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MOLECULAR CHARACTERIZATION, EXPRESSION PATTERNS AND METHYLATION ANALYSIS OF ATP-BINDING CASSETTE SUB-FAMILY G MEMBER 2 (*ABCG2*) IN BROILERS

J. Y. Xing*, Y. J. Zhang, W. Q. Jing, W. L. Li and Y. M. Yu

School of Life Sciences, Linyi University, Linyi 276000, China

*Corresponding author email: jinyixing02@126.com

ABSTRACT

As a member of the ATP-binding cassette (ABC) transporter superfamily, ATP-binding cassette sub-family G member 2 (*ABCG2*) plays an important role in clinical drug resistance and maintenance of cellular folate homeostasis. In this work, our aim was to clone and characterize the chicken *ABCG2* (*cABCG2*) gene, investigate its mRNA expression levels in different tissues and relationship between expression and methylation. Our results showed that the chicken cDNA was 2155 bp (Genbank Accession No: KU351683) with a 2106 bp open reading frame, which encoded 701 amino acids. The cABCG2 amino acid sequence showed 55.92%- 97.43% identity with other animal species. A phylogenetic tree revealed that cABCG2 had the closest relationships with chicken and turkey. qPCR analysis indicated the highest *cABCG2* expression levels were detected in liver, whereas the lowest expression levels were found in spleen. In addition, the expression levels of *cABCG2* in liver of offspring, not only 21 days old but also 42 days old chicken, were significantly upregulated by dietary folate deficiency group during laying eggs ($P \leq 0.05$). Bisulfite sequencing results revealed that no significant differences were observed between normal folate group and folate deficient group ($P > 0.05$). These studies will be helpful for future research *cABCG2*precise function and structure.

Keywords: *ABCG2*, broilers, cloning, folate, methylation.

INTRODUCTION

The ATP-binding cassette sub-family G member 2 (ABCG2) was first discovered in doxorubicin-resistant breast cancer cells, so it was also termed breast cancer resistance protein (BCRP) (Doyle *et al*., 1998), being important in clinical drug resistance. Studies have subsequently reported that expression of human *ABCG2* mRNA has been detected in variety of tissues, including liver, spinal cord, placenta, brain, small intestine, colon, prostate, adrenal gland, kidney, uterus, testes, and blood-brain barrier (Doyle *et al*., 1998;Jonker *et al*., 2002; Gutmann *et al*., 2005; Krishnamurthy *et al*., 2006; Hilgendorf *et al*., 2007; Tucker *et al*., 2012). *ABCG2* was found to regulate the homeostasis of nutrients and certain hormones, and plays a role in nutrient absorption of the gastrointestinal tract (Gutmann *et al*., 2005). It was also reported that *ABCG2* plays an important physiological role in multidrug resistance in cancer (Padmanabhan *et al*., 2012; Stacy *et al*., 2013) and in bile acid transport in placenta(Blazquez *et al*., 2012). Other work has indicated that *ABCG2* deficiency can generate dysfunctional mitochondria in hepatocytes (Lin *et al*., 2013), and ABCG2 protein is expressed at lower levels in the elderly, but its levels do not differ regionally in adult human livers (Riches *et al*., 2015).

ABCG2 expression has also been found to be regulated by histone acetylation and methylation. Demethylation of the promoter increased *ABCG2* mRNA and protein expression in human multiple myeloma cells (Turner *et al*., 2006), and DNA methylation-dependent formation of a repressor complex in the CpG island contributes to inactivation of *ABCG2*(To *et al*., 2006). DNA methyltransferase inhibitor treatment of human small lung cancer PC-6 cells induced*ABCG2* re-expression at the mRNA and protein levels, and the results indicated that there was an inverse correlation between promoter methylation of *ABCG2* gene and *ABCG2* expression in both SCLC and NSCLC cells (Nakano *et al*., 2008). Additionally, To *et al* reported that histone modifications play an important role in the regulation of *ABCG2* expression in multidrug-resistant cells (To *et al*., 2008).

Until now, although the *ABCG2* gene sequences and functions have been reported in human and mouse, the sequences of chicken *ABCG2* (*cABCG2*) gene was only predicted by automated computational analysis, the structure and function of chicken *ABCG2* (*cABCG2*) generemain unclear. Chicken as a model animal, plays a key role both as experimental animal for medicine and as farm animal for human life. It is, therefore, highly important toclarify*cABCG2* sequences and expression patterns, not only to improve understanding of the functional basis of *ABCG2*, but also to provide theoretical basis for researching transgenosis animal of *ABCG2*.

In the current study, we describe the cloning and characterization of cDNA encoding the complete open reading frame for the *cABCG2* from chicken and its

mRNA tissue distribution. Furthermore, we also report for the first time that the *cABCG2* gene expression of offspring in liver was affected by folate deficiency during laying eggs.

MATERIALS AND METHODS

Experimental design: This experiment was conducted with the Arbor Acres strain broiler breeder in cages, and, with the approval of Linyi University Institutional Animal Care and Use Committee. At 30 weeks of age, one hundred and twenty Arbor Acres female broiler breeders were randomly allotted to two diet groups: normal folate group (NF, control group) with 2.0 mg/kg of added folate in basal diet and folate deficient group (FD, treatment group) without folate in basal diet. Each group had five replicate cages with 10 birds per cage. Standard basal diet containing corn and soybean meal were fed as mash, and the composition of the basal diet was formulated to meet the nutrition requirements for female broiler breeders at the National Research Council (NRC, 1994). All male broiler breeders were fed with the same standard diet (NRC, 1994). Semen samples were collected for artificial insemination. After 12 weeks, 90 hatching eggs were collected in each group in last three days, and then hatched for offspring. Eighty chicks from each group (group was same as above NF and FD group) were selected for feeding, respectively. Each group consisting of five pen replicates of 16 chicks. Two groups were provided with same commercial diet (Supplied by Liuhe Group, Shandong province, China; Supplementary table 1) for 1-21 days and 22-42 days, respectively. The chicks were kept in floor pens and given free access to feed and water with conventional vaccination and natural lighting during 1-42 days of age.

Sample collection, total RNA extraction and cDNA synthesis: At 21 and 42 days of age, five birds per group (one bird each pen) were randomly chosen to slaughter by bleeding from jugular vein. Liver, kidney, lung, spleen, heart, abdominal adipose, pectoralis muscle and biceps femoris muscle were collected and snap-frozen in liquid nitrogen and stored at -80℃ for total RNA extraction. Meanwhile, liver from 42-day-old broilers were also used to extract genomic DNA for methylation analysis.

Total RNA was isolated by using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions, and then treated with DNase I to remove DNA contamination. First-strand cDNAs were synthesized with ~2μg total RNAs by using a cDNA Synthesis kit (Promega, Madison, USA) according to the manufacturer's protocol.

Isolation and sequencing of *cABCG2* gene: To clone the *cABCG2* cDNA, primers were designed according to the sequences are predicted by automated computational analysis (Genbank accession: XM_004942108) (Table 1). PCR were carried out by the following program: 95 ℃ for 5 min, 35 cycles of 94 ℃ for 30 s, 62.5 ℃ for 40 s, and 72 ℃ for 2 min 30 s and final extension at 72 ℃ for 10 min.

RT-PCR products were purified with QIA quick PCR Purification Kit (Qiagen, Beijing, China) and inserted into pMD18-T Vector (TaKaRa, Dalian, China) according to the manufacturer's protocol. The recombinant plasmid was transformed into competent *E. coli* DH5α cells. At least three positive recombinant clones were sequenced (Sangon, Shanghai, China).

Table 1. Sequences of PCR primers, annealing temperature and PCR products.

Primer name	Primer sequence (5'→3')	Annealing temperature (℃)	Usage	Product size (bp)
AB -F	TCTGGAGTAGATGGGAACTGCT	62.5	Cloning	2155
AB -R	TAAGACCTGAGAGGCTGTAAAGC			
EXP-F	AGTGGCTGATGCTAAGGTAG	58	Expression	171
EXP-R	CTTCAAGAGGATGAGGACAG			
ACT-F	GTGACATCAAGGAGAAGC	55	Internal control	105
ACT-R	CATCAGGGAGTTCATAGC			
M-F1	GTTGTTTTAGTAGATGGGAATTGTTTAA	55	Methylation	291
M-R1	TTATAAAAAATCTTCTTTTCCACAATCTT			
M-F2	AAATAATAGTTATTTTAGAAATTTTTATG	55		260
M-R2	AAAAAATCTTCTTTTCCACAATCTTC			

Bioinformatics analysis: Sequence analysis, open reading frame searching and amino acid deduction were performed with the program of DNAMAN version 6.0. The protein prediction and analysis were conducted with the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST). The protein molecular mass (Mw) and isoelectric point (pI), protscale, TMHMM, protein subcellular localization prediction, the secondary structure and the three-dimensional structure of cABCG2 were determined by ExPASy

(http://www.expasy.org/). A phylogenetic tree was constructed by neighbor-joining (NJ) method based on the alignment of cABCG2 amino acid sequences with Mega 5.1 software. The branches were tested with 1,000 bootstrap replicates.

Expression analysis: Primers for c*ABCG2* and mRNA amplification were EXP-F and EXP-R, ACT-F and ACT-R (Table 1), respectively. The housekeeping gene *β-actin* was used as an internal control for the relative quantification of c*ABCG2*mRNA expression. Real-time PCR (qPCR) was performed using SYBR® Premix Ex Taq™ II (TliRNaseH Plus) (TaKaRa, Dalian, China) in optical 96-well reaction plates (Roche, Mannheim, Germany) on a LightCycler480 real-time system (Roche, Mannheim, Germany). All samples were run in duplicate. PCR conditions were as followed: 95℃ for 2 min,40 cycles of 95 ℃ for 10 s, 58 (55) ℃ for 10 s, 72 ℃ for 1 s, and then the melting curve. Ct value, i.e., cycle number to reach threshold, was recorded and normalized by the $2^{-\Delta\Delta Ct}$ method.

Methylation analysis: Genomic DNA was extracted from liver of 21-day-old and 42-day-old broilers using Genomic DNA Purification Kit (Qiagen, Beijing, China) according to the manufacturer's protocol. For each group, equal quantity of DNA from the five samples which were used for qPCR analysis of *cABCG2* gene was mixed together according to its concentration to form two DNA pools. And then each DNA pool was treated using EZ DNA Methylation-Gold™ Kit （Zymo Research, California, USA). Primers (Table 1) for *cABCG2* gene CpG methylation assay were designed using the Meth Primer program (http://www.urogene.org/methprimer/) and selected to contain at least three to four transformed uracil residues (C→U) for specific amplification of successfully converted DNA strands only, and did not contain any CpG sites within their sequences. PCR reaction was performed by touchdown nested PCR. The first round PCR (using primers M-F1 and M-R1, Table 1), conditions for the reaction were 95 ˚C for 2 min, 16 cycles of 95 ˚C for 30 s, 68 ˚C for 30 s (the annealing temperature was lowered 1 ˚C every cycle beginning at 68 ˚C and ending at 53 ˚C), 72˚C for 1 min; followed by 30 cycles of 95 ˚C for 30 s, 55 ˚C for 30 s, 72 ˚C for 1 min; finally 10 min at 72 ˚C. The second round PCR used 1 µl of the first round PCR product as template with primer pair M-F2 and -R2 (Table 1) to amplify the 260 bp including 8 CpGs. Amplification conditions consisted of 95 ˚C for 2 min, 40 cycles of 95 ˚C for 30 s, 55 ˚C for 30 s, and 72 ˚C for 1 min, with a final extension at 72 ˚C for 10 min. Purification, cloning and sequencing of PCR products were as the same as above described. For each group, ten clones were sequenced. At each CpG site, the CpG of genomic DNA template was considered as methylated when the sequence obtained was TpG.

Data analysis: Data were shown as mean ± standard error. Tukey test and two-sample t-test were employed to compare mRNA levels of different tissues, the same tissue between NF and FD groups and methylation levels between NF and FD groups, respectively at $P \leq 0.05$, the difference was considered as significant.

RESULTS

Isolation and molecular characterization of *cABCG2*: After RT-PCR, a 2155 bp fragment of *cABCG2* cDNA (Genbank accession:KU351683) was obtained from broiler liver, while the open reading frame was 2106 bp. There are only 2 bp differences compared with the sequences predicted by automated computational analysis (Genbank accession: XM_004942108). In addition, the ORF encoded a predicted 701 amino acids, with a theoretical pIand a molecular mass of 8.81 and 77.4 kDa, respectively. Transmembrane region predicted by the TMHMM program showed that the protein has five potential membrane proteins which are located at 431-453, 515-534, 544-566, 571-593 and 670-692, respectively. The signal peptide prediction indicated that this polypeptide contained no signal peptide in its amino acids. Hydropathy analysis showed that cABCG2 protein was a hydrophobic protein and its average of hydrophobicity was 0.1394. Analysis of the amino acid sequences of cABCG2 with the PSORT II program indicated that the probability of cABCG2 residing in the endoplasmic reticulum, plasma membrane, nuclear, mitochondrial, vesicles of secretory system were 47.8%, 30.4%, 8.7%, 8.7% and 4.3%, respectively. And the ratio of alpha helices, extended strand, random coil and beta turns in its secondary structure was 41.51%, 21.84%, 28.82% and 7.85%, respectively. The three-dimensional structure model was based on template 4f4c. 1. A (3.40 Å), and sequence similarity was 32% with a coverage range of 61% (from 104 to 554) (Fig. 1).

In addition, amino acids sequences of cABCG2 showed 58.77%, 56.35%, 55.92%, 57.20%, 57.06%，56.92%, 56.35%，97.43%, 91.03%, 90.46%, 95.15%, 78.77%, 56.05%，60.49%, 60.06% and 57.93% identity to those of human, mouse, rat, pig, sheep, goat, cattle, turkey, mallard, adelie penguin, Japanese quail，rock pigeon, ansercygnoides domesticus, aquilachrysaetos canadensis, tropical clawed frog and atlantic salmon, respectively. To study phylogenetic relationship between cABCG2 and known ABCG2 of other animal, phylogenetic tree was constructed by comparing amino acids sequences of cABCG2 with those of ABCG2 from other species (Fig. 2). The cABCG2 was found to be close to turkey and Japanese quail.

Expression patterns of the *cABCG2*genein different tissues: The tissue distribution pattern of *cABCG2* was investigated in 8 tissues by qPCR. As shown in Fig. 3, the

highest expression level of *cABCG2* mRNA was found in liver, whereas that of *cABCG2* was detected lowest in spleen (P≤0.01). It showed moderate expression level in the other tissues (Fig. 3).

The *cABCG2* gene expression of offspring affected by folate deficiency during laying eggs: The*cABCG2* expression level in the liver and abdominal adipose tissue of 21-day-old and 42-day-old broilers were analyzed by folate deficiency during laying eggs and shown in Fig. 4. Compared with control (NF group), in dietary folate deficiency group during laying eggs, the mRNA expression levels of *cABCG2* in liver of offspring, not only 21 days old but also 42 days old, were significantly increased (Fig. 4, NF group vs FD group) (Fig. 4A). Further analysis during different development stages in the same group indicated that there was a trend to increase the *cABCG2* expression level from 21 days old to 42 days old, however, the difference was not significant (*P*>0.05) (Fig. 4, 21 days old vs 42 days old) (Fig. 4A).

Moreover, in the abdominal adipose tissue, expression levels of *cABCG2* in FD group of 21-day-old offspring broilers were higher than those in NF group; on the contrary, in 42-day-old offspring broilers, expression levels of *cABCG2* in FD group were lower than those in NF group, however, there was no significant difference (*P*>0.05) (Fig. 4 B). In addition, the expression levels of *cABCG2* in the abdominal adipose tissue of same group (NF or FD) increased significantly from 21 to 42 days of age (*P*≤0.05) (Fig. 4 B).

The *cABCG2* gene DNA methylation of offspring affected by folate deficiency during laying eggs: To determine whether folate deficiency during laying eggs affects DNA methylation of *cABCG2* of offspring, the methylation profile of 8 CpG dinucleotides lied at position +15 ~ +274 of exon 2 (numbering from the +1 *cABCG2* translation start site) of *cABCG2* gene in the liver of offspring was detected using bisulfite sequencing. The results demonstrated that, for all the CpG sites analyzed, the overall methylation percentage was 91.3%and 87.5% for NF group and FD group at 21 days of age, respectively (Fig. 5A, B); however, no significant differences were observed between NF group and FD group (*P*>0.05). Meanwhile, higher methylation percentage occurred mainly from 2nd to 8th sites while the 1st site was less methylated (Fig. 5A, B).

Then, in 42-day-old offspring, although the overall methylation percentage of FD group (80.0%) has a trend to decrease, the difference was not significant compared to NF group (86.3%) (*P*>0.05). To further analyze different CpG site, results indicated that, in the FD group, CpG methylation occurred mainly at 4th, 5th, 7th and 8th, but higher methylation was observed from 2nd to 8th in NG group (Fig. 5C, D).

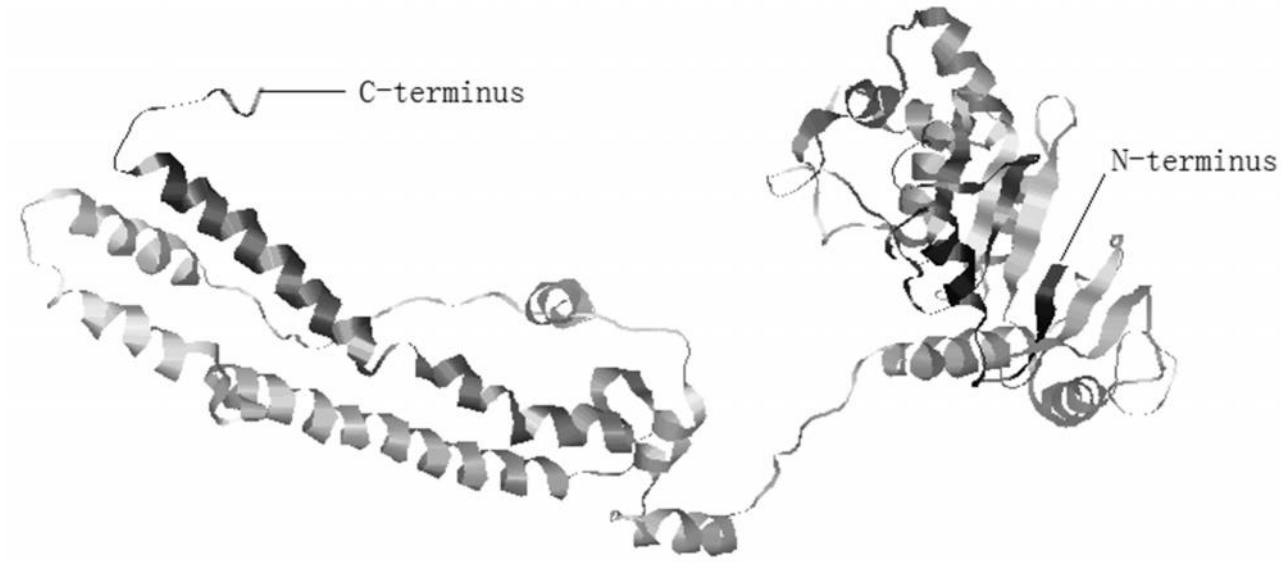

Fig. 1. cABCG2 protein three-dimensional model predicted by the SWISS-MODEL Workspace and RasMol software.

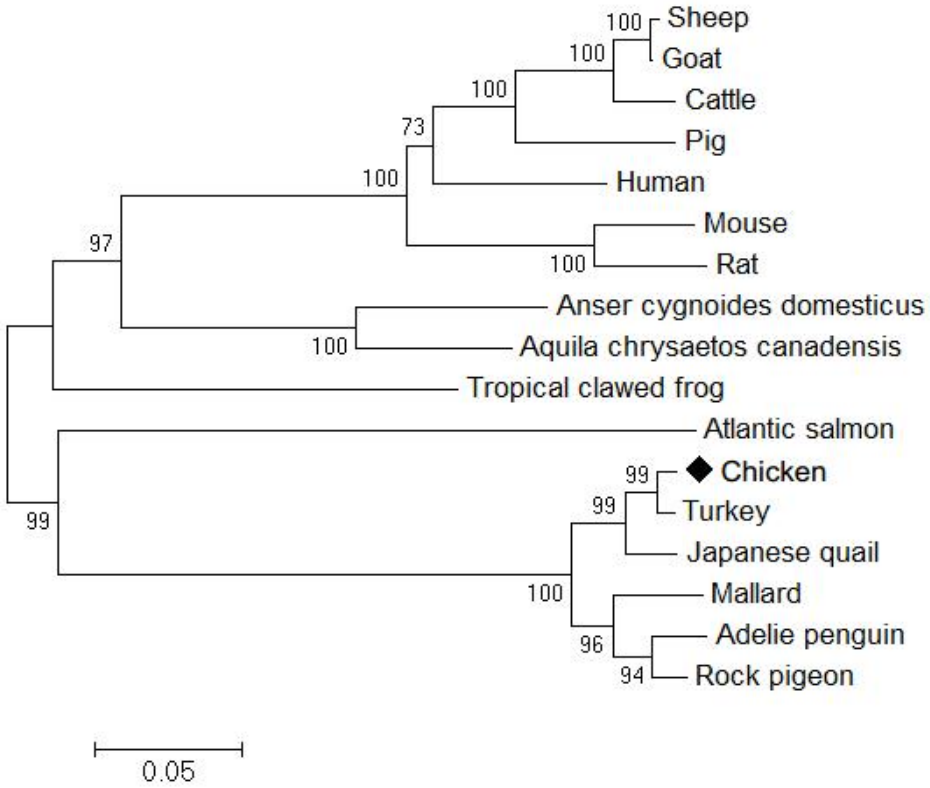

Fig. 2.Phylogenetic tree of cABCG2 amino acid and other animal species. The scale bar is 0.05 representing genetic distance. The number at each node indicates the percentage of bootstrapping after 1,000 replications. The broiler amino acid is labeled with asterisk (◆). The Genbank accession of the sequences used to build the phylogenetic tree are as follows: broiler (KU351683), human (AAG52982), mouse (NP_036050), rat (NP_852046), pig (NP_999175), sheep (NP_001072125), goat (NP_001272636), cattle (NP_001032555), turkey (XP_010712742), Japanese quail(XP_015722206), mallard (XP_005025255), adelie penguin (XP_009320667), rock pigeon (XP_013223959), aquilachrysaetos canadensis (XP_011599110), ansercygnoides domesticus (XP_013046088), tropical clawed frog (NP_001039227) and Atlantic salmon (XP_014064984).

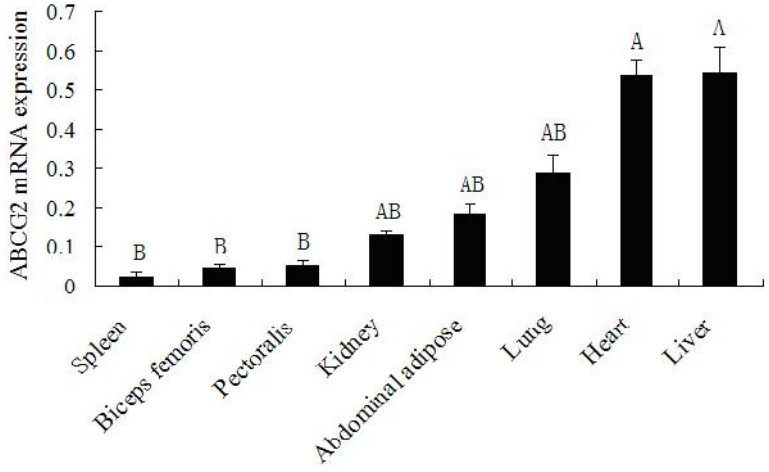

Fig. 3.The c*ABCG2* gene mRNA expression patterns in different tissues of the broiler. Data are represented as means ±std error (n=5). The uppercase super scripts indicate statistical difference among different tissues (*P*≤0.01).

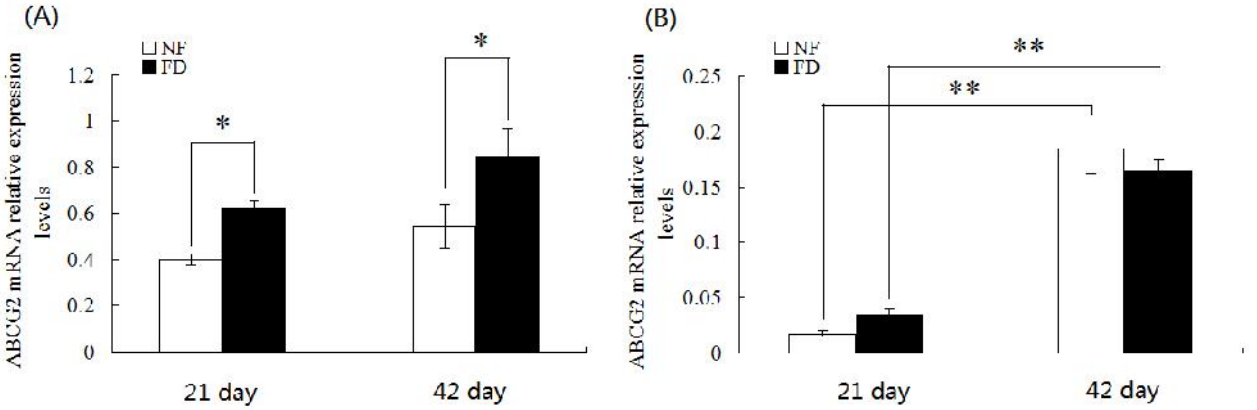

Fig. 4.Effect of dietary folate deficiency during laying eggs on mRNA expression of *cABCG2* in liver (A) and abdominal adipose (B) of the offspring. Data are represented as mean ± std error (n=5). The single asterisk (*) and double asterisk (**) show statistical difference (*P*≤0.05 and *P*≤0.01), respectively.

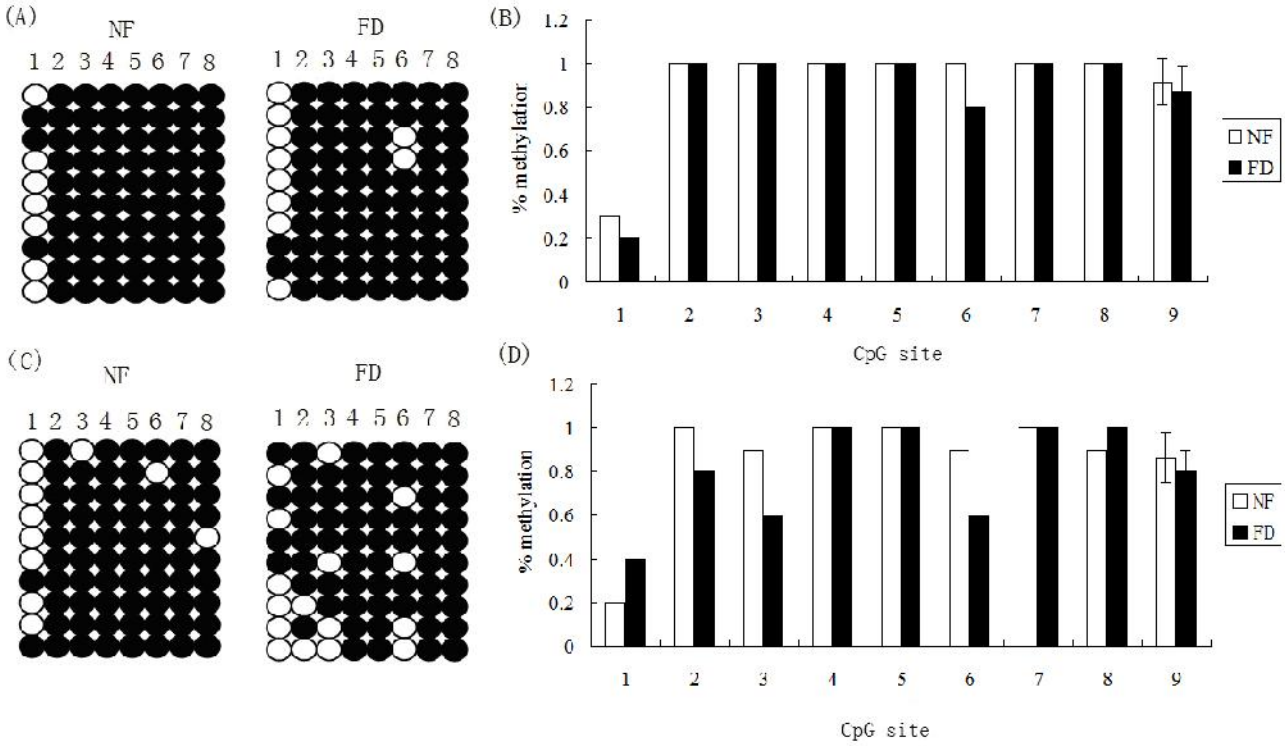

Fig. 5.DNA methylation status of *cABCG2* exon 2 in offspring during laying eggs. Bisulfite sequencing in DNA from liver of 21-day-old broilers (A) and 42-day-old broilers (C). Each horizontal line represents independent clone and each circle represents single CpG dinucleotide. Solid circles and open circles indicate methylated and unmentylated CpG sites, respectively. Bar graph represents ratio of methylation at each CpG site (1-8) and total methylation ratio of *cABCG2* exon 2 of 21-day-old broilers (B) and 42-day-old broilers (D).

DISCUSSION

In humans, physiological functions, structure, biogenesis of *ABCG2* have been observed, especially its role in multidrug resistance to tumor cells and affecting stem cell biology (Doyle and Ross, 2003; van Herwaarden and Schinkel, 2006; Padmanabhan *et al*., 2012; Stacy *et al*., 2013; Szepesi *et al*., 2015; Ding *et al*., 2016). Due to the broiler plays an important role both as an agriculture animal and experimental medical animal, it is necessary to study the structure and functions of *cABCG2*. According to the literature, only sequences of the *cABCG2*was predicted by automated computational analysis. In this study, the 2155 bp partial cDNA of

cABCG2 was isolated and sequenced for the first time to our knowledge. The putative amino acid sequences of *cABCG2* shares high identity with turkey, Japanese quail, mallard, and adelie penguin. Moreover, the phylogenetic analyses also indicated that cABCG2 has the highest sequence similarity to turkey (Fig. 2).

Our data also demonstrate that liver and heart indicated higher transcript abundance of *cABCG2*, this is agreement with the previous finding that expression level of *cABCG2* in the broiler liver is higher (Su *et al*., 2014); but in turkey, the highest expression levels of *ABCG2* were measured in the small intestines (Haritova *et al*., 2008). Meanwhile, the expression levels of *ABCG2*were found to be different among the various segments of intestine in rat, mouse, human and broiler (Maliepaard *et al*., 2001; Su *et al*., 2014; Tanaka *et al*., 2005). On the contrary, our results showed that low transcript levels were detected in spleen, biceps femoris and pectoralis (Fig. 3). Recent study in hens found that high-level expression of *cABCG2* were associated with brown, white and generally lighter eggshell colors, for example, *cABCG2* expression in spleen was significantly higher in brown-eggshell hens than thoseofwhite- or pink-eggshell hens (Zheng *et al*., 2014). In contrast, previous work reported that, in normal human tissues, high levels of *ABCG2* expression has been found in the placenta and kidney (Allikmets *et al*., 1998; Doyle *et al*., 1998; Maliepaard *et al*., 2001), but low levels of *ABCG2* in liver, small intestine, colon, ovary, spleen, skeletal muscle, kidney and heart have been also reported (Doyle *et al*., 1998). The differences in the *ABCG2* expression patterns are likely due to physiological differences between birds and mammals.

Folate, as methyl donor, plays a key role in the interactions between nutrition, fetal programming, and epigenomics, and maternal folate status influences DNA methylation, growth and development in offspring (Gueant *et al*., 2013). In order to determine the effect of folate deficiency during laying eggs on *cABCG2* expression of offspring, we compared the expression patterns of the *cABCG2* gene between NF group and FD group in liver and abdominal adipose. Our data revealed that *cABCG2* mRNA levels in liver of offspring were significantly increased by folate deficiency during laying eggs. Similarly, previous study has demonstrated that the folate deprivation induced an increase in *ABCG2* in colorectal cell lines (Lemos *et al*., 2008; Lemos *et al*., 2009a; Lemos *et al*., 2009b) and prostate cancer cell lines (Sobek, 2013), which was consistent with our study. In contrast, in the breast cancer cell line, folate deprivation decreased *ABCG2* gene expression (Ifergan *et al*., 2004). These studies suggested that folate deprivation can regulate *ABCG2* expression but the induction or repression of expression is likely tissue specific or possibly cell specific (Sobek, 2013). As a whole, *ABCG2* plays a key role in the maintenance of cellular folate homeostasis (Ifergan *et al*., 2004; Ifergan *et al*., 2005; Lemos *et al*., 2009a; Natarajan *et al*., 2012).

DNA methylation at the 5′-position of cytosine of CpG dinucleotide is an epigenetic process influenced by the environment in which heritable changes in the coding pattern of DNA occur(Klutke *et al*., 2010). So, we also carried out studies on the relationship between CpG methylation patten in *cABCG2* exon 2 and *cABCG2* mRNA expression because folate also acts as methyl donor. Our data showed that overall methylation percentage have no significant differences not only at 21 days of age but also at 42 days of age between NF group and FD group. However, a different CpG methylation distribution profile was observed in the *cABCG2* exon 2 between NF group and FD group. For example, at 42 days of age, among the 8 CpG sites, percentage of methylation of FD group at 2^{nd}, 3^{rd} and 6^{th} was decreased as compared to control group (NF group). Previous study reported that DNA methylation-dependent formation of a repressor complex in the GpG islands contributes to inactivation of *ABCG2* in renal cell carcinoma cell lines (To *et al*., 2006). Similar findings were reported in human multiple myeloma cell lines by Turner *et al*, who found that a decrease in promoter methylation led to an increase in *ABCG2* expression at the mRNA and protein level (Turner *et al*., 2006). A recent study discovered that LNCaP cells grown in folate deficient environment caused the *ABCG2* promoter to become hypomethylated (Sobek, 2013). Similarly, our results indicated that the *cABCG2* exon 2 methylation of offspring liver was also decreased by folate deficiency during laying eggs. These results suggest that folate may regulate *ABCG2* expression by altering *ABCG2* methylation modification patterns.

Taken together, methylation of cytosines associated with a repressed chromatin state and inhibition of gene expression (Siegfried and Simon, 2010), and high levels of gene expression often associated with low promoter menthylation (Kass *et al*., 1997), however, recent studies reported that CpG islands and island shores exhibited strong correlations with gene expression, but this was not true for island shelves (Martino and Saffery, 2015), and human body epigenome maps also revealed non-canonical DNA methylation variation (Schultz *et al*., 2015), suggesting that the relationship between genetic variation, DNA methylation and expression is more complex (van Eijk *et al*., 2012).There is still a lot to be explored these mechanisms.

This study described for the first time the isolation, cloning and characterization of a full length*ABCG2* encoding sequence and part of its non coding regions from chicken. The *cABCG2* expression patterns in chicken eight tissues and folate regulating its expression level were also analyzed. Moreover, the *cABCG2* gene DNA methylation affected by folate deficiency was investigated by using bisulfite sequencing

PCR.

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