenged control fish (denoted as day 0) [33].

**Abbreviations**

Ig: Immunoglobulin; IgH: Immunoglobulin heavy chain; ISH: In situ hybridization; MALT: Mucosa-associated lymphoid tissues; GALT: Gut-associated lymphoid tissue; wpf: Weeks post fertilization; dpf: Days post fertilization

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Not applicable.

**Authors’ contributions**

GWY, LGA and FMZ participated in the design of the study, MIL, CL, GCW, CW and YMW performed the experiments including gene clone, RACE, Real-time quantitative PCR, in situ hybridization and collected data, FMZ wrote the manuscript text and prepared figures and tables. All authors reviewed and approved the final manuscript.

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**Availability of data and materials**

The dataset supporting the conclusions of this article is available in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore/1806102469) and the accession number is MN170744.1.

**Declarations**

**Ethics approval and consent to participate**

For all experiments on live animals, we confirm that all methods were carried out in accordance with relevant guidelines and regulations. The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSDNU2017004). All efforts were made to minimize suffering. The study was carried out in compliance with the ARRIVE guidelines.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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