



# Molecular characterization, expression, and immunological response analysis of the *TWEAK* and *APRIL* genes in grass carp, *Ctenopharyngodon idella*

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Genet. Mol. Res. 13 (4): 10105-10120 (2014)

Received November 4, 2013

Accepted January 27, 2014

Published December 4, 2014

DOI <http://dx.doi.org/10.4238/2014.December.4.5>

**ABSTRACT.** *TWEAK* and *APRIL* are important members of the TNF superfamily, which play a crucial role in several diseases. Here, we describe the identification of grass carp (*Ctenopharyngodon idella*) homologs of *TWEAK* and *APRIL* (designated *gcTWEAK* and *gcAPRIL*, respectively) and their response to *Aeromonas hydrophila* and *Aquareovirus* infection. The *gcTWEAK* cDNA sequence contains 2273 bases with an open reading frame of 753 bases encoding 250-amino acid residues. The *gcTWEAK* protein contains a predicted transmembrane domain, a putative furin protease cleavage site, 3 conserved cysteine residues, and a typical TNF homology domain. The *gcAPRIL* cDNA sequence contains 1408 bases with an open reading frame of 747 bases encoding 248-amino acid residues. The *gcAPRIL* protein contains a

predicted transmembrane domain, a putative furin protease cleavage site, 2 conserved cysteine residues, and a typical TNF homology domain corresponding to other, known *APRIL* homologs. Reverse transcription-polymerase chain reaction analysis shows that both *gcTWEAK* and *gcAPRIL* transcripts are predominantly expressed in the skin, spleen, and head kidney, and they are significantly upregulated in most immune tissues by *A. hydrophila* and *Aquareovirus* infections. Our results demonstrate that liver is the most responsive tissue against bacterial infection, whereas gill is the most responsive tissue against viral infection. The association of increased *gcTWEAK* and *gcAPRIL* expression after bacterial and viral infections suggests that they play a potentially important role in the immune system of fish.

**Key words:** Grass carp; *TWEAK*; *APRIL*; *Aeromonas hydrophila*; *Aquareovirus*; mRNA expression

## INTRODUCTION

The tumor necrosis factor (TNF) superfamily of cytokines represents a wide group of proinflammatory cytokines, which activate cellular signaling pathways involved in cell survival, inflammation, apoptosis, lymphocyte homeostasis, and cellular differentiation (Wiens and Glenney, 2011). At present, about 19 members of this superfamily have been reported in mammals (Zhu et al., 2013). The TNF-like weak inducer of apoptosis (*TWEAK*; also named TNFSF12, Apo3L, and CD255) and a proliferation-inducing ligand (*APRIL*; also named TNFSF13a, TALL-2, TRDL-1, and CD256) proteins are important members of the TNF superfamily. *TWEAK* acts both as a homotrimeric type II transmembrane protein and a cleaved soluble molecule. It is cleaved at the cell surface by furin, a convertase that processes many inactive precursors including hormones, growth factors, and receptors (Molloy et al., 1999). This processing at the furin cleavage site, which is located on the N-terminal side of the TNF homology domain, permits the release of a soluble form of *TWEAK*, which weakly induces apoptosis and plays a role in angiogenesis (Jakubowski et al., 2002). *TWEAK* acts as a ligand to a TNF receptor superfamily member, fibroblast growth factor-inducible molecule 14 (Fn14). The *TWEAK* protein in vertebrates contains a transmembrane domain, a furin cleavage site, and 3 conserved cysteine residues. When not fused to *APRIL*, the full-length *TWEAK* is a multifunctional cytokine, regulating cell proliferation, differentiation, migration, apoptosis, angiogenesis, and inflammation (Winkles, 2008). Based on the cell type, *TWEAK* induces apoptosis through caspase-dependent or -independent pathways and can provoke cathepsin B-mediated necrosis as well (Gao et al., 2009).

*APRIL* is a homotrimeric type II transmembrane protein that also exists in a soluble form that is derived from the intracellular cleavage of the full-length protein (Hahne et al., 1998). *APRIL* does not exist in a membrane-bound form, but it is processed within the Golgi apparatus by a furin pro-protein convertase before secretion of the biologically active form (López-Fraga et al., 2001). The *APRIL* protein in vertebrates contains a transmembrane domain, a furin cleavage site, and 2 conserved cysteine residues (Hahne et al., 1998; Cui et al., 2012; You et al., 2012). It is closely related to the B-cell activating factor (BAFF), which is another member of the TNF superfamily. Both *APRIL* and BAFF bind with high affinity to 2

members of the TNF-receptor superfamily, transmembrane activator and cyclophilin ligand interactor and B-cell maturation antigen (Mackay et al., 2003; Bossen and Schneider, 2006). *APRIL*, BAFF, and their receptors (termed the BAFF/*APRIL* system) play important immunological roles in vertebrates, especially in the B-cell arm of the immune system (Mackay et al., 2003, 2007). Although *APRIL* is expressed in macrophages, monocytes, dendritic cells, and lymphoid cells, it is also abundantly expressed in cells outside the immune system, including osteoclasts and tumor tissues where it is directly secreted without cell-surface expression (Hahne et al., 1998; Moreaux et al., 2004). *APRIL* has the ability to stimulate the proliferation of various tumor cell lines including Jurkat T cells and MCF-7 carcinoma cells. It also stimulates the proliferation of B and T cells, and it serves an important role in immunological responses, such as B-cell survival, immunoglobulin secretion, isotype switching, and T-independent antibody responses (Castigli et al., 2004; Dillon et al., 2006; Mackay et al., 2007). In human, the *TWEAK* gene is positioned 878 bp upstream of the *APRIL* gene. *APRIL* can be attached to the cell surface with *TWEAK*, which is called TWE-PRIL, and share receptors (Zhu et al., 2013). These factors form a network of mediators that interact with an overlapping set of receptors.

Grass carp (*Ctenopharyngodon idella*) is one of the most important aquaculture species in the world. However, this fish is highly susceptible to bacterial and viral pathogens, especially *Aeromonas hydrophila* and *Aquareovirus* (Jang et al., 2010; Zhang et al., 2010c). A better understanding of the immune defense mechanisms of grass carp and the identification of crucial genes might be the key steps to the development of management strategies for disease control and long-term sustainability of grass carp farming. Although *TWEAK* and *APRIL* play important immune roles in mammals, their disease-specific roles in teleost fishes have not been well studied. In this study, we identified the grass carp ortholog of the mammalian *TWEAK* and *APRIL* genes and analyzed their expression profiles in response to bacterial and viral infections. We demonstrate that the grass carp *TWEAK* and *APRIL* genes are predominantly expressed in the immune tissues such as skin, spleen, and head kidney, and they are important immune genes against *A. hydrophila* and *Aquareovirus* infections.

## MATERIAL AND METHODS

### Fish rearing, microbial challenge, and sampling

Adult and juvenile grass carps were obtained from the Nanhui Fish Farm of Shanghai and acclimated in a circulating water system at 25°C for 1 week. For full-length cDNA cloning and expression analysis, various tissues including gill, brain, muscle, skin, liver, trunk kidney, head kidney, heart, spleen, intestine, and blood were collected from 4 adult grass carp that were about 2500 g in size. All animal experiments were performed with the approval of the Shanghai Ocean University Animal Care and Use Committee and in full compliance with its ethical guidelines.

Challenge experiments were conducted as previously described by Liu et al. (2010). For the bacterial challenge experiment, 32 juvenile fish that were about 100 g in size were evenly divided into 2 groups (challenged and control) and cultured under similar conditions in a circulating water system at 25°C. Fish from the challenged group were injected intraperitoneally with formalin-killed *A. hydrophila* S2 (obtained from the Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of  $2.0 \times 10^6$  cells suspended in 100  $\mu$ L

phosphate-buffered saline (PBS) per fish; fish from the control group were injected with 100  $\mu$ L sterile PBS per fish. Four fish were sampled 4 h and 1, 3 and 7 days post-injection from each group. Liver, kidney, spleen, gill, and skin samples were collected from each fish for total RNA isolation.

For the viral challenge experiment, 32 juvenile fish were evenly divided into 2 groups (challenged and control) and cultured under the same conditions in a circulating water system at 25°C. Fish from the challenged group were injected intraperitoneally with *Aquareovirus* (GCRV-JX01) (obtained from the Aquatic Pathogen Collection Centre of Ministry of Agriculture, China) at a dose of  $1.0 \times 10^7$  TCID<sub>50</sub>/mL suspended in 200  $\mu$ L PBS per fish; fish from the control group were injected with 200  $\mu$ L sterile PBS per fish. Four fish were sampled 4 h and 1, 3, and 7 days post-injection from each group. Liver, kidney, spleen, gill, and skin samples were collected from each fish for total RNA isolation.

All tissues were flash frozen in liquid nitrogen during collection followed by storage at -80°C until RNA extraction. The total RNAs were isolated using TRIZOL reagent (Invitrogen, USA) following the manufacturer protocol. All RNA samples were treated with RNase-free DNase (TaKaRa, Japan), quantified on a Nanodrop 2000C spectrophotometer (Thermo Scientific, USA), and stored at -80°C.

### Cloning of full-length *gcTWEAK* and *gcAPRIL* cDNA

First-strand cDNA was synthesized from the total spleen mRNA using the High Fidelity PrimeScript reverse transcription-polymerase chain reaction (RT-PCR) Kit (TaKaRa). A pair of primers for each gene (i.e., F: 5'-CAGGTGAATATGCAGCGTAAG-3' and R: 5'-GACTCTGGTTCTCATTGAAGTG-3' for *gcTWEAK* and F: 5'-TCTCCATCTGGTTCCTGTGTC-3' and R: 5'-TCAGCACCTGACTGTCAATG-3' for *gcAPRIL*) were designed based on the conserved coding region of the full-length cDNA sequences of zebrafish (GenBank accession No. NM\_001076607 and NP\_001161936, respectively) using the Primer Premier 6 program. The PCR program for *gcTWEAK* was as follows: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and 1 cycle of 72°C for 10 min. The PCR program for *gcAPRIL* was as follows: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 2 min; and 1 cycle of 72°C for 10 min. The PCR products were ligated into the pGEM-T easy vector (Promega, USA), transformed into competent *Escherichia coli* DH5 $\alpha$  cells, plated on Luria broth-agar medium and incubated overnight at 37°C. Positive clones containing the insert with the expected size were identified by colony PCR. Five of the positive clones for each gene were picked and sequenced on an ABI PRISM 3730XL Automated Sequencer using BigDye terminator v3.1 (Applied Biosystems, USA).

To obtain the full-length cDNA sequence, 3'-rapid amplification of cDNA ends (RACE) and 5'-RACE were performed by nested PCR using gene-specific primers and universal primers (Table 1). The RT- and RACE-PCRs were conducted with the SMART RACE cDNA Amplification Kit and Advantage 2 PCR Kit (Clontech, Palo Alto, CA, USA). The PCR conditions for the 3'-RACE was 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and 1 cycle of 72°C for 10 min. Similarly, the PCR conditions for the 5'-RACE was 5 cycles of 94°C for 30 s and 72°C for 3 min; 5 cycles of 94°C for 30 s, 70°C for 30 s, and 72°C for 3 min; and 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. PCR products were cloned and sequenced as described above.

## Bioinformatic analysis

The nucleotide and amino acid sequence identity were performed using the Basic Local Alignment Search Tool program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The open reading frame (ORF) of *gcTWEAK* and *gcAPRIL* cDNAs were predicted using the ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>), and the predicted transmembrane domain was based on the TMHMM Server v. 2.0 program (<http://www.cbs.dtu.dk/services/TMHMM/>). The putative furin cleavage site was identified using ProP 1.0 Server (<http://www.cbs.dtu.dk/services/ProP/>). The needle program (<http://www.ebi.ac.uk/Tools/emboss/align/>) was used to determine the identities between different sequences. Multiple-sequence alignments were performed using the CLUSTALW 1.8 program (Jeanmougin et al., 1998). The phylogenetic tree was constructed based on the deduced full-length amino acid sequences using the neighbor-joining algorithm in MEGA version 4.1, and the analysis reliability was assessed by 1000 bootstrap replicates.

## Real-time quantitative (q)PCR analysis

For real-time qPCR, first-strand cDNA was synthesized from total spleen RNA using the PrimerScript First-Strand cDNA Synthesis Kit (TaKaRa) following manufacturer instructions. Primers were designed based on the *gcTWEAK* and *gcAPRIL* cDNA sequences presented in this study (Table 1). A 10-fold dilution series of cDNAs containing target gene fragments was used to construct standard curves. The amplification efficiency was measured from the regression slope of the standard curve. Only the primer pairs that had a single peak in the melting curve analysis and displayed an amplification efficiency close to the theoretical 100% were retained for further use. The amplification of grass carp  $\beta$ -actin mRNA was performed to confirm the steady-state level of expression of a housekeeping gene and to serve as an internal control for the *gcTWEAK* and *gcAPRIL* gene expression analysis. The reaction consisting of the same reaction mixture without template was conducted as a negative control. Real-time qPCR was performed in triplicate for each sample using SYBR Premix EX Taq (TaKaRa) on a CFX96™ Real-Time System (BIO-RAD, USA). The PCR parameters for both genes were as follows: 95°C for 3 min, 39 cycles at 95°C for 5 s and 60°C for 30 s, and a dissociation curve analysis of 5 s per step from 65° to 95°C. Relative expression analyses were based on the level of *gcTWEAK* and *gcAPRIL* gene expression normalized against  $\beta$ -actin according to the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

**Table 1.** Oligonucleotide primers used to amplify the *gcTWEAK* and *gcAPRIL* genes.

Primer	<i>gcTWEAK</i> sequence (5'-3')	<i>gcAPRIL</i> sequence (5'-3')	Application
F	CAGGTGAATATGCAGCGTAAG	TCTCCATCTGGTTCCTGTGTC	Amplification of cDNA
R	GACTCTGGTTCATTGAAGTG	TCAGCACCTGACTGTCAATG	Amplification of cDNA
3-RACE1	GAGTCAGAGGTTCCGCAGAGAT	TCTCCATCTGGTTCCTGTGTCCTC	3'-RACE outer PCR
3-RACE2	GAGTCATTAAGGATGGACTGA	CACTGTTCTGTCTTGGGCACTAGG	3'-RACE inner PCR
5-RACE1	GGTGCCGGTCTCTGGAITATAATG	CAGCACCTGACTGTCAATGAAGTAG	5'-RACE outer PCR
5-RACE2	ATTTCCTTTCCTCCCTCCACATC	TGCGAGGACACAGGAACCAGATG	5'-RACE inner PCR
RT-F	ACGCAAGTGCCTTCCATTA	ATAACATGGACAGCCTTGC	Real-time PCR
RT-R	GCAGCCAGACCGACAGAGTA	CAACTTGCCAGTGTTC AAC	Real-time PCR
$\beta$ -actin-F	GCTATGTGGCTCTTGACTTCG	GCTATGTGGCTCTTGACTTCG	Real-time PCR (control)
$\beta$ -actin-R	CGAAAGACTCCATACCCAAG	CGAAAGACTCCATACCCAAG	Real-time PCR (control)

## Statistical analysis

Data from the real-time qPCR were analyzed statistically by analysis of variance using SPSS (version 16.0) statistical software (SPSS Inc., USA). Differences between means were considered to be significant at the 95% confidence level ( $P < 0.05$ ). All data are reported as means  $\pm$  standard error (SE).

## RESULTS

### Analysis of gcTWEAK and gcAPRIL cDNA

Full-length cDNAs encoding gcTWEAK and gcAPRIL from *C. idella* were obtained through RT-PCR and RACE (GenBank Accession Nos. KF267443 and KC759144, respectively). The gcTWEAK cDNA consisted of 2273 nucleotides, which encoded a protein of 250 amino acids with a calculated molecular mass of about 28.663 kDa and theoretical isoelectric point (pI) of 10.22. The gcTWEAK protein contained a predicted transmembrane domain (TMD) of 23 amino acids (residues 29-51), a putative furin cleavage site (RFRR; position 98), 3 conserved cysteine residues (Cys165, Cys191, and Cys212), and a TNF domain spanning amino acid residues 120 to 249 (Figure 1A).

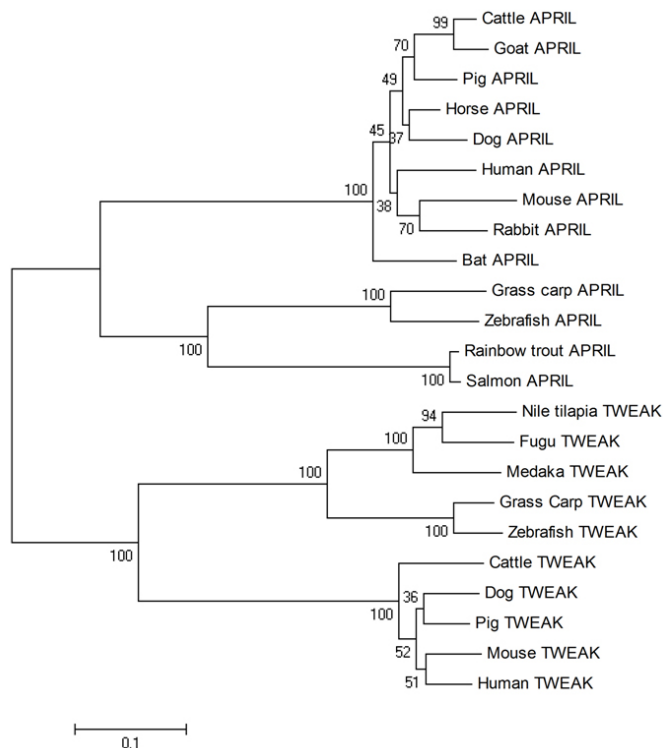
The full-length cDNA of gcAPRIL consisted of 1408 nucleotides, which encoded a protein of 248 amino acids with a calculated molecular mass of about 28.131 kDa and pI of 9.26. The gcAPRIL protein contained a predicted TMD of 23 amino acids (residues 21-43), a putative furin cleavage site (RKRR; position 108), 2 conserved cysteine residues (Cys194 and Cys209), and a TNF domain spanning amino acid residues 114 to 248 (Figure 1B).

### Multiple-sequence alignment and phylogenetic analysis

In order to study the evolutionary relationship of gcTWEAK and gcAPRIL with other known teleost TWEAK and APRIL proteins, amino acid sequences corresponding to representative orthologs from several species were aligned with those of gcTWEAK and gcAPRIL using the ClustalW software. The deduced amino acid sequence of gcTWEAK showed high homology with the TWEAK sequences of other teleosts in the GenBank database, such as *Danio rerio* (91.0% similarity), *Oryzias latipes* (74.0% similarity), and *Takifugu rubripes* (73.0% similarity). Similarly, the deduced amino acid sequence of gcAPRIL showed high homology with *D. rerio* (73.0% similarity), *Salmo salar* (63.0% similarity), and *Oncorhynchus mykiss* (63.0% similarity).

The phylogenetic tree was divided into 2 different groups with one containing TWEAK sequences and the other containing APRIL sequences. Each of the TWEAK and APRIL groups also had 2 distinct clades with one containing all teleost fish sequences and the other containing mammalian and avian sequences. gcTWEAK and gcAPRIL were clustered with orthologous teleost proteins, and they were most similar to the *D. rerio* orthologs (Figure 2).





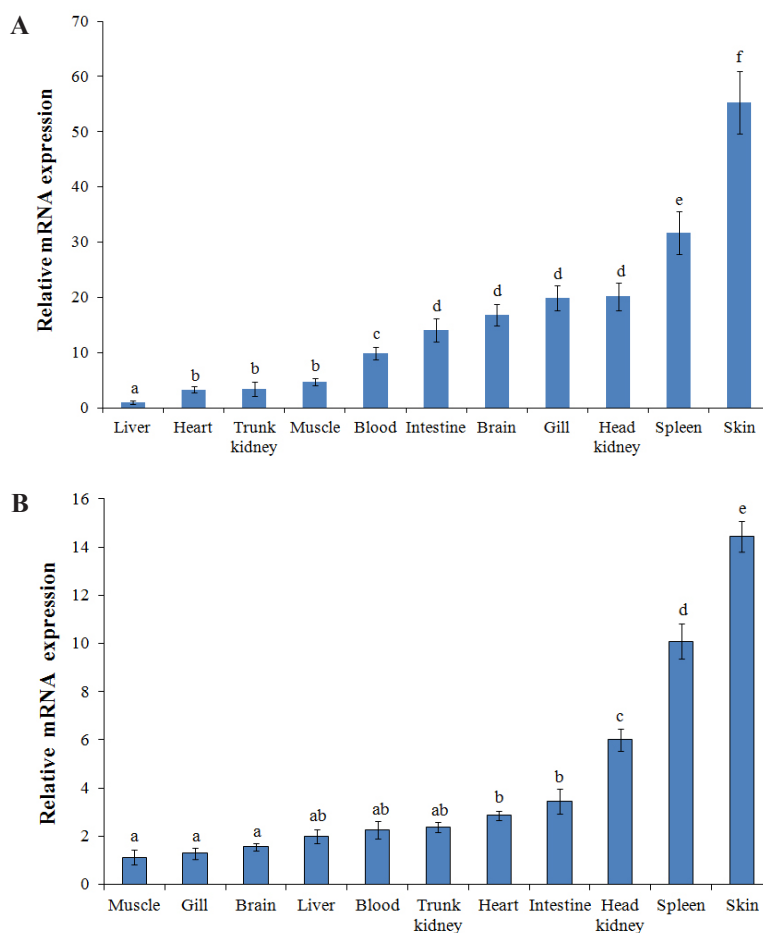
**Figure 2.** Phylogenetic tree showing the relationship of the *gcTWEAK* and *gcAPRIL* amino acid sequences with other identified *TWEAK* and *APRIL* sequences. The tree was constructed by the neighbor-joining method (Tree Top Program) using ClustalW and MEGA 4.1 packages and was bootstrapped 1000 times. The GenBank accession numbers of the sequences used for the phylogenetic analysis are as follows: Cattle (*Bos taurus*) *APRIL*, ACT66298.1; Goat (*Capra hircus*) *APRIL*, ADC55525.1; Pig (*Sus scrofa*) *APRIL*, NP\_001106160.1; Horse (*Equus caballus*) *APRIL*, ADZ54789.1; Dog (*Canis lupus familiaris*) *APRIL*, ACG70196.1; Human (*Homo sapiens*) *APRIL*, AAC61312.1; Mouse (*Mus musculus*) *APRIL*, AAG22534.1; Rabbit (*Oryctolagus cuniculus*) *APRIL*, NP\_001099146.1; Bat (*Vespertilio sinensis*) *APRIL*, AFB81870.1; Zebrafish (*Danio rerio*) *APRIL*, NP\_001161936.1; Rainbow trout (*Oncorhynchus mykiss*) *APRIL*, NP\_001118143.1; Salmon (*Salmo salar*) *APRIL*, NP\_001135076.1; Cattle *TWEAK*, NP\_001232883.1; Dog *TWEAK*, NP\_001192097.1; Pig *TWEAK*, NP\_001106161.1; Mouse *TWEAK*, NP\_035744.1; Human *TWEAK*, AAC51923.1; Zebrafish *TWEAK*, NP\_001070075.1; Nile tilapia *TWEAK*, XP\_003449424.1; Fugu *TWEAK*, XP\_003966992.1; and Medaka *TWEAK*, XP\_004079870.1.

### Expression of *gcTWEAK* and *gcAPRIL* mRNA in different tissues

The *gcTWEAK* and *gcAPRIL* mRNA expression levels in 11 tissues of normal fish were examined using real-time PCR normalized against  $\beta$ -actin levels. A dissociation curve showing a single peak at the melting temperature that was expected for that amplicon suggested specific amplification. Expression was determined as fold-increase  $2^{-\Delta\Delta C_t}$  levels relative to the tissue with the lowest expression set to 1. As shown in Figure 3A, the *gcTWEAK* mRNA transcripts were expressed in a wide range of tissues with a varied pattern of expression. The highest expression was observed in the skin (55.3-fold), followed by spleen (31.7-fold) and head kidney (20.2-fold) ( $P < 0.05$ ); its expression was very low in the liver (the calibrator), heart (3.3-fold), and trunk kidney (3.4-fold) (Figure 3A). Similarly, the *gcAPRIL* mRNA tran-



scripts were highly expressed in the skin (14.4-fold), followed by spleen (10.1-fold) and head kidney (6.0-fold) ( $P < 0.05$ ); its expression was very low in the muscle (the calibrator), gill (1.3-fold), and brain (1.6-fold) (Figure 3B).



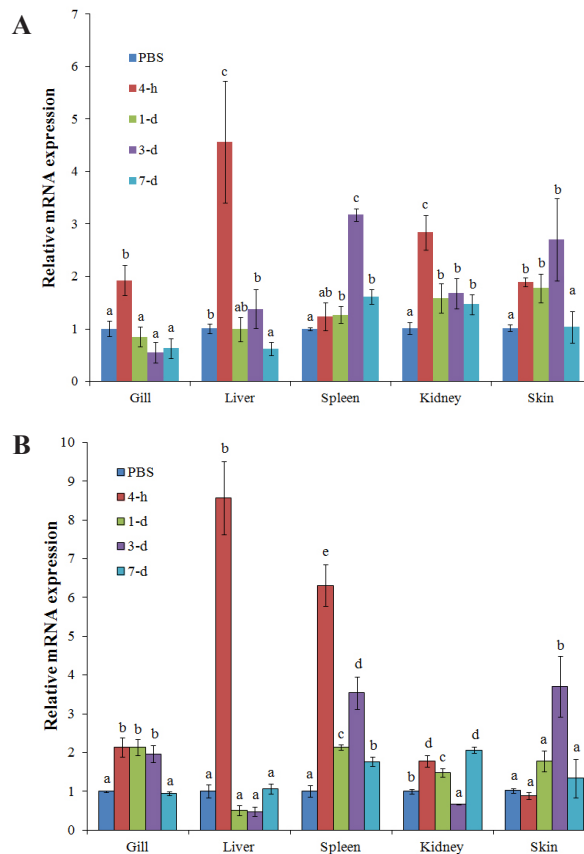
**Figure 3.** Tissue expression profiles of the *gcTWEAK* (A) and *gcAPRIL* (B) genes. Amounts of mRNA levels obtained by quantitative real-time polymerase chain reaction (qPCR) are indicated. The relative expression of each particular gene transcript was calculated based on the standard curve and normalized to the  $\beta$ -actin mRNA level. Data from qPCR are reported as means  $\pm$  SE. Results shown are the mean of 4 individual RNA samples of fish ( $N = 4$ ). The data with different letters indicate a significant difference at  $P < 0.05$ .

### Expression of the *gcTWEAK* and *gcAPRIL* mRNA in different tissues after challenge with *A. hydrophila*

The expression patterns of *gcTWEAK* and *gcAPRIL* mRNA were investigated in the gill, liver, spleen, kidney, and skin of grass carp 4 h and 1, 3, and 7 days post-injection with *A. hydrophila* using real-time PCR. The relative expression was determined as fold-changes relative to the PBS-injected control samples. After infection with *A. hydrophila*, both *gcTWEAK* and

*gcAPRIL* mRNAs were upregulated in a tissue-specific manner. The *gcTWEAK* expression was significantly upregulated in gill, liver, kidney, and skin 4 h post-challenge ( $P < 0.05$ ), whereas it was significantly upregulated in spleen 3 days post-challenge (Figure 4A). The highest up-regulation was observed in liver (4.6-fold) and spleen (3.2-fold) 4 h and 3 days post-challenge, respectively. In most tissues, the high level of *gcTWEAK* mRNA expression continued up to 3 days after the challenge and returned to almost normal levels after 7 days.

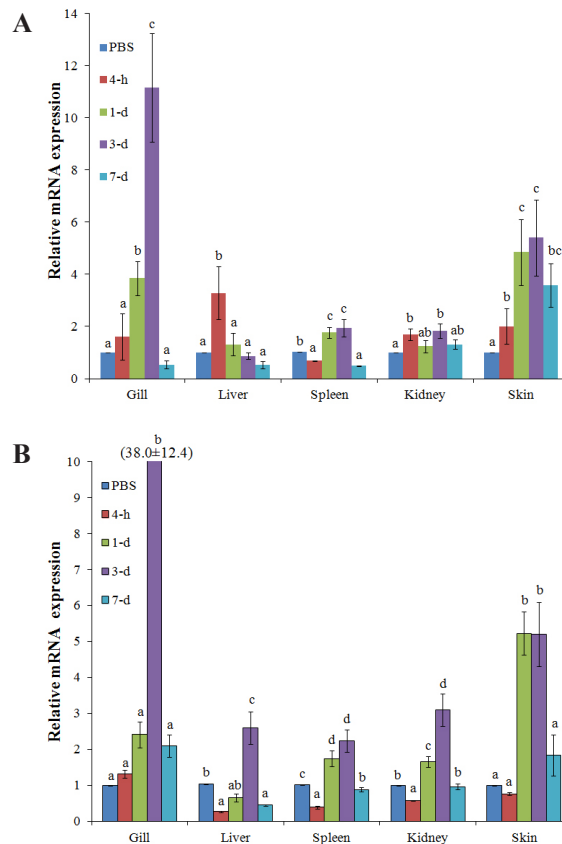
A similar pattern of expression of *gcAPRIL* mRNA was observed in the gill, liver, spleen, kidney, and skin of grass carp. Its expression was significantly upregulated in gill, liver, spleen, and kidney 4 h post-challenge ( $P < 0.05$ ), whereas it was significantly upregulated in skin 3 days post-challenge (Figure 4B). The highest upregulation was observed in liver (8.6-fold) and spleen (6.3-fold) 4 h post-challenge. In most tissues, the high level of *gcAPRIL* mRNA expression continued up to 3 days after the challenge and returned to almost normal levels after 7 days.



**Figure 4.** Quantitative expression profiles of the *gcTWEAK* (A) and *gcAPRIL* (B) genes after challenge with *Aeromonas hydrophila*. Control samples were injected with phosphate-buffered saline (PBS). The relative expression of each particular gene transcript is shown as a fold-change over PBS-injected control samples as normalized to changes in expression in the  $\beta$ -actin mRNA level. Data from qPCR are reported as means  $\pm$  SE. Results shown are the mean of 4 individual RNA samples of fish ( $N = 4$ ). The data with different letters within the same tissue group indicate a significant difference at  $P < 0.05$ .

### Expression of the *gcTWEAK* and *gcAPRIL* mRNA in different tissues after challenge with *Aquareovirus*

The expression patterns of *gcTWEAK* and *gcAPRIL* mRNA were investigated in the gill, liver, spleen, kidney, and skin of grass carp 4 h and 1, 3, and 7 days post-injection with *Aquareovirus* using real-time PCR. The relative expression was determined as fold-changes relative to the PBS-injected control samples. A significant upregulation of *gcTWEAK* expression was observed in all tissues tested after *Aquareovirus* infection ( $P < 0.05$ ; Figure 5A); however, the expression pattern was different among tissues. In gill, spleen, and skin, the level of *gcTWEAK* transcript was significantly upregulated 1 and 3 days post-challenge, whereas it was significantly upregulated in liver and kidney 4 h post-challenge. The highest upregulation was observed in gill (11.2-fold) and skin (5.4-fold) 3 days post-challenge. In most tissues, the gene expression returned to almost normal levels 7 days post-challenge.



**Figure 5.** Quantitative expression profiles of the *gcTWEAK* (A) and *gcAPRIL* (B) genes after challenge with *Aquareovirus* (GCRV-JX01). Control samples were injected with PBS. The relative expression of each particular gene transcript is shown as a fold-change over PBS-injected control samples as normalized to change in expression in the  $\beta$ -actin mRNA level. Data from qPCR are reported as means  $\pm$  SE. Results shown are the mean of 4 individual RNA samples of fish ( $N = 4$ ). The data with different letters within the same tissue group indicate a significant difference at  $P < 0.05$ .

A significant upregulation of *gcAPRIL* mRNA expression was observed in all tissues tested after *Aquareovirus* infection ( $P < 0.05$ ; Figure 5B); however, the expression pattern was different among tissues. In liver, spleen, kidney, and skin, the level of *gcAPRIL* transcript decreased 4 h post-challenge, and then it was significantly upregulated 1 and 3 days post-challenge. The highest upregulation was observed in gill (38.0-fold) and skin (5.2-fold) 3 days post-challenge. In most tissues, the gene expression returned to almost normal levels 7 days post-challenge.

## DISCUSSION

*TWEAK* and *APRIL* are important members of the TNF superfamily, which have a wide range of immune functions and contribute to the pathogenesis of several diseases through the upregulation of autoantibody production and maintenance of autoimmune phenomena. Although the cloning and functional analysis of *TWEAK* and *APRIL* genes have been well characterized in mammals (Hahne et al., 1998; Guan et al., 2007; Shui et al., 2008; Luo et al., 2011; Zhang et al., 2010a,b, 2011; You et al., 2012;), very limited studies have been conducted on these genes in bony fishes (Du et al., 2012; Min et al., 2012). In this study, we report the cloning, expression analysis, and disease response of the *TWEAK* and *APRIL* genes from grass carp, a commercially important cyprinid fish. To our knowledge, this is the first cloning of the *TWEAK* and *APRIL* genes from grass carp, and we report a novel finding of their roles in *A. hydrophila* and *Aquareovirus* infections in fish. The *gcTWEAK* cDNA consists of an ORF of 753 bases with a coding capacity of 250 amino acids. A comparison of the *gcTWEAK* protein sequence with sequences from other vertebrates showed that many of the structurally and functionally important features were conserved in *gcTWEAK*, such as a predicted TMD of 23 amino acids, a putative furin cleavage site, and 3 conserved cysteine residues (Zhang et al., 2011). The ORF cDNA of *gcAPRIL* consists of 747 bases encoding a polypeptide of 248 amino acids. Like *gcTWEAK*, the protein encoded by *gcAPRIL* contains a predicted TMD of 23 amino acids and a putative furin cleavage site; however, it contains only 2 conserved cysteine residues. By amino acid sequence alignments, *gcAPRIL* shares about 33% sequence identity in the TNF domain with grass carp BAFF; 32% identity was shared in the human orthologs (Schneider et al., 1999). Similarly, *gcAPRIL* shares about 22% sequence identity in the TNF domain with *gcTWEAK*. The amino acid sequence comparison revealed that *gcTWEAK* and *gcAPRIL* are more similar to *TWEAK* and *APRIL* from zebrafish than from other teleosts. The absence of a signal peptide suggests that both *gcTWEAK* and *gcAPRIL* are type II transmembrane proteins, which is typical of the members of the TNF ligand family.

Phylogenetic tree analysis, based on the amino acid sequence alignment, showed that *gcTWEAK* and *gcAPRIL* proteins are clustered primarily with other corresponding teleost *TWEAK* and *APRIL* molecules, respectively (Figure 2). This is consistent with the results of previous studies in zebrafish (Du et al., 2012; Min et al., 2012). Interestingly, for both *TWEAK* and *APRIL*, grass carp and zebrafish form a separate clade, indicating the unique characteristics of the Cypriniformes order of the teleosts. Based on the molecular characterization, multiple alignment, and phylogenetic results, we confirmed that the newly identified *gcTWEAK* and *gcAPRIL* proteins are the members of the TNF superfamily.

A protein's physiological function can be inferred from its tissue distribution pattern. Therefore, the constitutive mRNA expression patterns of *gcTWEAK* and *gcAPRIL* in different tissues were analyzed by real-time PCR normalized against  $\beta$ -actin levels. Interestingly,

a similar expression pattern of *gcTWEAK* and *gcAPRIL* was observed in many tissues. The highest expression of both genes was observed in the skin, followed by the spleen and head kidney ( $P < 0.05$ ; Figure 3A and B). The high expression of these genes in immunologic tissues such as skin, spleen, and head kidney indicates that *TWEAK* and *APRIL* in grass carp, as in mammals, might play a complex biological role *in vivo*. In teleost fish, the head kidney and spleen are considered to be the primary source of T and B cells (Salinas et al., 2011). Thus, it is reasonable that these genes are highly expressed in spleen and head kidney. A similar expression pattern of *TWEAK* was reported in zebrafish (Du et al., 2012). Because this is the first study to investigate the tissue-specific expression profiles of *APRIL* in fish, we could not compare our result with expression in other fish species. However, this result is consistent with results in human and most mammalian species, where higher expression of *TWEAK* and *APRIL* was observed in spleen (Hahne et al., 1998; Luo et al., 2011; Cui et al., 2012; You et al., 2012). The higher expression of *APRIL* in head kidney in grass carp is distinct from the observation in most mammals. The most interesting finding in this study is the demonstration of the highest expression of *gcTWEAK* and *gcAPRIL* in skin. In our previous experiment, a similar high expression level of *BAFF* was observed in the skin of grass carp (Pandit et al., 2013). The skin of fish is the first line of defense against several pathogens (Esteban, 2012). The presence of B cells and their subsets (e.g., IgM) has also been reported in the skin of carp, catfish, and rainbow trout (Salinas et al., 2011). Therefore, it is possible that *TWEAK* and *APRIL* is strongly present in the fish skin. Although there is no published information about the expression of *TWEAK* and *APRIL* in skin in teleost fishes, we also cannot explain why their expression should be so high in grass carp skin. Further studies are necessary to gather immunocytochemical evidence of the presence of *TWEAK*- and *APRIL*-expressing cells or their subsets in the skin. In this study, the *gcTWEAK* and *gcAPRIL* mRNA levels in liver were the lowest among the tissues tested. While liver is a known immune tissue, we cannot explain why *TWEAK* and *APRIL* expression was so low in grass carp liver.

The biological role of the *TWEAK* and *APRIL* proteins in fish is still unclear. Previous studies in higher vertebrates showed that *APRIL* provides survival and activation signals to normal B and T cells (Mackay and Ambrose, 2003). The involvement of *TWEAK* in angiogenesis and inflammation is well known (Winkles, 2008). In addition, these genes are highly expressed in several tumor tissues and stimulate the growth of tumor cells *in vitro* and *in vivo*. In order to identify the immune response of *gcTWEAK* and *gcAPRIL* in fish, we examined the expression profiles of these genes in 5 immune tissues after infection with *A. hydrophila* and *Aquareovirus*. The fold-change was calculated relative to the corresponding PBS-injected control samples. Our results demonstrated that the expression patterns of *gcTWEAK* and *gcAPRIL* mRNA varied in the tissues tested following challenge. Both *gcTWEAK* and *gcAPRIL* transcripts were significantly upregulated in gill, liver, trunk kidney, spleen, and skin after *A. hydrophila* challenge (Figure 4A and B). However, the upregulation of *TWEAK* was weaker than that of *APRIL*. The highest upregulation of both *gcTWEAK* and *gcAPRIL* transcripts was observed in the liver (4.6- and 8.6-fold, respectively) 4 h post-challenge. This indicates that liver is the most responsive tissue against bacterial infection. In most tissues, the high level of *gcTWEAK* and *gcAPRIL* gene expression continued up to 3 days and returned to almost normal levels 7 days post-challenge. Similar elevated mRNA and protein levels of *TWEAK* were reported in tissue from periodontal inflammatory disease in human (Kataria et al., 2010).

The expression of *gcTWEAK* and *gcAPRIL* mRNAs was also induced after infection with *Aquareovirus* in all tissues tested. However, the upregulated expression appeared to be

slower in response to *Aquareovirus* infection than *A. hydrophila* infection, and the highest response was observed 1 to 3 days post-challenge (Figure 5A and B). The highest upregulation of both *gcTWEAK* and *gcAPRIL* transcripts was observed in the gill (11.2- and 38.0-fold, respectively), followed by skin (5.4- and 5.2-fold, respectively) 3 days post-challenge. As in *A. hydrophila* infection, the *gcTWEAK* and *gcAPRIL* gene expression returned to almost normal levels 7 days post-challenge with *Aquareovirus* infection. These results of the highest upregulation of both *gcTWEAK* and *gcAPRIL* transcripts in the gill indicate that the gill is the most responsive organ against viral infection. The results that *gcTWEAK* and *gcAPRIL* expression levels are increased by bacterial and viral infection suggest that they are important immune genes that act against bacterial and viral pathogens. However, this study could not rule out the possibility that the higher expression levels of *gcTWEAK* and *gcAPRIL* are due to cell migration or any other physiological process. No previous reports on the tissue-specific expression of the *TWEAK* and *APRIL* genes after bacterial and viral challenge in fish are available; however, these expression patterns are similar to those observed in our previous investigation of grass carp *BAFF* expression after bacterial and viral challenge (Pandit et al., 2013). He et al. (2003, 2006) also reported that *APRIL* expression is upregulated by lymphoma-associated viruses, such as Epstein-Barr virus and human immunodeficiency virus. Similarly, in humans, increased levels of the *APRIL* protein in serum or target tissues have been described in a number of autoimmune conditions and are often correlated with disease progression (Mackay and Schneider, 2009). Moreover, the observation that some viruses (e.g., poxvirus and adenovirus) encode proteins that antagonize TNF activity suggests that this cytokine plays a key role in antiviral immunity (Benedict and Ware, 2001).

In summary, we successfully cloned the full-length cDNAs of *TWEAK* and *APRIL* from grass carp, analyzed the structure of the proteins, examined their phylogenetic relationship, and demonstrated their expression profiles in normal tissues and against bacterial and viral infections. Our study demonstrated that there are many structural and functional similarities between the *gcTWEAK* and *gcAPRIL* proteins. It also suggested that the *gcTWEAK* and *gcAPRIL* genes play a potentially important role in the immune system of grass carp. This article may provide the basis for investigations about the immune roles of *TWEAK* and *APRIL* genes in teleost fishes. Further studies are recommended for critical analyses, such as functional studies using recombinant protein.

## ACKNOWLEDGMENTS

Research supported by the Earmarked Fund for China Agriculture Research System (#CARS-46-04), the National Key Technology R&D Program (#2012BAD26B02), the Agricultural Seed Development Program of Shanghai City (#2012NY10), and the Shanghai Universities First-Class Disciplines Project of Fisheries.

## REFERENCES

- Benedict CA and Ware CF (2001). Virus targeting of the tumor necrosis factor superfamily. *Virology* 289: 1-5.
- Bossen C and Schneider P (2006). BAFF, APRIL and their receptors: Structure, function and signaling. *Semin. Immunol.* 18: 263-275.
- Castigli E, Scott S, Dedeoglu F, Bryce P, et al. (2004). Impaired IgA class switching in APRIL-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 101: 3903-3908.
- Cui XW, Xiao W, Ji CB, Tian AY, et al. (2012). Gene cloning, expression and functional characterization of a proliferation-

- inducing ligand (APRIL) from hedgehog (*Erinaceus europaeus*). *Mol. Biol. Rep.* 39: 6123-6132.
- Dillon SR, Gross JA, Ansell SM and Novak AJ (2006). An APRIL to remember: novel TNF ligands as therapeutic targets. *Nat. Rev. Drug Discov.* 5: 235-246.
- Du M, Liang Z, Xu X, Li J, et al. (2012). Molecular cloning, phylogenetic analysis, tissue distribution, and expression of the cytokine TWEAK in zebrafish (*Danio rerio*). *Biochem. Genet.* 50: 318-324.
- Esteban MA (2012). An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunol.* 2012, 853470. <http://dx.doi.org/10.5402/2012/853470>.
- Gao HX, Campbell SR, Burkly LC, Jakubowski A, et al. (2009). TNF-like weak inducer of apoptosis (TWEAK) induces inflammatory and proliferative effects in human kidney cells. *Cytokine* 46: 24-35.
- Guan ZB, Shui Y and Zhang SQ (2007). Two related ligands of the TNF family, BAFF and APRIL, in rabbit: molecular cloning, 3D modeling, and tissue distribution. *Cytokine* 39: 192-200.
- Hahne M, Kataoka T, Schröter M, Hofmann K, et al. (1998). APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J. Exp. Med.* 188: 1185-1190.
- He B, Raab-Traub N, Casali P and Cerutti A (2003). EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching. *J. Immunol.* 171: 5215-5224.
- He B, Qiao X, Klasse PJ, Chiu A, et al. (2006). HIV-1 envelope triggers polyclonal Ig class switch recombination through a CD40-independent mechanism involving BAFF and C-type lectin receptors. *J. Immunol.* 176: 3931-3941.
- Jakubowski A, Browning B, Lukashev M, Sizing I, et al. (2002). Dual role for TWEAK in angiogenic regulation. *J. Cell Sci.* 115: 267-274.
- Jang S, Liu H, Su J, Dong F, et al. (2010). Construction and characterization of two bacterial artificial chromosome libraries of grass carp. *Mar. Biotechnol.* 12: 261-266.
- Jeanmougin F, Thompson JD, Gouy M, Higgins DG, et al. (1998). Multiple sequence alignment with Clustal X. *Trends Biochem. Sci.* 23: 403-405.
- Kataria NG, Bartold PM, Dharmapathi AA, Atkins GJ, et al. (2010). Expression of tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor-inducible 14 protein (Fn14), in healthy tissues and in tissues affected by periodontitis. *J. Periodontol. Res.* 45: 564-573.
- Liu F, Li JL, Yue GH, Fu JJ, et al. (2010). Molecular cloning and expression analysis of the liver-expressed antimicrobial peptide 2 (LEAP-2) gene in grass carp. *Vet. Immunol. Immunopathol.* 133: 133-143.
- Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408.
- López-Fraga M, Fernández R, Albar JP and Hahne M (2001). Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *EMBO Rep.* 2: 945-951.
- Luo CH, Ai HX, Zhou XM, Min C, et al. (2011). Molecular structure, expression analysis and functional characterization of APRIL (TNFSF 13) in goat (*Capra hircus*). *Gene* 485: 63-68.
- Mackay F and Ambrose C (2003). The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev.* 14: 311-324.
- Mackay F and Schneider P (2009). Cracking the BAFF code. *Nat. Rev. Immunol.* 9: 491-502.
- Mackay F, Schneider P, Rennert P and Browning J (2003). BAFF and APRIL: a tutorial on B cell survival. *Annu. Rev. Immunol.* 21: 231-264.
- Mackay F, Silveira PA and Brink R (2007). B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. *Curr. Opin. Immunol.* 19: 327-336.
- Min C, Liang Z, Cui X, Wang Q, et al. (2012). Molecular cloning and partial functional characterization of a proliferation inducing ligand (APRIL) in zebrafish (*Danio rerio*). *Dev. Comp. Immunol.* 37: 202-206.
- Molloy SS, Anderson ED, Jean F and Thomas G (1999). Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. *Trends Cell Biol.* 9: 28-35.
- Moreaux J, Legouffe E, Jourdan E, Quittet P, et al. (2004). BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood* 103: 3148-3157.
- Pandit NP, Shen Y, Wang W, Chen Y, et al. (2013). Identification of TNF13b (BAFF) gene from grass carp (*Ctenopharyngodon idella*) and its immune response to bacteria and virus. *Dev. Comp. Immunol.* 39: 460-464.
- Salinas I, Zhang YA and Sunyer JO (2011). Mucosal immunoglobulins and B cells of teleost fish. *Dev. Comp. Immunol.* 35: 1346-1365.
- Schneider P, MacKay F, Steiner V, Hofmann K, et al. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189: 1747-1756.
- Shui Y, Guan ZB, Zhang JX and Zhang SQ (2008). cDNA cloning, genomic structure, expression analysis and biological activity of porcine A Proliferation-Inducing Ligand (APRIL). *Vet. Immunol. Immunopathol.* 125: 190-197.
- Wiens GD and Glenney GW (2011). Origin and evolution of TNF and TNF receptor superfamilies. *Dev. Comp. Immunol.*

- 35: 1324-1335.
- Winkles JA (2008). The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat. Rev. Drug Discov.* 7: 411-425.
- You F, Zhou L, Liu X, Fan J, et al. (2012). Molecular structure, expression analysis and functional characterization of APRIL (TNFSF13) gene in bat (*Vespertilio superans* Thomas). *Gene* 498: 196-202.
- Zhang JX, Ma HW, Sang M, Hu YS, et al. (2010a). Molecular structure, expression, cell and tissue distribution, immune evolution and cell proliferation of the gene encoding bovine (*Bos taurus*) TNFSF13 (APRIL). *Dev. Comp. Immunol.* 34: 1199-1208.
- Zhang JX, Sang M, Zhao W, Ai HX, et al. (2010b). Molecular characterization of the canine cytokine TWEAK (CD255) and its receptor, Fn14 (CD266). *Vet. Immunol. Immunopathol.* 137: 172-175.
- Zhang JX, Sang M, Li JF, Zhao W, et al. (2011). Molecular structure and characterization of the cytokine TWEAK and its receptor Fn14 in bovine. *Vet. Immunol. Immunopathol.* 144: 238-246.
- Zhang L, Luo Q, Fang Q and Wang Y (2010c). An improved RT-PCR assay for rapid and sensitive detection of grass carp reovirus. *J. Virol. Methods* 169: 28-33.
- Zhu LY, Nie L, Zhu G, Xiang LX, et al. (2013). Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Dev. Comp. Immunol.* 39: 39-62.