

## The cSNP scanning and expression analysis of the duck *FTH1* gene

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**Abstract:** Ferritin, heavy polypeptide 1 (*FTH1*) is a kind of ferritin complex that catalyzes the conversion of Fe(II) into Fe(III) to protect the cell from oxidative damage. Recent research has shown that *FTH1* is also associated with several disease-related processes, such as inflammation and tumor progression and so on. In this study, the coding sequence (CDS) of *FTH1* in Jinding ducks was cloned. The CDS sequence revealed an open reading frame of 546 nucleotides encoding a protein with 181 amino acids. Based on this sequence, the duck *FTH1* protein is predicted to have conserved domains typical of eukaryotic ferritin. Only one synonymous mutation (c.447T > C) of the coding sequence of the duck *FTH1* gene was identified in ducks. Quantitative RT-PCR results revealed that *FTH1* expression in the liver and spleen was significantly downregulated after duck hepatitis virus 1 infection. Meanwhile, in DF-1 cells transfected with an *FTH1* plasmid, expression of the antiviral marker gene *Mx1* was significantly upregulated. These results suggest that the coding sequence of the duck *FTH1* gene was highly conserved and *FTH1* plays an important role in antiviral activity.

**Key words:** Duck, heavy polypeptide 1, gene cloning, expression analysis

### 1. Introduction

Iron, one of the most abundant essential microelements, participates in metabolic processes critical to life and helps maintain dynamic equilibrium in vivo (1). Inside the body, iron is stored in a soluble, nontoxic form called ferritin (2); along with transferrin, ferritin is one of the major iron metabolism proteins. As the largest iron storage organ, the liver is vital in iron metabolism. The balance among iron, ferritin, and transferrin plays an important role in maintaining the iron stability of the internal environment. Numerous studies have shown that ferritin occurs as a soluble protein in plants and animals and is related to processes that can lead to disease, including oxidative stress, tissue and cell damage, and inflammatory response (3–5). Furthermore, ferritin is a biomarker used to diagnose a large number of illnesses (6). For example, it has been found that a low level of ferritin is associated with a good prognosis in breast cancer patients (7). Ferritin is composed of a variable array of heavy-chain (FHC) and light-chain (FLC) subunits, encoded by two distinct genes (8). Despite a high degree of amino acid homology, the FHC and FLC subunits have different functions: the FHC possesses ferroxidase activity and is involved in rapid iron uptake and release, while the FLC is devoid of enzymatic activity and contributes to long-term iron storage (9).

The ferritin, heavy polypeptide 1 (*FTH1*) gene is regulated by iron regulatory proteins, encoding a 21-kDa

protein that catalyzes the conversion of Fe(II) into ferric Fe(III) and allows for the intracellular storage of inert Fe(III) (10). Thus, the major function of *FTH1* is protecting the cell from oxidative damage (11). Recent data indicated that the *FTH1* gene is also associated with several disease processes, such as inflammation, tumor progression, and so on (12,13).

In our previous study, we screened 70 differentially expressed genes related to the duck hepatitis virus (DHV) using the suppression subtractive hybridization (SSH) cDNA library of 3-day-old ducklings treated with DHV-1 and we found that the *FTH1* gene is crucial among them (14,15). However, little is known about the regulation mechanism and disease-resistance mechanism of duck *FTH1*. Thus, in this study, we cloned the duck *FTH1* and scanned its coding single nucleotide polymorphism (cSNP). Then we examined the *FTH1* mRNA expression in livers and spleens challenged with DHV-1. The expressions of *FTH1* and *Mx1* were also analyzed after the eukaryotic expression vector was transfected into DF-1 to further study the function of *FTH1* in antiviral activity.

### 2. Materials and methods

#### 2.1. Ethics statement

All procedures involving the care and use of animals conformed to US National Institutes of Health guidelines

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(NIH Pub. No. 85-23, revised in 1996) and were approved by the Laboratory-Animal Management and Experimental-Animal Ethics Committee of Yangzhou University.

## 2.2. Experimental animals, sample collection, cell line, and culture conditions

All the healthy Jinding ducks were obtained from the Chinese Waterfowl Germplasm Resource Pool (Taizhou, China). The tissue samples (liver) were collected after the healthy adult ducks were euthanized and dissected, then snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until total RNA extraction. In addition, 80 healthy Jinding ducklings of 3 days old were intramuscularly injected with either 0.4 mL of allantoic liquid containing DHV-1 or saline (as a control). After injection, tissue samples (spleen and liver) were collected immediately when the classical clinical signs appeared in infected ducklings. The tissue samples were also snap-frozen in liquid nitrogen immediately after being collected and stored at  $-80^{\circ}\text{C}$ .

DF-1 cells were purchased from the cell bank of the Chinese Academy of Science and cultured in complete growth medium: Dulbecco's modified Eagle's medium (DMEM, HyClone, Shanghai, China) and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The culture conditions were  $38^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  and 95% air incubator. If DF-1 cells are in good condition, they will be adherent with a fibroblast-like morphology.

## 2.3. Cloning, sequencing, and sequence analysis of duck *FTH1*

Total RNA was extracted from the tissue samples using the RNAiso plus reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions and the total RNA concentration was determined using a NanoDrop 1000 spectrophotometer V3.7 (Thermo, Shanghai, China). First-strand cDNA was synthesized with 1  $\mu\text{g}$  of total RNA as a template with the Prime Script First Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. Based on the published *Anas platyrhynchos* *FTH1* coding sequence (CDS), one pair of primers (FTH1 F and FTH1 R; Table) was designed to amplify the CDS

of *FTH1*. The mRNA isolated from the liver was used as a template to synthesize the *FTH1* cDNA. Polymerase chain reaction (PCR) was performed with the following cycling parameters:  $94^{\circ}\text{C}$  for 5 min; 35 cycles of  $94^{\circ}\text{C}$  for 40 s,  $65^{\circ}\text{C}$  for 40 s, and  $72^{\circ}\text{C}$  for 30 s; and a final extension of  $72^{\circ}\text{C}$  for 10 min. PCR products were purified from the gel and cloned into the pMD19-T-simple vector (TaKaRa), then sequenced. The NCBI CDD program (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) was used to predict the conserved domain of the amino acid sequences.

## 2.4. cSNP scanning for the duck *FTH1* gene

RNA extraction and cDNA preparation were performed from another 10 ducks. Cloning of the *FTH1* CDS was conducted as above, and SNPs were obtained by sequencing. The multiple sequence alignment was performed using MEGA 6.0.

## 2.5. Construction of eukaryotic expression vector and transient transfection

The *FTH1* was cloned into the BamHI and EcoRI sites of the pEGFP-C1 vector (Invitrogen, Shanghai, China) to produce the plasmid named pEGFP-C1-FTH1. The day before transfection, DF-1 cells in good condition were digested with trypsin to obtain a single-cell suspension and then seeded at  $1 \times 10^5$ /well (24-well plate). The DF-1 cells (grown to 90% confluence) were transfected with 2  $\mu\text{L}$  of Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China) and 0.8  $\mu\text{g}$  of plasmid DNA (with pEGFP-C1 used as a control) according to the manufacturer's instructions. Six hours later, the complete growth medium was replaced.

## 2.6. Quantitative real-time PCR analysis (qRT-PCR)

The mRNA expression levels were measured via qRT-PCR using the Ultra SYBR Mixture with ROX (CW Biotech, Beijing, China). Real-time PCR as carried out on an Applied Biosystems 7500 Real-Time PCR System with the following program: incubated for 10 min at  $95^{\circ}\text{C}$ , followed by 40 cycles of 15 s at  $95^{\circ}\text{C}$  and 1 min at  $60^{\circ}\text{C}$ . The primers designed for real-time PCR are shown in the Table. Relative expression of mRNA was determined

**Table.** Primers used for gene cloning and expression analysis.

Primer name	Sequence (5'→3')	Note
FTH1F FTH1R	CGGGATCCATGGCTACGCCTCTCTCCC CGGAATTCTCAGCTCTCGTCACTGTGC	Coding sequence amplification
FTH1Y F FTH1Y R	TACTTCCTGCACCAGTCCCA CCAGTCATCACGATCCGGTT	RT-qPCR
GAPDH-F GAPDH-R	GCCATCAATGATCCCTTCAT CCAACAGAACAGAGAATGGAAGAA	RT-qPCR

using the  $2^{-\Delta\Delta Ct}$  method. All cDNA samples were tested three times and the results were normalized to the levels of duck *GAPDH* expression.

### 2.7. Statistical analysis

Differences in relative gene expression between the DHV-1-challenged group and the control group were analyzed using GraphPad Prism 5 (San Diego, CA, USA). Statistical analyses were performed with one-way ANOVA and Tukey's tests (SPSS 22.0, IBM Corp., Armonk, NY, USA).  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Cloning and bioinformatics analysis of *FTH1*

The CDS of *FTH1* was synthesized from the total RNA of duck liver using RT-PCR. The sequence obtained was identical to that of the duck (*Anas platyrhynchos*) sequence in GenBank using DNASTAR software. The coding sequence was 546 bp in length and encoded 181 amino acid residues.

The conserved domain of duck *FTH1* deduced from the amino acid sequence was one member of the ferritin-like domain, which contains ferritins and other ferritin-like proteins such as members of the DPS family and bacterioferritins (Figure 1). It was the eukaryotic ferritin (Euk\_Ferritin) domain that included seven ferroxidase diiron centers, four ferrihydrite nucleation centers, and three iron ion channels.

### 3.2. Variation analysis of duck *FTH1* CDS

Multiple sequence alignment revealed that the coding sequence of the duck *FTH1* gene was highly conserved, and only one variant (c.447T > C) was identified in ducks, which belonged to a synonymous mutation at the amino acid level (Figure 2). Sequence analysis further showed a point mutation (c.447T > C) beyond the three above domains.

### 3.3. Expression of *FTH1* gene by qRT-PCR after DHV-1 infection

To investigate the function of the *FTH1* gene in antiviral innate immunity, we injected 3-day-old healthy ducklings with DHV-1. After injection, spleen and liver samples of the infection group and control group were collected (with three ducklings analyzed per group). The mRNA expression levels of *FTH1* in different groups were

measured by qRT-PCR (Figure 3). The results were similar in the spleen and liver; relative expression of *FTH1* in the infection group was significantly lower than that in the control group ( $P < 0.05$ ).

### 3.4. Expression of *FTH1* and *Mx1* genes by qRT-PCR after plasmid transfection

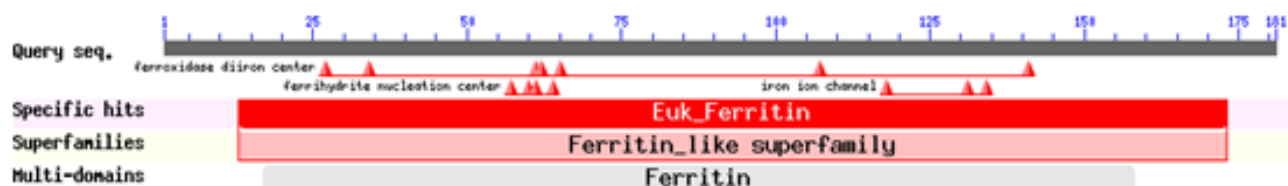
We performed a real-time PCR assay to investigate the change of *FTH1* and *Mx1* mRNA expression levels with *FTH1* plasmid transfection (Figure 4). The results showed that *FTH1* mRNA expression was very significantly higher than that of the control ( $P < 0.01$ ), and the antiviral gene *Mx1* increased significantly compared to the control ( $P < 0.05$ ).

## 4. Discussion

DHV is the causative agent of duck viral hepatitis, an acute, rapidly spreading, and fatal disease of young ducklings. Three serotypes of DHV (DHV types 1, 2, and 3) have been described, and no antigenic relationships have been found among them (16,17). DHV-1 has a worldwide distribution and can cause mortality higher than 80% in ducklings younger than 3 weeks, making it a threat to all duck-growing farms (18,19). In order to study the molecular mechanism of DHV-1 injection, we previously used the SSH cDNA library of 3-day-old ducklings infected with DHV-1 to screen differentially expressed genes, and we found that the *FTH1* gene is very crucial. This result was consistent with the research of Nair et al. (15).

In this study, we cloned the coding sequence of *FTH1*, which is 546 bp in length and encodes 181 amino acid residues. It matched completely with the *FTH1* sequence of *Anas platyrhynchos* in GenBank. Based on conserved domains, we found that duck *FTH1* was the eukaryotic ferritin containing seven ferroxidase diiron centers, four ferrihydrite nucleation centers, and three iron ion channels, similar to mammalian ferritin (12,20–24). Its coding sequence and the domains of the duck *FTH1* gene were very conservative, which suggested that *FTH1* might have a stable biological function.

We then examined the expression of *FTH1* in the liver and spleen after DHV-1 infection to reveal the function of the duck *FTH1* gene. The mRNA expression of *FTH1* decreased very significantly ( $P < 0.01$ ) in the liver after

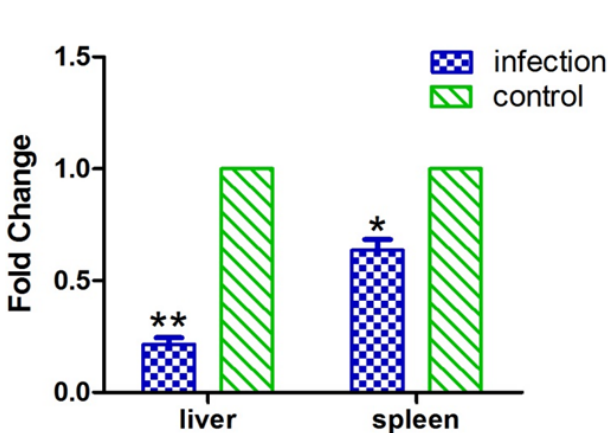


**Figure 1.** The prediction of duck *FTH1* conserved domain. “Ferritin” represents the ferritin-like domain, “Ferritin\_like superfamily” represents the ferritin-like superfamily of diiron-containing four-helix-bundle proteins, “Euk\_Ferritin” represents the eukaryotic ferritin, and the red triangle represents the residues involved in the conserved feature of eukaryotic ferritin.

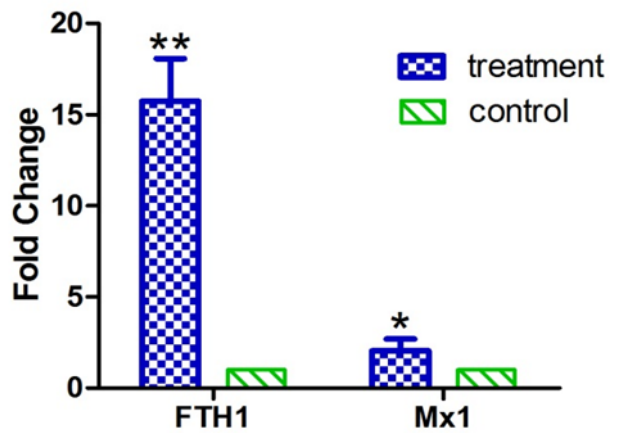




**Figure 2.** Alignment of the coding sequence of duck *FTH1* among individuals. The asterisk indicates that the bases are identical among all individuals, while the arrows indicate not identical. The bottom line indicates the deduced amino acid sequence.



**Figure 3.** The mRNA relative expression of the duck *FTH1* gene in livers and spleens was detected after infection with DHV-1 compared to controls. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ . The relative mRNA levels of individual *FTH1* were normalized with respect to housekeeping gene *GAPDH*.



**Figure 4.** Relative expression of *FTH1* and *Mx1* were detected after transfection. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ . “Treatment” is the recombinant plasmid group and “control” is the empty plasmid group. The relative mRNA levels of individual *FTH1* and *Mx1* were normalized with respect to housekeeping gene *GAPDH*.

infection, while the expression level was also reduced significantly ( $P < 0.05$ ) in the spleen and we easily found that *FTH1* mRNA expressions were similar in both organs. The results indicated that *FTH1* expression was downregulated by DHV-1 infection. Research by Lee et al. (25) also suggested that *FTH1* expression was downregulated in swine livers after infection with the hepatitis virus.

We also showed that the expression of the *FTH1* mRNA increased very significantly in DF-1 cells, and we demonstrated that *FTH1* was overexpressed successfully by recombinant plasmid transfection. Furthermore, the expression of *Mx1* was consistent with the *FTH1*, and therefore we concluded that overexpressed duck *FTH1* gene upregulated the expression of *Mx1*.

*Mx* acts as a marker gene of viral resistance, induced by type I interferon (IFN) to play a key role in the innate immune response to viral infection (26,27). *Mx* proteins have been shown to provide resistance to a wide range of both DNA and RNA viruses in vertebrates (28,29). In this study, the expression of *Mx1* was significantly upregulated

( $P < 0.05$ ) by transfection of the *FTH1* plasmid. Therefore, our data indicate that *FTH1* may play an important role in hepatic diseases, such as duck virus hepatitis.

In summary, duck *FTH1* was cloned and one synonymous mutation (c.447T > C) of the CDS was identified in ducks, revealing that mRNA expression was significantly downregulated in the liver and spleen after DHV-1 infection. Moreover, overexpressed duck *FTH1* could upregulate the expression of the antiviral marker gene *Mx1*. Our findings reveal that the CDS of the duck *FTH1* gene is highly conserved and *FTH1* plays an important role in antiviral activity.

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