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Cloning, molecular characterization, and tissue differential expression of connective tissue growth factor (*ctgf*) of grass carp

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Abstract Connective tissue growth factor (*ctgf*) is involved in the proliferation, migration, adhesion of cell, and the constituent of extracellular matrix, which plays an important role in embryogenesis, angiogenesis, wound repair, and fibrosis diseases. In this study, the cDNA sequence of grass carp *ctgf* gene was cloned by rapid amplification of cDNA ends (RACE) method; then, the characteristics of this gene and the predicted protein sequence were analyzed by bioinformatics methods, and the tissue differential expression pattern was detected by the quantitative real-time PCR. The results showed that the grass carp *ctgf* gene has a full-length of 2223 bp, encoding 343 amino acids. The deduced CTGF protein is a hydrophilic and secretary protein with a molecular mass of 37,978.2 Da and an isoelectric point of 8.22. The signal peptide locates between residue positions 1 and 22 of the polypeptide chain. The protein contains α-helix, β-strand, and loops. The CTGF protein of grass carp shows a homology of 98%, 96%, 91%, and 91% with Wuchang bream (*Megalobrama amblycephala*), zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*), and Mexican tetra (*Astyanax mexicanus*). The grass carp *ctgf* gene expressed significantly higher in blood and spleen than that in other tissues ($P < 0.05$). The low expression tissues included the heart, gill, skin, muscle, kidney, brain, and intestinal, and the lowest expression tissue was the liver. The results are consistent with the function of this gene.

Keywords *ctgf* · RACE · Tissue differential expression · Gene clone · Grass carp

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Introduction

Connective tissue growth factor (*ctgf*) is a member of the CCN gene family which includes *ctgf*, *cyr61* (cysteine-rich 61), and *nov* (nephroblastoma overexpressed) proteins. Early studies showed that CCN gene was expressed in various tissues of human (Brigstock et al. 1997), mice (Lasky et al. 1998), rat (Brunner et al. 1991), cattle (Brigstock 1999), pig (Ball et al. 1998), and *Xenopus* (Ying and Ling 1996). CCN proteins have extensive biological functions in cell proliferation, migration, adhesion and differentiation, and play an important role in

angiogenesis (Babic et al. 1999), cartilage formation (Nishida et al. 2000; Nakanishi et al. 2000), wound healing (Igarashi et al. 1993), and cancer treatment (Grotendorst et al. 1996). It was speculated that the CCN family came from a unique gene 40 million years ago (Su and Cai 2002). The CCN proteins have a conservative structure containing four domains: an insulin-like growth factor binding protein (IGFBP) domain, a von Willebrand factor type C repeat (vWC) domain, a thrombospondin type 1 repeat (TSP1) domain, and a carboxyl-terminal (CT) domain.

Ctgf, also known as CCN2, was identified in human umbilical vein endothelial cells (HUVEC) in 1991 (Lau and Lam 1999). It can bind different signal factors from different signal pathways to regulate the growth and development of the body. The study of *ctgf* has been successfully conducted in vertebrates including human (Brigstock et al. 1997), rats (Brunner et al. 1991), mice (Lasky et al. 1998), and *Xenopus* (Ying and Ling 1996).

Among the four conservative domains of *ctgf* protein, the vWC domain and CT domain can promote the CTGF protein to combine with TGFβ and BMP4, respectively (Mercurio et al. 2004). The in vitro study indicated that the combination of CTGF with TGFβ induced the skin fibrosis in mouse (Mori et al. 1999). When injecting CTGF and TGFβ into the subcutaneous tissue of rat, TGFβ just started the fibrosis, and the combination of CTGF and TGFβ further promoted the tissue fibrosis (Takehara 2003). After injecting CTGF into the ventral mesenchymal cells of *Xenopus*, the BMP4 antibody was induced to inhibit the combination of BMP4 with its receptor and block BMP4 signaling pathways, resulting in the secondary axis and embryonic development deformity (Abreu et al. 2002). CTGF protein in microvascular endothelial cells could combine with VEGF165 (vascular endothelial growth factor) through TSP domain and then inhibit the combination of VEGF165 with its receptor, and the activity of VEGF165 to generate blood vessel was also inhibited (Hashimoto et al. 2002; Dean et al. 2007). The CT domain of CTGF protein could combine with integrin αvβ3, promote the deposition of extracellular matrix, and then induce the adhesion of HSC (hepatic stellate cells) in rat (Gao and Brigstock 2004; Chen et al. 2004). The CT domain of *Xenopus*'s CTGF protein could combine with LRP-6 (Wnt synergy receptor LDL receptor related protein) directly and inhibit Wnt signaling pathway (Mercurio et al. 2004) and then result in the enlargement of cement gland and the damage of eyes.

There are many *ctgf* gene sequences and amino acid sequences of aquatic animals in GenBank, such as zebrafish (*Danio rerio*, AAI15210.1, *ctgf*), medaka(*Oryzias latipes*, XP_004084039.1), Wuchang bream (*Megalobrama amblycephala*, AIZ09082.1), common carp (*Cyprinus carpio*, KTG05492.1), Mexican tetra (*Astyanax mexicanus*, XP_007233529.1), Atlantic herring (*Clupea harengus*, XP_012682112.1), Atlantic salmon (*Salmo salar*, NP_001133471.1), fugu rubripes (*Takifugu rubripes*, XP_003971661.1), and half-smooth tongue sole (*Cynoglossus semilaevis*, XP_008311045.1), but the related literatures were so limited; only zebrafish (Fernando et al. 2010), Wuchang bream (Wang 2014), and common carp (Sun 2012) have been reported.

It is worth noting that zebrafish had two copies of *ctgf* (*ctgf*a NM_00101504 and *ctgf*b NM_001102573.1) in GenBank, and Wuchang bream also had two copies of *ctgf*. Wang (2014) pointed out that the coding regions homology of *ctgf*a and *ctgf*b of Wuchang bream was only 57%. The *ctgf* gene of common carp had four copies, *ctgf-A1*, *ctgf-A2*, *ctgf-B1*, and *ctgf-B2*, but there was no corresponding sequence in GenBank (Kong et al. 2008). However, other fishes such as medaka, silver carp, Mexican tetra, Atlantic herring, Atlantic salmon, fugu rubripes, and half-smooth tongue sole had only one copy of *ctgf* gene. The *ctgfa* gene, but not the *ctgfb* gene of zebrafish and Wuchang bream, showed a high homology with the *ctgf* gene of human, rat, *Xenopus*, and other species (Wang 2014).

Sun (2012) cloned six genes of CCN family of common carp (*cyr61*, *ctgf*, *nov*, *wisp1*, *wisp2*, and *wisp3*) and analyzed the tissue differential expression pattern of those genes. Kong studied the variation of *ctgf* gene sequence of cyprinid fish, analyzed the evolution rate and evolutionary pressure among groups, and found that CTGF proteins of cyprinid fish kept a high conservation during evolution (Kong et al. 2008). In Kong's study, the primer was designed according to the conservative region of zebrafish *ctgf* gene, but the *ctgf* type used was not pointed out. In Wuchang bream, *ctgfa* gene was expressed in tissues except the heart, liver, and gonad during embryonic period, while in adult fish, *ctgfa* gene was expressed in all tissues, but the expression quantity of *ctgfb* was much lower than that of *ctgfa*, which indicated that the *ctgf* gene had different expression patterns in different periods (Wang 2014). At present, the *ctgf* gene of grass carp has not been reported. So, in this study, the full-length sequence of *ctgf* gene of grass

carp was cloned, and bioinformatics information of this gene and tissue differential expression pattern was analyzed. The results will be used to explain the collagen synthesis of grass carp regulated by *ctgf* gene.

Materials and methods

Samples collection

The grass carp was collected from Binhai Breeding Center of Shanghai Ocean University (Shanghai, China). A fish with body weight of 154 g was used for full-length cDNA cloning, and the fish with body weight of 78 ± 0.3 g were used for tissue differential expression analysis. After sterilizing the fish body surface with alcohol, the dorsal muscle above the lateral line, and the blood, liver, spleen, kidney, heart, brain, foregut, gill, muscle, and skin were collected and deposited at − 80 °C for full-length cDNA cloning and tissue differential expression analysis, respectively.

Cloning the full-length cDNA of *ctgf*

Total RNA extraction and reverse transcription of muscle tissue

The total RNA in muscle was extracted according to the instructions of TaKaRa RNAiso Plus RNA kit (TaKaRa, BIO, Dalian, China), and the RNA quality and concentration were tested with agarose gel electrophoresis and protein and nucleic acid analyzer(Smart SpecTM Plus; Bio-Rad Laboratories, Inc. Foster City, CA, USA). The total RNA with two complete bands of 28 s and 18 s and a $OD_{260/280}$ value between 1.8 and 2.1 were selected and transcribed into cDNA by PrimeScript™ Reverse Transcriptase kit (TaKaRa, BIO, Dalian, China) and then stored at − 20 °C.

Primer design

According to the nucleotide sequences of *Danio rerio* (BC115209.1), *Salmo salar* (BT045041.1), *Takifugu rubripes* (XM_003971612.2), *Oreochromis niloticus* (XM_003440779.3), and *Megalobrama amblycephala* (KM874828.1), and the referring gene sequence of zebrafish (BC115209.1), the degenerate primers *ctgf*-F and *ctgf*-R were designed by Primer premier 5.0 software to obtain the *ctgf* fragment of grass carp (Table 1). Then, the specific primers *ctgf*-3′ and *ctgf*-5′ of the RACE were designed (Table 1) according to the cloned fragment sequence.

Ctgf fragment cloning

A 20-μL reaction system for PCR amplification was conducted, which included 1 μL muscular cDNA sample, 0.5 μL (10 μmol L^{-1}) forward primer, 0.5 μL (10 μmol L^{-1}) reverse primer, 10 μL Premix Taq™ (Ex Taq™ Version 2.0 plus dye), and 8 μL RNase-free ddH_2O. The PCR conditions were showed as follows: 95 °C 3 min for predenaturating; 95 °C30 s for denaturating, 52 °C 30 s for annealing, 72 °C 1 min for extending, 34 cycles; and 72 °C 10 min extending. PCR products were analyzed by agarose gel electrophoresis and dyed by GoldView (SBS gene, BIO, Shanghai, China). After being evaluated in specificity and brightness, the target bands were purified by agarose gel DNA purification kit (CWBIO, BIO, Beijing, China).

Ctgf RACE amplification

One microgram of total RNA of muscle tissue was used to synthesize the 3′ and 5′ RACE-Ready cDNA according to the instruction of SMART RACE kit (Clontech BIO, Beijing, China). The 3′ and 5′ specific primers showed in Table 1 (*ctgf*-3′, *ctgf*-5′) were used to perform 3′ and 5′ RACE PCR amplification by Advantage two PCR kit.

The total PCR reaction was 25 μL system, including 1.25 μL 3′/5′ RACE-Ready cDNA, 2.5 μL 10× Universal Primer A Mix, 0.5 μL *ctgf*-3′/*ctgf*-5′ primer, 17.25 μL PCR-Grade Waster, 2.5 μL 10× Advantage IPCR Buffer, 0.5 μL dNTP Mix, and 0.5 μL 50× Advantage 2 Polymerase Mix.

The 3′ RACE PCR and 5′ RACE PCR were conducted as follows: predenaturated for 3 min at 95 °C, denaturated for 30 s at 95 °C, annealed for 30 s at 62 °C for 3′ RACE or 61 °C for 5′ RACE, and extended for 3 min at 72 °C, 34 cycles, extended for 10 min at 72 °C. After evaluating specificity and brightness, the target bands of PCR products were purified by agarose gel DNA purification kit.

Ligation, transformation and sequencing of the PCR products

Using the TA cloning method, the purified products and pMD19-T vector(TaKaRa, BIO, Dalian, China) (mole

Table 1 Primers in the experiment

The name of the primer	Sequence (5′–3′)	Usage
ctgf-F	AGGTCTTTACTGTGACTACGGC	PCR
ctgf-R	GGAAATCTCAAACTTCATGGGT	PCR
ctgf-3′	CCAATGCCCCGTAGGGTGAAAG	3′ RACE
ctgf-5′	AAGTGGCACCTTCTCTGGCTGT	5′ RACE
ctgf-QF	CAAGCTGAGAGAAGGTGGAA	qRT-PCR
ctgf-QR	CAGGAGGAGCCAGATAGTCA	qRT-PCR
18s-F	GGAATGAGCGTATCCTAAACCC	qRT-PCR
18s-R	CTCCCGAGTCCAACTACAAGC	qRT-PCR

ratio of 3:1) were kept ligating for 2 h at 16 °C, and the ligation products were transformed into *Escherichia coli* (*E. coli*) DH5α (TIANGEN BIO, Shanghai, China). After vibrate-culturing for 1 h at 37 °C (200 r min^{-1}), the bacteria liquid was evenly coated on LB solid medium which contained ampicillin, IPTG, and X-gal. After cultivating at 37 °C for 14 h, the positive single colony was selected and vibrate-cultured in ampicillin liquid medium for 8 h at 37 °C. Then, the cultured bacterial liquid was tested by PCR, and the target fragment-inserted bacterial liquid was sequenced by Sangon Biotech, Shanghai, China. Finally, the sequences of *ctgf* fragment and RACE were joined together into a full-length cDNA.

Sequence analysis

ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used to obtain the ORF nucleotides of gene and the amino acid sequence of protein. Conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) tool of NCBI was used to analyze the domains of CTGF protein, and the amino acid sequence homology was analyzed by BLAST of NCBI. The evolutionary homology of CTGF amino acid sequences among grass carp and other species was analyzed by ClustalW tool of MEGA6. Then, the *ctgf* protein phylogenetic tree was constructed by MEGA6 software.

ProtParam tool at ExPASy (http://expasy.org/tools/protparam) was used to analyze the physicochemical parameters of the deduced *ctgf* protein. The online ProtScale program (http://web.expasy.org/protscale) and SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) were used to analyze the protein hydropathy profile and predict the signal peptide, respectively.

PredictProtein tool (http://www.predictprotein.org.predictprotein.org) was used to predict the secondary structure of the *ctgf* protein, and Swiss model (http://www.swissmodel.expasy.org/) was used to predict the three-dimensional structure of the deduced *ctgf* protein.

Expression analysis of *ctgf* by real-time quantitative PCR

According to the cloned fragment sequence and *18s* rRNA (EU047719.1), the real-time quantitative PCR primers of grass carp *ctgf* gene were designed by the Primer Premier 5.0 software. Primers *ctgf*-QF and *ctgf*-QR and *18s*-F and *18s*-R are shown in Table 1.

The total RNA in tissue differential expression was extracted using the same extraction method described previously. The reverse transcription reactions were performed following the manufacturer's instructions of PrimeScript RT Reagent kit with gDNA Eraser.

According to the instructions of the SYBR Premix Ex Taq kit(TaKaRa, BIO, Dalian, China), the real-time quantitative PCR was conducted with 20 μL reaction volume, which included 1 μL cDNA template, 0.5 μL upstream primer (10 μmol L^{-1}), 0.5 μL downstream primer (10 μmol L^{-1}), 10 μL SYBR Premix Ex Taq (2×), and 8 μL ddH_2O.

The real-time quantitative PCR reaction conditions were shown as follows: predenatured at 95 °C for 30 s, denatured at 95 °C for 10 s, and annealed at 60 °C for 30 s, 39 cycles. The melting curve was created after the extension at 60–95 °C, and the plate temperature was increased by 0.5 °C every 5 s. The relative expression of *ctgf* mRNA of

different tissues was calculated by $2^{-\triangle\triangle Ct}$ method, and the liver tissue with the lowest minimal $\triangle Ct$ value was employed for the calibration.

Results

Total RNA extraction and quality evaluation

The electrophoresis of total RNA showed that the sample had a good integrity (Fig. 1a). The $OD_{260/280}$ value was 1.93, and the purity concentration was 1271.11 μg mL^{-1}, which indicated that the RNA sample had a high purity and it could be used for reverse transcription and cloning.

Electrophoresis analysis of *ctgf* RACE products

The electrophoresis of *ctgf* PCR amplification products showed that the bands of target gene fragments and products of 5′ RACE, 3′ RACE were clear, bright, and single. The lengths of the three bands were 580 bp, 650 bp, and 1700 bp, respectively (Fig. 1b–d).

Nucleotide sequence analysis

The sequences of *ctgf* gene fragment, 5′ RACE, and 3′ RACE products were joined together to obtain a 2223-bp full-length cDNA sequence, and the GenBank accession number is KY024218. This sequence contained a 240-bp length 5′-untranslated region (5′ UTR), a 951-bp length 3′-untranslated region (3′ UTR), and a 1272-bp length open reading frame (ORF), encoding a 343-amino-acid peptide with an ATG start codon and a TGA stop codon. The 3′ UTR contained one canonical polyadenylation signal (AATAAA) which located at the 24-bp distance before the 30 bp poly (A) tail. This information indicated that the obtained sequence was a full-length cDNA (Fig. 2).

This grass carp *ctgf* protein contained four domains (Fig. 3), an insulin-like growth factor binding protein (IGFBP), a von Willebrand factor type C (vWC), a

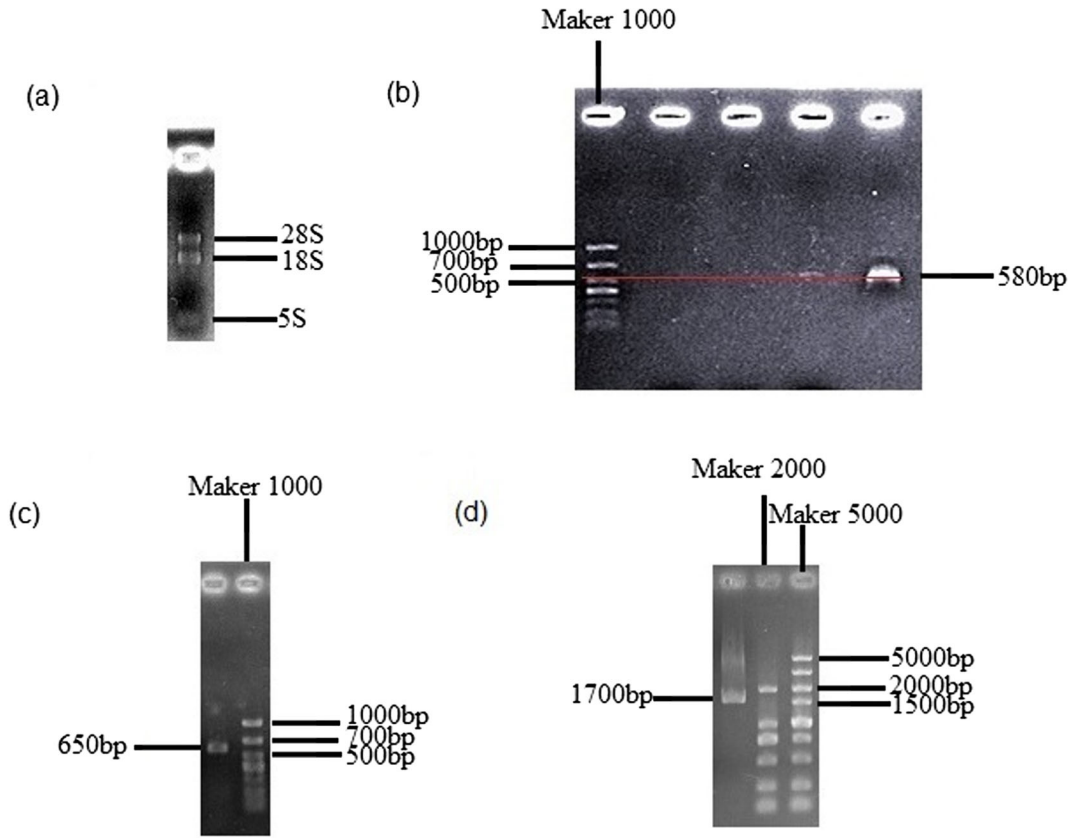

Fig. 1 The electrophoresis of total RNA and PCR products. **a** Total RNA. **b** Fragment of *ctgf*. **c** Product of 5′RACE. **d** Product of 3′ RACE

Fig. 2 The sequences of cDNA and predicted amino acid of grass carp *ctgf* gene. The uppercase letters are open reading frame, ATG is start codon, TGA is stop codon, AATAAA is polyadenylation signal, and the polyA tail is underlined

thrombospondin type-I repeat (TSP-1), and a carboxy-terminal cystine knot (CT) domain.

Homology and phylogenetic analysis

The amino acid homology analysis showed that the amino acid sequence of grass carp *ctgf* protein exhibited a homology of 98%, 96%, 91%, 91%, 90%, 88%, 85%, 85%, 84%, 84%, 84%, 83%, 82%, and 82% with that of Wuchang bream (AIZ09082.1), zebrafish (AAI15210.1), common carp (KTG05492.1), Mexican tetra (XP_007233529.1), Atlantic herring (XP_012682112.1), Atlantic salmon (NP_001133471.1), fugu rubripes(XP_003971661.1), *Stegastes partitus* (XP_008280009.1), half-smooth tongue sole (XP_008311045.1), swordtail fish (XP_005808383.2), *Nothobranchius furzeri* (XP_015803465.1), medaka (XP_004084039.1), *Tilapia nilotica*(XP_003440827.1), and *Astatotilapia burtoni* (XP_005917672.1), respectively. However, the amino acid sequence of grass carp *ctgf* protein showed a low homology of 79%, 77%, 77%, and 77% with that of human (AAH87839.1), mice (NP_034347.2), machin (NP_001271856.1), and cattle (AAI13280.1).

The *ctgf* protein phylogenetic tree (Fig. 4) showed that swordtail fish (*X. maculates*), *Nothobranchius furzeri* (*N. furzeri*), *Tilapia nilotica* (*O. niloticus*), and *Astatotilapia burtoni* (*H. burtoni*) clustered into a separate branch in turn. Then, this branch clustered together with the branch of *Stegastes partitus* (*S. partitus*), half-

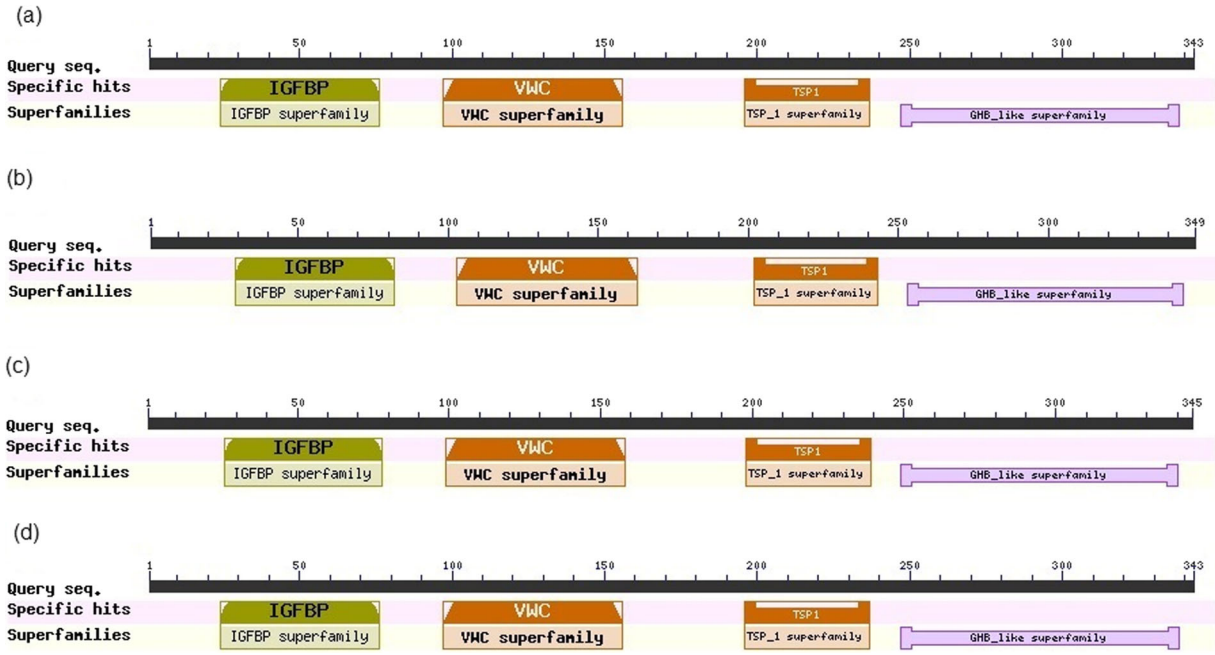

Fig. 3 The analysis of protein domains. **a** Grass carp. **b** *Homo sapiens.* **c** *Danio rerio.* **d** *Megalobrama amblycephala*

smooth tongue sole (*C. semilaevis*), medaka (*O. latipes*), and fugu rubripes (*T. rubripes*). Grass carp (*C. idella*), Wuchang bream (*M. amblycephala*), zebrafish (*D. rerio*), common carp (*C. carpio*), Mexican tetra (*A. mexicanus*), Atlantic herring (*C. harengus*), and Atlantic salmon (*S. salar*) clustered into one branch in

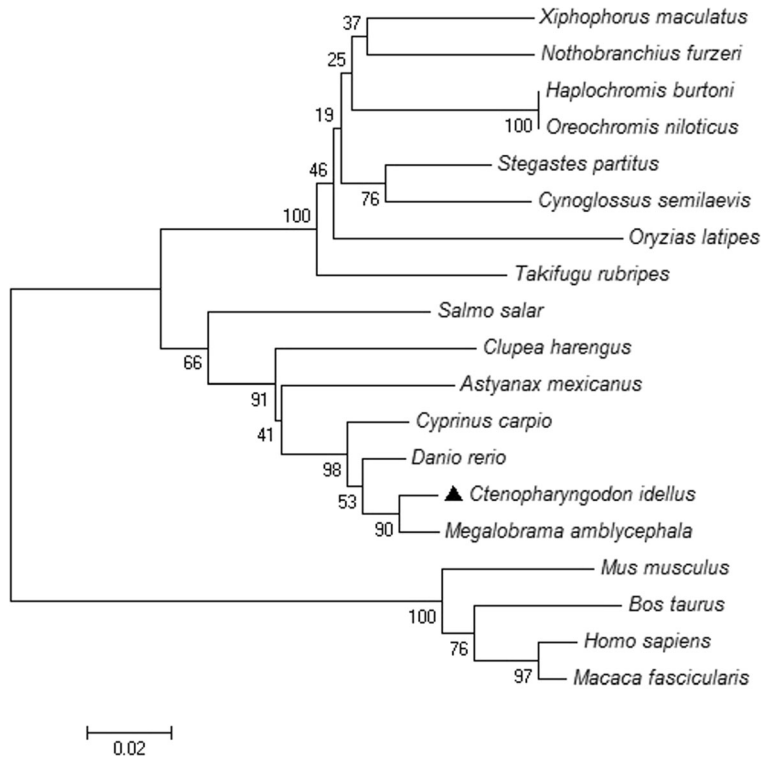

Fig. 4 Phylogenetic tree drawn with Neighbor-Joining method based on the amino acid sequences of *ctgf* protein

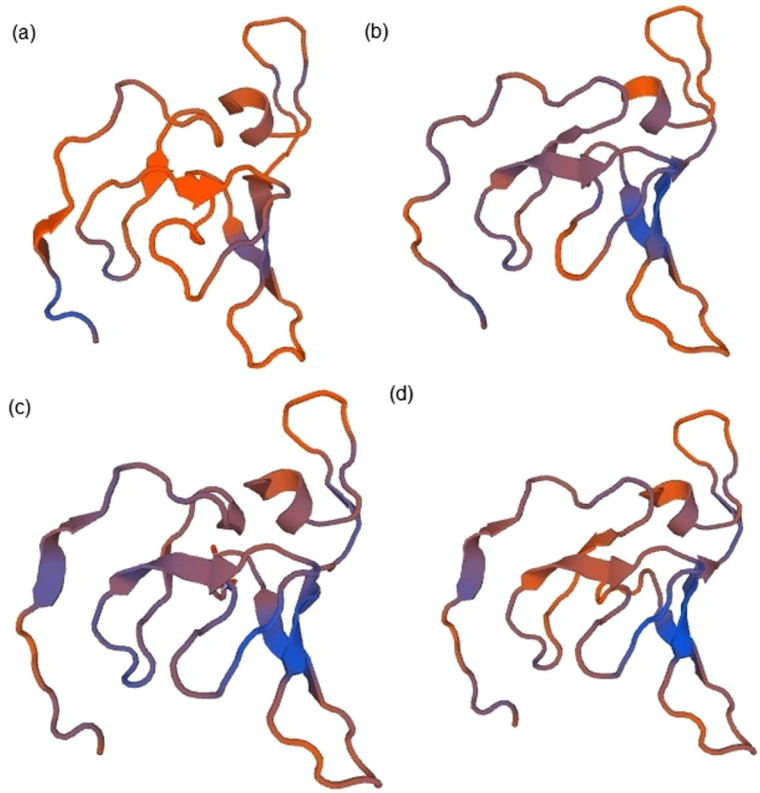

Fig. 5 Predicted tertiary structure of *ctgf* proteins. **a** Grass carp. **b** *Homo sapiens.* **c** *Danio rerio.* **d** *Megalobrama amblycephala*

turn. At last, the two branches above clustered into a big branch and then clustered together with the branch of human (*H. sapiens*), machine (*M. fascicularis*), cattle (*B. taurus*), and mice (*M. musculus*). The above result is consistent with the classification and evolutionary status of these species.

Analysis of CTGF protein

Physico-chemical properties

ProtParam showed that grass carp CTGF protein has a molecular formula of $C_{1610}H_{2578}N_{462}O_{490}S_{54}$, 343 amino acid residues, 103,699.8 Da molecular mass, and a theoretical PI of 8.22. The deduced protein has 36 negatively charged residues (Asp + Glu), 43 positively charged residues (Arg + Lys), and the instability index and aliphatic index are 54.63 and 58.78, respectively.

Hydrophobic analysis

The hydropathy profile analyzed by ProtScale showed that leucine (L) of residue 11 and cysteine (C) of residue 12 of the *ctgf* polypeptide exhibited the highest hydrophobicity (hydrophobic parameter 2.900) and arginine (R) of residue 245 exhibited the highest hydrophily (hydrophobic parameter − 2.556). The entire polypeptide chain presented a hydrophilic property.

Signal peptide analysis

The C value of residue 22 of the polypeptide was the highest, which indicated that the splice site located at the residue 22 of the polypeptide. The polypeptide values of S and D from residue 1 to 22 were high, which showed that the CTGF protein was a secreted protein, and the signal peptide located between residues 1 and 22 of this polypeptide.

Secondary and tertiary structure prediction

The predicted secondary structure of *ctgf* protein indicated that several amino acid residues participate in the secondary structures including a-helix, β-strand, and loops. The spatial conformation of the *ctgf* protein predicted by Swiss model is shown in Fig. 5. The deduced protein had an α-helix/β-

strand/loop complex, and it could be compressed into a highly compact spherical structure to exert the physiological activity. The grass carp CTGF protein showed a similar structure to that of zebrafish and Wuchang bream.

Tissue differential expression of *ctgf* gene

The liver was used as the calibrator for its lowest expression among tissues; then, the relative expression of different tissues was calculated. The *ctgf* mRNA expressed in all examined tissues, including the blood, liver, spleen, kidney, heart, brain, foregut, gill, muscle, and skin (Fig. 6). The highest expression tissue was blood ($P < 0.05$), and the second was spleen ($P < 0.05$). The expression level in the heart and gill were higher than that in the liver ($P < 0.05$). There was no significant difference among the other tissues including the kidney, brain, foregut, muscle, skin, and liver.

Discussion

Gene sequence, amino acid homology, and phylogenetic tree of *ctgf*

It was the first time to obtain the full-length cDNA sequence of *ctgf* gene of grass carp. The ORF of this sequence contains 1029 bp, encoding 343 amino acids. The amino acid sequence of grass carp CTGF protein shows a relatively high conservation with other species, which has a homology over 91% with cyprinid fishes including Wuchang bream, zebrafish, common carp, a homology of 82–90% with Atlantic herring, Atlantic salmon, and a homology of 74–79% with human and other mammals.

The phylogenetic tree of CTGF proteins showed that grass carp has a closely genetic relationship with cyprinid fishes including *Megalobrama amblycephala*, zebrafish, carp, and a relatively far distance with other fishes and farther distance with mammals, such as mice, human, cattle, and machine (Fig. 4). These molecular evolution results match with the taxonomic status of these species.

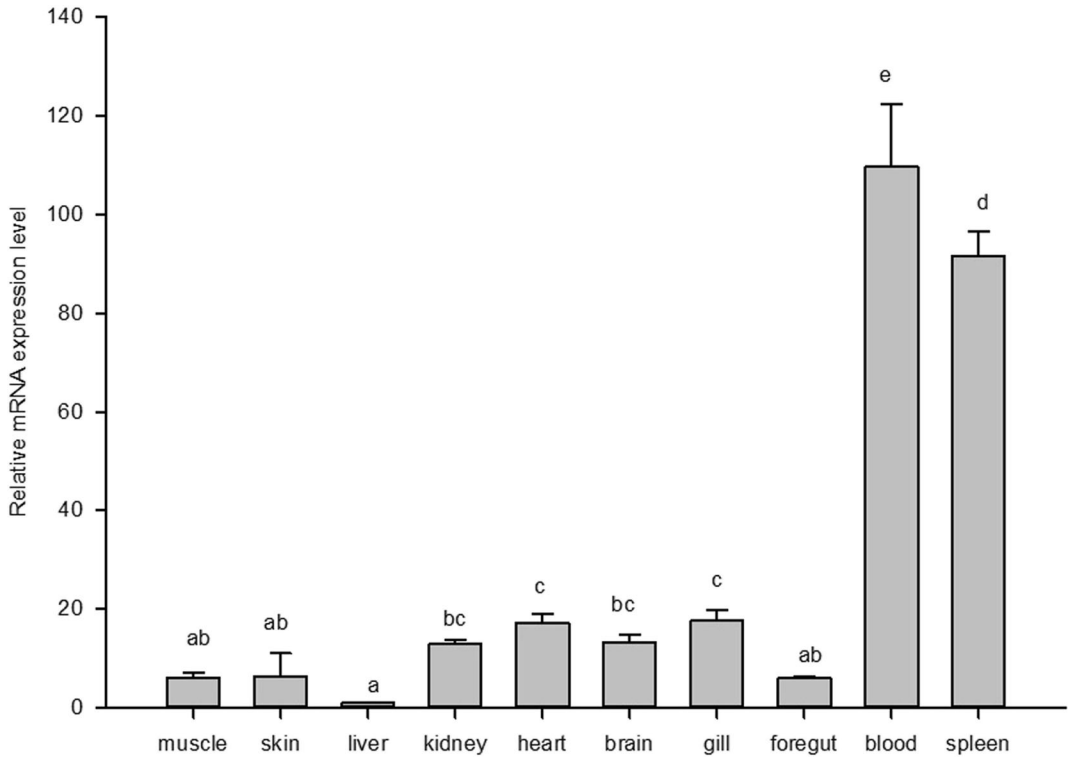

Fig. 6 The tissue differential expression pattern of *ctgf* of grass carp**.** All data and the difference values between means were analyzed under the SPSS18.0 software by one-way ANOVA. Difference values with *P* values less than 0.05 were considered significant ($P < 0.05$)

The structure of grass carp CTGF protein

The CTGF protein of grass carp has a high similarity with other species in protein domain, secondary structure, and tertiary structure. Like other species, the grass carp CTGF protein has an insulin-like growth factor binding protein (IGFBP) domain, a von Willebrand factor type C repeat (vWC) domain, a thrombospondin type 1 repeat (TSP1) domain, and a carboxyl-terminal (CT) domain. The structure and binding site of grass carp CTGF protein are in accordance with those of CTGF of human and zebrafish and also show a similarity to the CTGFa protein of Wuchang bream, which indicates that the CTGF protein in animals has the similar function. The predicted secondary and tertiary structure of grass carp CTGF protein has a-helix, β-strand, and loops, which is similar to the CTGF protein of human, zebrafish, and to the CTGFa protein of Wuchang bream. The result indicated that CTGF protein in different species had a high conservation.

The CTGF protein of grass carp, human, and zebrafish and the CTGFa protein of Wuchang bream all show a hydrophilic property, which means a high solubility in water. The CTGF protein of grass carp, human, and zebrafish is a secreted protein with a signal peptide.

Tissue differential expression of grass carp *ctgf*

In this study, the grass carp *ctgf* gene was expressed in all tissues especially high in the blood and spleen and then in the heart, gill, brain, and kidney. This result was accordance with other species including human, mice, zebrafish, common carp, and Wuchang bream. *Ctgf* of human had a high expression in bone, ovarian, and testis where rich blood vessels exist (Brigstock et al. 2003). The *ctgf* of adult mice was expressed strongly in mesenchymal cells of the cardiovascular system and gonad but expressed lowly in the kidney, liver, intestines, and heart during the embryo period (Sönke et al. 2005). In zebrafish, *ctgfa* was expressed in the heart and the developing axial vasculature during the embryo period (Fernando et al. 2010).

In common carp, *ctgf-A1* was expressed in all tissues, highly in the heart, spleen, and kidney, and *ctgf-A2* had a strong expression in gill (Kong et al. 2008). The *ctgfa* of Wuchang bream was expressed higher in the heart, gill, and intestines. All these tissues with a high expression had a common character of abundant blood capillaries. This may be related to its function in angiogenesis.

Like the *ctgf-A1* of common carp (Kong et al. 2008), the grass carp *ctgf* gene was expressed lower in the skin and meat and lowest in the liver. *Ctgfa* of Wuchang bream also had a lower expression in meat and no expression in the liver. However, there was no a reasonable explanation.

Ctgf, *ctgfa*, and *ctgfb*

In GenBank, there are several *ctgf* sequences of zebrafish, but only one literature pointed out that this gene had two copies, *ctgfa* and *ctgfb* (Fernando et al. 2010). Wuchang bream also possesses duplicated genes, *ctgfa* and *ctgfb*. The phylogenetic tree of *ctgf* protein showed that the *ctgfa* of the two fishes had a highly homology with other cyprinid fishes, while the *ctgfb* of the two fishes just shared homology with each other (Wang 2014). The study by Hui showed that common carp had four sequences, *ctgf-A1*, *ctgf-A2*, *ctgf-B1*, and *ctgf-B2* (Kong et al. 2008), but no sequence information was found in GenBank.

Meyer thought that the entire genome of ray-finned (actinopterygian) fishes had one more duplication than vertebrates (3R hypothesis), leading, at least initially, to up to eight copies of the ancestral deuterostome genome (Meyer and Schartl 1999). The term fish-specific genome duplication, 3R, was also applied to all, even sarcopterygian (lobe-finned fishes and tetrapods). Therefore, the genome of actinopterygian and sarcopterygian possessed originally only half as many genes compared to the derived fishes (Meyer and Peer 2005). Most duplicated genes were secondarily lost, yet some evolved new functions (Fernando et al. 2010). The *ctgfa* of zebrafish and Wuchang bream had a closer relationship with the *ctgf* of human, mice, and xenopus than *ctgfb*. It was speculated that *ctgf* gene of zebrafish and Wuchang bream might exist genetic mutations during the evolution process (Wang 2014).

In this study, the cloned *ctgf* gene of grass carp showed a high homology with the *ctgf* of human, machine, cattle, mice, and other bunch of aquatic animals. Especially, after blasting the *ctgf* sequence of grass carp with the *ctgfa* and *ctgfb* of zebrafish and Wuchang bream, and with the *ctgf*-A1, *ctgf*-A2, *ctgf*-B1 and *ctgf*-B2 of common carp, we found that the *ctgf* sequence of grass carp had a high homology with the *ctgfa* of zebrafish, Wuchang bream, and the *ctgf*-A1 and *ctgf*-

A2 of common carp but showed no homology with *ctgfb* of zebrafish and Wuchang bream and with *ctgf*-B1 and *ctgf*-B2 of common carp.

The function of *ctgf* gene

The *ctgf* gene is involved in the proliferation, migration, adhesion of cell, and the remodeling of extracellular matrix through various signal pathways (Leask and Abraham 2006), and it also plays an important role in the angiogenesis (Lau and Lam 1999), the constituent of collagen (Grotendorst et al. 1996), and the formation of bone and cartilage (Nishida et al. 2000; Nakanishi et al. 2000). *Ctgf* could induce the fibrosis and the expression of collagen as the cofactor of TGFβ via the induction of the transcription factors of Smad family (Perbal and Takigawa 2005). *Ctgf* can also promote the synthesis of extracellular matrix and collagen without the Smad signaling pathway in mouse embryonic fibroblast cells (Leask and Abraham 2006; Mori et al. 2008).

The expression of *ctgf* showed a significantly positive correlation with the fibrosis degree (Ihn 2002). The overexpression of *ctgf* cooperated with TGFβ was found in many fibrotic diseases (Moussad and Brigstock 2000; Beddy et al. 2006) including skin fibrosis (Igarashi et al. 1995), liver fibrosis (Tamatani et al. 1998), and intestinal fibrosis (Dammeier et al. 1998). In the *ctgf* blocked liver of rat, the liver fibrosis induced by chemical drug was effectively prevented (Li et al. 2006).

Ctgf has a physiological significance in the repair of wounded tissue and the improvement of pathological tissue fibrosis (Igarashi et al. 1993). The overexpression of *ctgf* was considered as a symbol of tissue fibrosis (Blom et al. 2001; Leask and Abraham 2006), but the overexpression happened only in a short period after the tissue fibrosis (Mori et al. 1999; Bonniaud et al. 2003). Igarashi found that the overexpression of *ctgf* occurred at the ninth day in the injured rat, while the overexpression of TGFβ occurred at the third day, which indicated that TGFβ started the wound repair and caused the *ctgf* overexpressed to repair the wounded tissue (Igarashi et al. 1993). *Ctgf* can promote the proliferation and the differentiation of osteoblast and chondrocyte (Nishida et al. 2000; Nakanishi et al. 2000). The *ctgf* knockout rat showed a hypertrophy of cartilage cells, a decrease of osteoblasts in cartilage, a thoracic deformity, and a quick death after birth (Ivkovic et al. 2003).The transgenic mice, which overproduced *ctgf* under the control of XI collagen promoter, could develop into dwarfism within a few months after birth due to the reduced bone density. The possible reason was that the overexpression of *ctgf* resulted in the ossification of cartilage cells before the normal maturation (Nakanishi et al. 2001).

As an effective angiogenesis factor, *ctgf* can promote the angiogenesis in the development of blood vessel (Zhang and Lin 2006). Via integrin αvβ3, *ctgf* can mediate the proliferation and adhesion of vascular endothelial cell and induce the formation of blood vessels (Shimo et al. 1998). Shimo found that the differentiation and migration of endothelial cells of normal blood vessel were inhibited when *ctgf* gene was knocked out in rat (Shimo et al. 1998).

High expression of *ctgf* has been shown to correlate with tumor stage and patient prognosis (Grotendorst et al. 1996). The overexpression of *ctgf* was found in various human malignant tumors such as breast cancer (Xie et al. 2001), lymphatic leukemia (Vorwerk et al. 2002), and hepatoma (Zeng et al. 2004). The antibody of rat *ctgf* can suppress the growth of subcutaneous tumor (Shimo et al. 2006).

Although functional analysis was not carried out in this study, the abundant literatures of other species showed the potential importance of *ctgf* in grass carp, which needs a further study in the future.

Conclusion

The *ctgf* gene of grass carp has a full length of 2223 bp, which encodes 343 amino acids. The CTGF protein is a hydrophilic and a secretary protein with a molecular mass of 37,978.2 Da and an isoeletronic point of 8.22. The signal peptide locates between residues 1 and 22 of the polypeptide chain. The *ctgf* gene has the highest expression in the blood and spleen and then the kidney, heart, gill, and brain. The lowest expression tissue is the liver. The results are consistent with the function of this gene, and the results will be helpful to study the muscular fibrosis in the future.

Authors' contributions WenQian Pan, XiangJun Leng and XiaoQin Li conceived, designed the experiments, analyzed data and wrote the manuscript together. WenQian Pan, JunPeng Wang, Zhihan Tu, Tian Gan, Jing Hu and Jing Wei collected the samples and performed the experiments.

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