Biochemical Genetics (2019) 57:159–169
https://doi.org/10.1007/s10528-018-9876-3

ORIGINAL ARTICLE

Altered Expression of Zinc Transporter ZIP12 in Broilers of Ascites Syndrome Induced by Intravenous Cellulose Microparticle Injection

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Received: 25 December 2017 / Accepted: 14 July 2018 / Published online: 2 August 2018
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Abstract

Ascites syndrome (AS) is a harmful disease in fast-growing broilers characterized by heart failure and serious fluid accumulation in the abdominal cavity. One of the known functions of zinc transporter ZIP12 is an important regulator in pulmonary hypertension (PH) in rat. Whether chicken ZIP12 is involved in the process of AS need to be explored. Here, chicken ZIP12 was sequenced and expression pattern and histological distribution were detected in broilers of AS induced by intravenous cellulose microparticle injection. Phylogenetic analysis showed that ZIP12 was significantly different between chicken and mammalian. The relative mRNA expression level of ZIP12 in the liver and lung in AS and pre-ascites (PAS) groups were significantly higher ($P < 0.01$) than that in control. The immunohistological staining using rabbit anti-chicken ZIP12 IgG and integrated optical density analysis showed the positive cells of ZIP12 distributed in detected tissues and the expression level of ZIP12 protein increased in AS and PAS groups compared to control. The results will provide the basic data of ZIP12 in the pathological process of AS in broiler chickens and offer an important reference for prevention and control of the disease.

Keywords Chicken · Ascites syndrome · ZIP12 · Expression pattern

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Introduction

Ascites syndrome (AS), also known as pulmonary hypertension syndrome (PHS), is a nutrition metabolic disease mainly occurring in fast-growing broilers characterized by right heart failure and serious fluid accumulation in the abdominal cavity (Julian 1998; Tan et al. 2007). AS caused approximately 25% of overall mortality in the broiler industry around the world (De Smit et al. 2005). Environmental hypoxia or low temperature could induce AS (Wideman et al. 2004; Li et al. 2011). In this pathological process, pulmonary artery remodeling (PVR) including thickness of pulmonary blood vessel wall and proliferation of smooth muscle cells plays a key role (Pan et al. 2008; Li et al. 2010).

Regulatory genes found in the host are crucial to get the molecular mechanism of AS for prevention and control. Recently, zinc transporter family member 12 (ZIP12) was found as a key regulator of hypoxia-induced pulmonary vascular remodeling which is the most important line in PH in rat (Zhao et al. 2015). In mammalians, ZIP12 encoded by the gene slc39a12 is a protein with several transmembrane domains (Liuzzi and Cousins 2004; Zhao et al. 2015) and plays an important role in the regulation of cellular zinc homeostasis (Liuzzi and Cousins 2004) by a zinc transporting aqueous cavity between transmembrane domains IV and V. Previous reports showed that ZIP12 highly expressed in brain relative peripheral tissues which was thought to be required for nervous system development and mental illness (Chowanadisai et al. 2013; Chowanadisai 2014; Scarr et al. 2016). ZIP12 can also highly expressed in pulmonary vascular smooth muscle cells which is hypoxia dependent and this process can be inhibit by disruption of ZIP12 expression attenuating the development of PH in rats (Zhao et al. 2015). AS of broilers has the similar pathophysiological features with mammalians, whether chicken ZIP12 participating in this process need to be explored. To date, data regarding the expression profiles of ZIP12 in AS of broilers are still conspicuously lacking.

Here, chicken ZIP12 were sequenced and the rabbit-origin polyclonal antibody was prepared. Expression and distribution of ZIP12 were investigated during the development of AS induced by intravenously injected with ion-exchange cellulose CM-32 suspension. Results will provide the basic data of ZIP12 in the pathological process of AS in chicken and offer an important reference for the prevention and control.

Materials and Methods

Chickens and How to Determine Their State of AS and PAS

Chickens were provided by the Centre of Laboratory Animals at South China Agricultural University. The animal experiments were approved by the Institutional Animal Care and Use Committee at South China Agricultural (Certification

Number: CNAS BL0011) and performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). Treatment of chickens was performed as our previous report (Li et al. 2013). Briefly, a total number of one hundred and twenty 14-day-old broilers were randomly divided into a test group (TG) ($n = 90$) and a control group (CG) ($n = 30$). All broilers were allowed to have free access to the same diet ad libitum and had a 24-h fluorescent illumination per day throughout the trial period. At 20 days old, chickens of TG group were intravenously injected with ion-exchange cellulose CM-32 suspension (Cellulose CM-32, average particle diameter between 24 and 63 mm, batch number 4035010; Whatman, Maidstone, Ken, UK). While, broilers in control were injected intravenously with the same volume of physiological saline with broilers in TG group.

At 42 days old, broilers in TG were measured the ascites heart index (AHI), the right ventricle (RV) weight, the total ventricle (TV) weight and the RV/TV ratio which were used to determined their state of AS and PAS (Forman and Wideman 1999; Li et al. 2017). The broilers were determined as AS showing signs including depression, dullness, open beak breathing, distended abdomen, cyanosis, more than 10 ml ascetic fluid in the peritoneum, generalized venous congestion and right ventricular weight to total ventricular weight ratio ≥ 0.30; while chickens were determined as PAS when they appeared symptoms of AS without ascetic fluid in the peritoneum and right ventricular weight to total ventricular weight ratio ≥ 0.27 (Forman and Wideman 1999; Li et al. 2017).

Tissues Preparation of AS, PAS, and Control Groups

Based on the RV/TV ratio, 13 chickens showed in the state of AS and 18 chickens in the state of PAS in TG. Heart, liver, spleen, lung, and kidney were obtained from five broilers of AS, PAS, and control, respectively. Two different parts of each tissue were used for RNA exaction and immunohistochemical detection.

Sequencing, Inducible Expression, and Purification of Chicken ZIP12

Amplification primers of chicken ZIP12 were 5′-AGGCCACAAAGCTAGATA CAC-3′ (forward) and 5′-ACCAGCAAGACAACCAAGGAT-3′ (reverse). All the work of amplification, cloning, and sequencing was performed according to our previous reports (Ning et al. 2012; Zhou et al. 2013; Ye et al. 2015).

Primers of 5′-CGTGGAGGATCCATGTGCTTTCTGAGCAAGC-3′ (forward) (the *Bam*H I restriction enzyme site) and 5′-AATATACTCGAGTCAGTGGTGGTG GTGGTG GTGAGTGCTGTAACCATAT-3′ (reverse) (the *Xho* I restriction enzyme site) were used to construct the expression vector pGEX-6P-1-ZIP12. Purification and detection of recombinant chicken ZIP12 were performed according to our previous researches (Zhou et al. 2013; Ye et al. 2015). GST-tagged inclusion protein purifying kit (Cwbiotech, Beijing, China) and anti-GST-tag rabbit antibody (Cat. no.cw0085a, Cwbiotech, Beijing, China) were used for purification and Western Blot detection of GST-tagged ZIP12.

Immunity of Rabbits and IgG Purification

The immunity of New Zealand white rabbits, detection of serum titers, and preparation of IgG were carried out according to our previous researches (Zhou et al. 2013; Ye et al. 2015).

Real-Time Quantitative PCR

Relative expression level of ZIP12 was detected by real-time quantitative PCR using the $2^{-\Delta\Delta Ct}$ method according to our previous researches (Huang et al. 2014; Zhou et al. 2013; Ye et al. 2015). The primers for ZIP12 were 5′-TGGTATGTTCCACTT TCCTGCT-3′ (forward) and 5′-AACATAGCATCTGGTTCAAGGC-3′ (reverse). Primers of 5′-TGAA CTCCCTGATGGTCAGGTC-3′ (forward) and 5′-ACCACA GGACTCCATACCCAAG-3′ (reverse) were used for β-actin.

Immunohistochemical Detection and Integrated Optical Density (IOD) Analysis

Immunohistochemical assay was performed as our previous reports (Huang et al. 2014; Yu et al. 2015). Basically, prepared IgG diluted 800-fold in PBS containing 1% BSA used as first antibody reacted with the tissue samples for 2 h at 37 °C and then HRP-conjugated mice anti-rabbit IgG (Dingguo, Beijing, China) used as second antibody reacted with the tissue samples for 40 min at 37 °C. DAB kit (ZSGQ-BIO, China), hematoxylin and GVA medium were used for coloration, counterstaining, and sealing. Control antibodies against nonspecific staining were pre-immune rabbit serum and normal IgG. Expression level of ZIP12 was assessed by measuring the average integrated optical density (IOD) using Image-Pro Plus 6.0 (IPP 6.0).

Data Analysis

One-way ANOVA and t test of Statistical Product and Services Solution, version 21.0 (SPSS, Cary, NC, USA) were used for statistical analysis and GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used for graphs. Data are expressed as the mean ± standard deviation (SD) from three separate experiments.

Results

Sequence Analysis of the Chicken ZIP12

Chicken ZIP12 was amplified and the sequence was deposited in GenBank (Accession No.: KU178951). The protein deduced according to nucleotide sequence consists of 613 amino acids and molecular weight is 69 kDa. Nucleotide sequence of ZIP12 was aligned by the software MegAlign (DNAstar, Madison, WI). The nucleotide homology of ZIP12 among chicken and gallus (Accession No.: XM_015282004.1), cattle (Accession No.: NM_001076878.1), goat

(Accession No.: XM_013968564.2), horse (Accession No.: XM_003364356.2), swine (Accession No.: XM_003130728.4), chimpanzee (Accession No.: XM_001153573.3), human (Accession No.: NM_001145195.1), rabbit (Accession No.: XM_002717411.3), mouse (accession no.: NM_001012305.2), and rat (Accession No.: NM_001106124.1) were 99.5, 71.2, 71, 71, 69.8, 71.2, 71.2, 69, 68.5, and 67%, respectively (Fig. 1). ZIP12 was conservative in mammalian but significantly different between mammalian and chicken.

Expression and Purification of Recombinant ZIP12

Double enzyme digestion and nucleotide sequence analysis showed that expression plasmid pGEX-6p-1-ZIP12 was successfully constructed. The recombinant GST-tagged ZIP12 was efficiently expressed and SDS-PAGE showed that the purified protein was consistent with its predicted size, approximately 69 kDa (Fig. 2a, b).

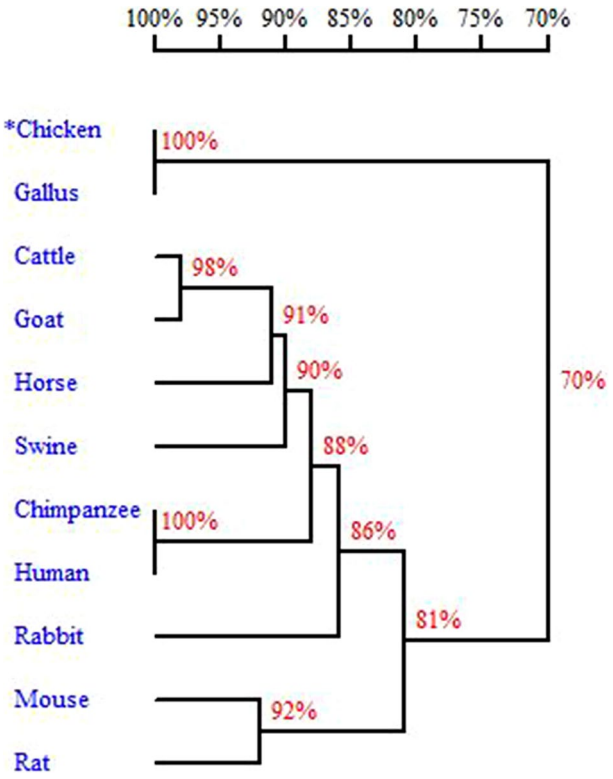

Fig. 1 Nucleotide homology analysis of chicken ZIP12. Nucleotide homology of ZIP12 between chicken and gallus, goat, horse, swine, chimpanzee, human, rabbit, mouse, and rat were analyzed

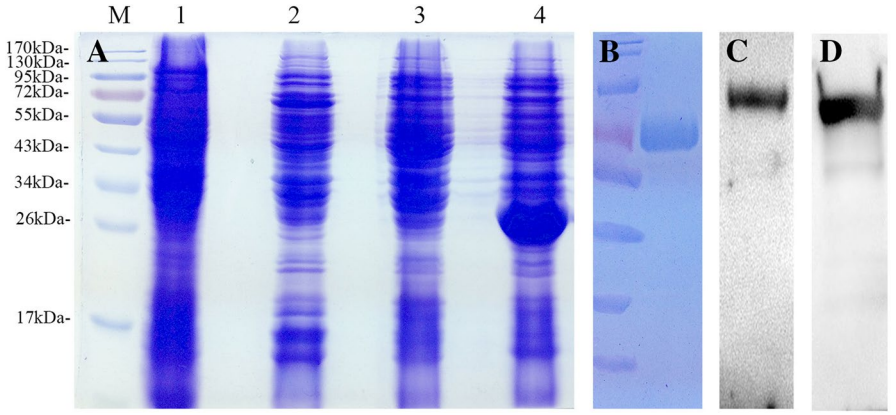

Fig. 2 Analysis of GST-tagged ZIP12 fusion protein expressed in *Escherichia coli* BL21 (DE3). **a** M, Genview Prestained Protein Marker I; 1, cell lysates of bacteria transformed with plasmid pGEX-6P-1-ZIP12 without IPTG induction; 2, cell lysates of bacteria transformed with plasmid pGEX-6P-1-ZIP12 under IPTG induction at 37 °C for 6 h; 3, cell lysates of bacteria transformed only with plasmid pGEX-6P-1 without IPTG induction; 4, cell lysates of bacteria transformed only with plasmid pGEX-6P-1 under IPTG induction at 37 °C for 6 h. **b** The purified GST-tagged ZIP12 protein. **c** Western Blot analysis of GST-tagged ZIP12 using monoclonal antibody against GST-tag. **d** Western Blot analysis of GST-tagged ZIP12 using purified IgG prepared

Purified IgG Preparation

Serum titers of all rabbits were higher than 1:1024. Purified IgG was obtained from total 30 ml blood. The concentration of purified IgG was 2 mg/ml. Western Blot detection using GST-tag antibody and prepared IgG confirmed that the fusion protein was ZIP12 (Fig. 2c, d).

Detection of the Expression of ZIP12 by Quantitative Real-Time PCR

Melting-curve analysis confirmed the specificity of primers for amplification of ZIP12 and β-actin gene fragments. The amplified gene fragments of ZIP12 and β-actin showed 100% homology with their original sequences. The relative expression level of ZIP12 in the heart, liver, spleen, lung, and kidney of the AS, PAS, and control were showed in Fig. 3.

In heart, expression level of ZIP12 increased in AS and PAS groups compared with the control and it was significantly higher in PAS group than that in control ($P < 0.01$). In liver, the expression level of ZIP12 in AS and PAS groups was significantly higher than that in control ($P < 0.01$). In spleen, expression level of ZIP12 was significantly higher in AS group than that in control ($P < 0.05$). In lung, the expression level of ZIP12 in AS and PAS groups was significantly higher than that in control ($P < 0.001$). In kidney, there was no significant difference in expression level of ZIP12 between the AS, PAS, and control groups.

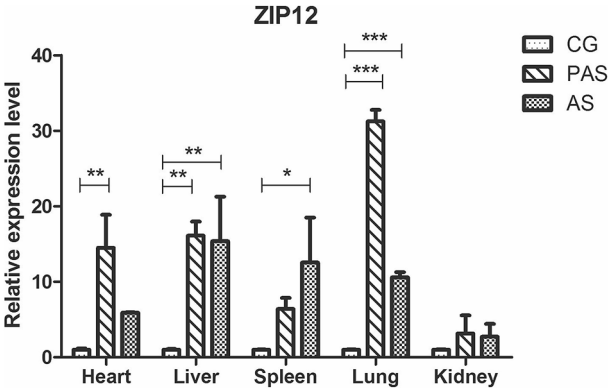

Fig. 3 Relative expression levels of ZIP12 in the heart, liver, spleen, lung, and kidney of control (CG), PAS and AS groups. *$P<0.05$; **$P<0.01$; ***$P<0.001$. Data are represented as mean ± standard deviation (SD) ($n=5$). All samples were tested in triplicate

Immunohistochemical Detection

Immunohistochemical detection was performed to show distribution of the ZIP12 in heart, liver, spleen, lung, and kidney (Fig. 4). The expression level of ZIP12 was analyzed by average IOD in the tissues of AS, PAS, and control groups (Fig. 5). In heart, cardiac myocytes of each group were strong positive (Fig. 4a–c). The average IOD in heart showed no significant difference between AS, PAS, and control (Fig. 5a). In liver, hepatic sinusoidal endothelial cells showed weak to medium positive in control and mild to strong in AS and PAS groups, while liver cells showed negative in all groups (Fig. 4d–f). The average IOD of AS and PAS groups were higher than that in control and the control was significantly lower than that in PAS group ($P<0.05$) (Fig. 5b). In spleen, endothelial cells showed weak positive in control, while medium positive in the same cell in AS and PAS groups (Fig. 4g–i). The average IOD of control was significantly lower than that in AS and PAS groups ($P<0.01$) (Fig. 5c). In lung, the ZIP12-positive cells were mainly in the epithelial cells of the pulmonary alveoli in control, while stronger positives in epithelial cells of the pulmonary alveoli as well as pulmonary vascular smooth muscle cells in AS and PAS groups (Fig. 4j–l). Average IOD of the control was significantly lower than that in PAS and AS groups ($P<0.01$) (Fig. 5d). In kidney, renal tubular epithelial cells of three groups showed weak to medium positive (Fig. 4m–o) and the average IOD in three groups showed no significant difference (Fig. 5e).

Discussion

Fast growth and high metabolic rate of broilers increased oxygen demand and this makes broilers easier to suffer AS (Wideman and Kirby 1995; Scheele 1997). ZIP12 had been identified as drug targets for pulmonary hypertension such

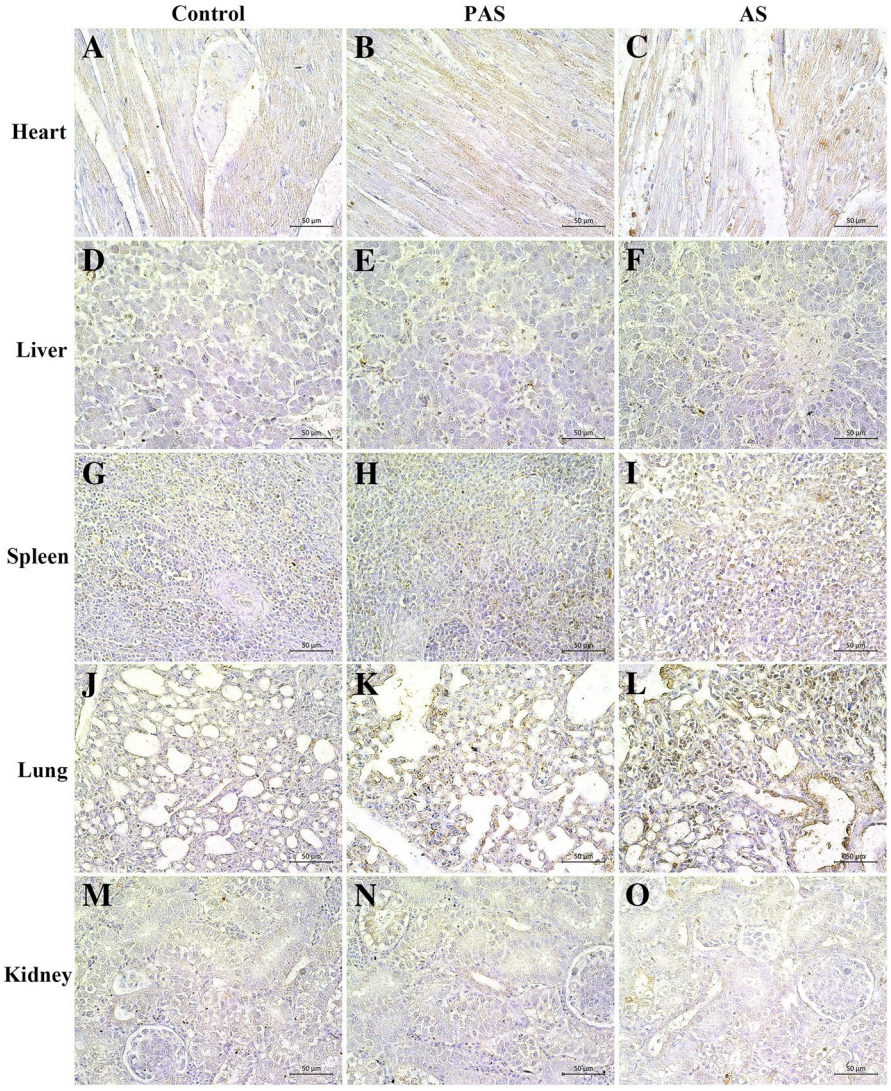

Fig. 4 Distribution of ZIP12 in the heart, liver, spleen, lung, and kidney of control, PAS, and AS groups. Scale bar = 50 μm. **a–c** Heart of control, PAS, and AS, respectively, **d–f** Liver of control, PAS, and AS, respectively, **g–i** Spleen of control, PAS, and AS, respectively, **j–l** Lung of control, PAS, and AS, respectively, **m–o** kidney of control, PAS, and AS, respectively

as phosphodiesterase type 5 (PDE5) (Zhao et al. 2001; Zhao et al. 2012). ZIP12 expression and PDE5 activity have close relationship with pulmonary vascular smooth muscle proliferation (Francis et al. 1994; Wharton et al. 2005). Zhao et al. proved that ZIP12 expression in pulmonary vascular smooth muscle cells is hypoxia dependent in rat (2015). And it had been confirmed that pulmonary vascular remodeling was associated with pulmonary artery smooth muscle cells excessive

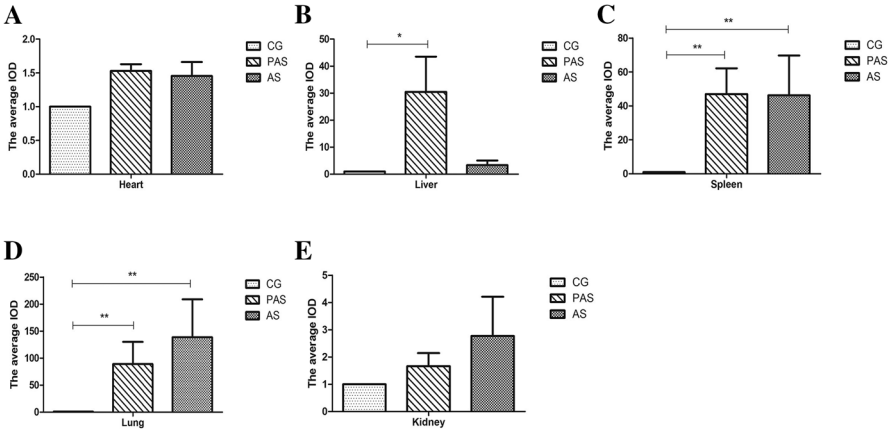

Fig. 5 Integrated optical density analysis of ZIP12 in the heart, liver, spleen, lung, and kidney of control, PAS, and AS groups. $*P<0.05$; $**P<0.01$. Data are represented as mean ± standard deviation (SD) ($n=5$)

proliferation and apoptosis reduced in broilers (Tan et al. 2005). Because of similar pathological process, we speculated that chicken ZIP12 may also participate in AS. In order to verify our speculation, AS model was constructed and target organs chosen were detected the expression pattern of ZIP12. In this research, AS in broilers induced by injecting cellulose microparticles intravenously was consistent with previous reports (Wideman et al. 2002; Tan et al. 2005). Previous reports showed that heart, lung, and liver were the victim organs in the AS (Olkowski 2007; Olkowski et al. 2007; Nain et al. 2009). Meanwhile, considering kidney and spleen may participate in this pathological process. So, these five organs were chosen as target detection organs.

Expression level of ZIP12 in detected organs changed in AS and PAS groups compared to control. In heart, expression level of ZIP12 of AS and PAS groups was higher than that in control. In liver and lung, the expression level of ZIP12 in AS and PAS groups was significantly higher ($P<0.01$) than that in control. Interestingly, expression level of ZIP12 in AS group was lower than PAS group in heart and lung and this need further explored. Increased expression level of ZIP12 in spleen may be associated with inflammatory reaction of AS. As the commercial antibody for chicken ZIP12 is not available, the purified IgG against chicken ZIP12 was prepared and it will be a valuable tool for future research. Results of immunohistochemical analysis showed that ZIP12 is cell-specific distribution in heart, liver, lung, spleen, and kidney in chicken and this protein may play an important role in these cells. There were some discrepancies in the expression of ZIP detected by real-time PCR and protein levels showed by average IOD in some tissues and this may be the difference of post-transcriptional or post-translational regulation in tissues of chicken.

Our results will provide the basic data on the function of ZIP12 in the pathological process of AS in chicken. This will offer an important reference for the prevention and control of AS in chicken and enrich the data of ZIP12 between birds and mammalian.

Acknowledgements This work was partially supported by the National Natural Science Foundation of China (31502131).

Compliance with Ethical Standards

Conflict of interest The authors have declared that no competing interests exist.

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