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Original study

Molecular cloning, sequence characterization, and gene expression profile of a novel water buffalo (Bubalus bubalis) gene: Na^+, K^+-ATPase β_2-subunit (*ATP1B2*)

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Abstract

The Na^+, K^+-ATPase is a transmembrane carrier protein which plays an important role in Na-K transport and energy metabolism. The aim of this study was to obtain the full-length coding sequence (CDS) of water buffalo Na^+, K^+-ATPase β_2-subunit (*ATP1B2*) using RT-PCR and to investigate the characterizations of its sequence and tissue expression patterns. Sequence analysis revealed that the CDS of water buffalo *ATP1B2* encodes an enzyme of 290 amino acid residues with a deduced molecular weight of 33.39 KDa and a PI of 8.37. Water buffalo *ATP1B2* was presumed to have a signal peptide, a strong hydrophobic region and to exert its function in the plasma membrane with high reliability. In addition, water buffalo *ATP1B2* has a conserved Na^+, K^+-ATPase β domain which belongs to Na^+, K^+-ATPase superfamily. The sequence of water buffalo *ATP1B2* gene shares 97.6, 97.4, 92.2, 93.2, 89.9, 93.2, 89.5 and 62.9 % identify with its homologous sequence of cattle, sheep, dog, human, mouse, baboon, rat and African clawed frog, respectively. Phylogenetic tree analysis based on the CDS of *ATP1B2* gene showed that water buffalo has a closer genetic relationship with cattle than with other species. The *ATP1B2* gene was widely expressed in the tissues examined, being high in the pituitary gland and brain, moderate in the muscle, spleen, liver, mammary gland, kidney and rumen, weak in the heart, small intestines and skin, and not expressed in the lung and adipose tissue. This study will establish a foundation for further insights into this novel water buffalo gene.

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Keywords: Water buffalo, Na^+, K^+-ATPase β_2-subunit (*ATP1B2*), bioinformatics analysis, tissue expression analysis

Abbreviations: CDS: coding sequence

Introduction

Heat stress has huge economic impact on the global dairy industry, and has adverse effects on a variety of productive traits including milk yield, milk composition, growth, carcass and reproduction of dairy cattle, while the heat tolerance can be improved for the high-yielding breeding (Nardone *et al.* 2000, Wheelock *et al.* 2010, Baumgard & Rhoads Jr 2013). That's to say, one possible way to increase milk yield of dairy cattle is to improve heat tolerance and ability of recovery from heat stress of animals. Previous studies showed that animals exposed to heat-stress in environment exhibited a significant change in Na^+, K^+-ATPase activity in a variety of tissues (Levy *et al.* 2005, Pearce *et al.* 2011). In addition, genetic association study on Na^+, K^+-ATPase β_2-subunit gene (*ATP1B2*) in Chinese Holstein cattle showed that the polymorphisms of *ATP1B2* are associated with milk yield, milk composition and heat tolerance (Wang *et al.* 2011). From the above studies, Na^+, K^+-ATPase is especially sensitive to heat stress and its function is to maintain the electrochemical gradient of Na^+ and K^+ ions across the cytomembrane, which provides energy for the membrane transport of metabolites, nutrients and ions. Therefore, the cattle Na^+, K^+-ATPase gene presents a plausible candidate responsible for heat tolerance traits.

Na^+, K^+-ATPase is a transmembrane carrier protein that uses the energy of ATP hydrolysis to transport Na^+ ions out of and K^+ ions into the cell (Köksoy 2002). The enzyme has been shown to be composed of three subunits. The α subunit has been implanted in the ion-pumping process (Köksoy 2002, Vague *et al.* 2004). The β subunit is a highly glycosylated protein that interacts with the α subunit and is involved in ion recognition (Köksoy 2002, Vague *et al.* 2004). The γ subunit is a specific, but dispensable component of functional Na^+, K^+-ATPase. Each subunit consists of multiple isoforms (Béguin *et al.* 1997, Barcroft *et al.* 2002). In the case of α subunit, four isoforms, α_1, α_2, α_3, and α_4 are present in mammal cells. The β subunit is a type II membrane protein and is necessary for maturation and membrane targeting of the enzyme. There are three isoforms of the β subunit, namely β_1, β_2, and β_3, each of them derived from a different gene (Köksoy 2002).

The structure of *ATP1B2* gene has been described in the mouse, human, and cattle (Emanuel *et al.* 1987, Hernando *et al.* 1994, Avila *et al.* 1998). The β_2 isoform gene of the mouse is located on chromosome 11, the human *ATP1B2* is located on chromosome 17, and the cattle *ATP1B2* is located on chromosome 19 (Yang-Feng *et al.* 1988, Vague *et al.* 2004). Study in cattle showed that the *ATP1B2* gene has six introns, seven exons and its total length is 4 310 bp, the CDS length of this gene is 873 bp, encoding a protein of 290 amino acid residues (Tokhtaeva *et al.* 2010, Wang *et al.* 2012). Study in Chinese Holstein cow showed that two novel SNPs, G2258A and C2833T, in the *ATP1B2* gene were associated with milk production trait. G2258A significantly affected the milk fat and milk yield, C2833T significantly affected the milk protein and milk yield (Wang *et al.* 2011).

Water buffalo contributes significantly to the agricultural economy and dairy industry in

the tropical and subtropical areas (Singh *et al*. 2000, Khan *et al*. 2011, Perera 2011). In tropical and subtropical countries, one of the problems to be solved in the dairy industry is the heat stress suffered from dairy cows under hot and humid weather condition. Buffalo is the second largest source of milk supply in the world, and buffalo milk contains less water and more fat, lactose, protein, and minerals than cow milk (Vijh *et al*. 2008, Mahmood & Usman 2010, Yindee *et al*. 2010). The Na^+, K^+-ATPase gene maybe represents a candidate gene for heat tolerance traits. Although *ATP1B2* gene has been studied in humans, mice and cattle, there is no report on *ATP1B2* gene in water buffalo. In this study, we firstly cloned the full-length coding sequence of water buffalo *ATP1B2* gene, and subsequently did the bioinformatics analysis based on the sequence of *ATP1B2* gene obtained, and finally examined its differential expression in tissues by semi-quantitative PCR. These may establish a primary foundation for understanding the mechanisms of heat tolerance in water buffalo.

Material and methods

Sample collection and cDNA preparation

All the procedures of sample collection were performed in accordance with the Guide for Animal Care and Use of Experimental animal approved by the Institutional Animal Care and Use Committee of Yunnan Agricultural University. Thirteen fresh tissue samples from the heart, pituitary gland, small intestines, muscle, spleen, liver, mammary gland, skin, lung, brain, kidney, stomach and adipose tissue were collected from three non-lactating water buffaloes (Binglangjiang buffalo) after they had been slaughtered, and another three mammary tissue samples were taken from three lactating water buffaloes (Binglangjiang buffalo). The sampled individuals had no blood relationships at the age of about 54 months. The samples were snap-frozen immediately in liquid nitrogen and then stored at −80 °C before processing for RNA isolation.

Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China) and treated with RNase-free DNase I (TaKaRa, China) following the manufacturers' instructions. The total RNA (1 µL) was checked by 2.0 % agarose gel electrophoresis containing ethidium bromine. The RNA (3 µg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Cloning and sequencing of water buffalo ATP1B2 CDS

The sequence of cattle *ATP1B2* gene (acc. no. NM_001035373) and its highly homologous expressed sequence tags, were used to design a set of primers to amplify the complete water buffalo *ATP1B2* CDS. The primers were: 5'-AATTGACAGCGGCTGCATATCT-3' (forward) and 5'-GGGTATTCCAGAAGCATCCGTA-3' (reverse). The PCR was performed to isolate the water buffalo *ATP1B2* gene using the pooled cDNAs from the mutiple tissue RNAs. The 25 µL reaction system contained 2.5 µL of 50 ng/µL cDNA, 2.0 µL of 1.25 mM dNTPs mixed (TaKaRa, China), 12.5 µL of 10x Ex-Taq DNA polymerase buffer ($Mg2^+$ Plus), 0.5 µL of 10 µM forward primer, 0.5 µL of 10 µM reverse primer, 0.25 µL of 5 U/µL Ex Taq DNA polymerase (TaKaRa, China), and 6.25 µL of sterile water. The reaction conditions included denaturation for 3 min at 94 °C, followed by 35 cycles of amplification at 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 2 min, and

then with a final extension at 72 °C for 5 min. The purified amplified fragment was subcloned into the pMD18-T vector (TaKaRa, China) and then sequenced. At least eight independent positive clones were sequenced. The cDNA segments obtained from sequencing were edited and assembled using DNAstar SeqMan program.

Bioinformatics and sequence analysis

The software on the ExPASy server (Walker 2005) was used to predict the physical and chemical properties of the putative *ATP1B2* protein. Conserved domain prediction was performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the NCBI server. ClustalW software (Thompson *et al.* 1994) was used for alignment of multiple sequences. Signal peptides were predicted using the SignalP 3.0 server (Dyrløv Bendtsen *et al.* 2004). ProtComp 9.0 (http://www.softberry.com) was employed to predict protein sorting signals and intracellular localization. Secondary structures of deduced AA sequences were predicted by SOPMA (Geourjon & Deléage 1995). TMHMM version 2.0 (Krogh *et al.* 2001) was used to predict transmembrane helices of the protein. Phylogenetic tree (neighbour-joining tree) was constructed based on *ATP1B2* gene sequences by applying MEGA v. 4.0 software (Tamura *et al.* 2007). Statistical significance of groups within phylogenetic trees was evaluated using the bootstrap method with 1 000 replications. The three-dimensional structures of the *ATP1B2* protein were predicted by the amino acids homology modelling on SWISS-MODEL (Guex & Peitsch 1997).

Semi-quantification PCR of water buffalo ATP1B2 gene

To characterize the differential expression, we assayed the mRNA levels of the *ATP1B2* gene in 13 tissues of non-lactating water buffalo by semi-quantitative RT-PCR, and to determine its functional role in the mammary gland, we further examined the *ATP1B2* gene expression in the mammary tissues of lactating water buffalo. The primers used for semi-quantitative RT-PCR were: 5'-CCGTCAAGATGGTCATTC-3' (forward) and 5'-ATCAAGCCTGGTGTAGCC-3' (reverse) (Wang *et al.* 2012). The RT-PCR were performed using Ex Taq DNA polymerase (TaKaRa, China) according to the manufacturer's protocol. The PCR reaction condition was as follows: predenaturation at 94 °C for 3 min; then 35 cycles of 94 °C for 15 s, 55 °C for 45 s, 72 °C for 1 min, followed by a 3 min extension at 72 °C, and finally to terminate the reaction at 4 °C. To eliminate the effect of cDNAs concentration, we repeated the RT-PCR five times using 1, 2, 3, 4, and 5 µL of the cDNAs as templates, respectively. In this study, water buffalo housekeeping gene *18S ribosomal RNA* was chosen as the internal reference and was detected with the primers, 5'-GGACATCTAAGGGCATCACAG-3' (forward) and 5'-AATTCCGATAACGAACGAGACT-3' (reverse). Results of semi-quantitative RT-PCR were expressed as the relative quantity of the *ATP1B2/18S ribosomal RNA*.

Results

Cloning and identification of water buffalo ATP1B2 complete coding sequence

Being consistent with the expectations, a PCR product of 1 167 bp was obtained (Figure 1). The sequencing and open reading frame prediction showed that the full-length coding sequence

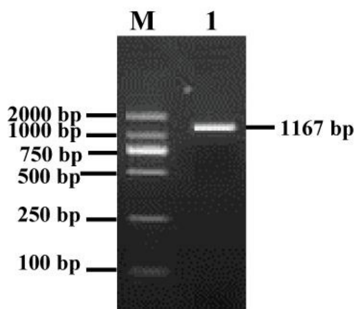

Figure 1
Gel image of RT-PCR product of water buffalo *ATP1B2* gene. 1, PCR product for water buffalo *ATP1B2* gene. M, DL2000 DNA marker.

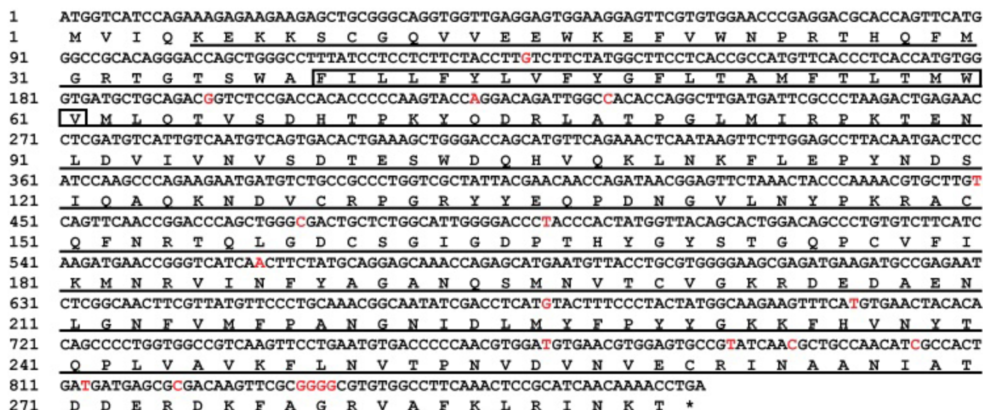

Figure 2
The complete CDS of water buffalo *ATP1B2* gene (acc. no. JX888716) and its encoding amino acid sequences. An asterisk indicates the stop codon; Conserved domain sequences of Na^{+} K^{+}-ATPase superfamily is underlined. Red characters donate nucleotide different sites of complete *ATP1B2* CDS between water buffalo and cattle (NM_174677).

of water buffalo *ATP1B2* gene is 873 bp which encoded 290 AA. The coding sequence was then submitted to the NCBI database (acc. no. JX888716). The complete CDS of the gene and the deduced AA are presented in figure 2. Comparison of the CDS sequences showed that *ATP1B2* gene is conserved throughout the mammals. BLAST analysis revealed that water buffalo *ATP1B2* gene shares high homology with *Bos taurus* (97.6 %), *Ovis aries* (97.4 %), *Canis lupus familiaris* (92.2 %), *Homo sapiens* (93.2 %), *Mus musculus* (89.9 %), *Papio anubis* (93.2 %), *Rattus norvegicus* (89.5 %), and has a lower homology with *Xenopus laevis* (62.9 %) at the nucleotide level. The coding region sequence of *ATP1B2* gene has an overall base composition of 25.43 % A, 27.72 % C, 25.09 % G, and 21.76 % T. There are 4 non-synonymous substitutions for the coding region sequences of the *ATP1B2* gene between water buffalo and cattle. They are c.560 A>G, c.678 G>A, c.684 T>C, c.835 G>C, and c.836 G>C, which lead to the corresponding deduced amino acid changes at p.187 (N>S), p.226 (M>I), p.256 (D>E) and p.279 (G>P), respectively.

Phylogenetic tree construction of ATP1B2

Phylogenetic tree was constructed based on the nucleotide sequences of the *ATP1B2* gene. The sequences from mammals form four subgroups: Bovidae family, primate, Canine family and rodent. Water buffalo has a closer genetic relationship with the species of Bovidae family (cattle and sheep).

Amino acid sequence analysis and protein characteristics

The molecular weight and theoretical pI for the deduced amino acid sequence of water buffalo *ATP1B2* are 33.39 kD and 8.37, respectively. Bioinformatic analysis showed that Water buffalo *ATP1B2* has a 53 AA N-terminal signal peptide (Figure 4A) and it functions in the plasma membrane with high reliability (score: 9.93). Conserved domains of Na^+, K^+ ATPase superfamily in water buffalo was displayed as figure 2 (AA 5-284). Hydrophobicity structure prediction showed that water buffalo *ATP1B2* contains a strong hydrophobicity domain (Figure 4B). Conserved domain prediction indicated that the *ATP1B2* has a Na^+, K^+-ATPase β domain which belongs to Na^+, K^+-ATPase superfamily (AA 5-284) (Figure 5). Two Na^+, K^+-ATPase β subunit signatures were found in the *ATP1B2*, which are 17-WkeFvWNprthqfMGRTgtsW-37 and 147-KraCQfnrtqLgdCSG-163, respectively. The putative amino acid sequence of the *ATP1B2* contains 5 potential N-Glycosylation sites. They are at p.96, p.118, p.197, p.238 and p.288 of the protein. The prediction of secondary structure by SOPMA indicated that the deduced *ATP1B2* of water buffalo contains 79 AA alpha helices, 53 AA extended strands, 6 AA beta turns and 152 AA random coils (Figure 6), which suggested that a significant portion of the amino acids of the *ATP1B2* are most likely to form random coils. The three-dimensional structure of the *ATP1B2* (AA 23-289) (Figure 7A) predicted by homology modeling is similar to that of pig *ATP1A1* (Figure 7B).

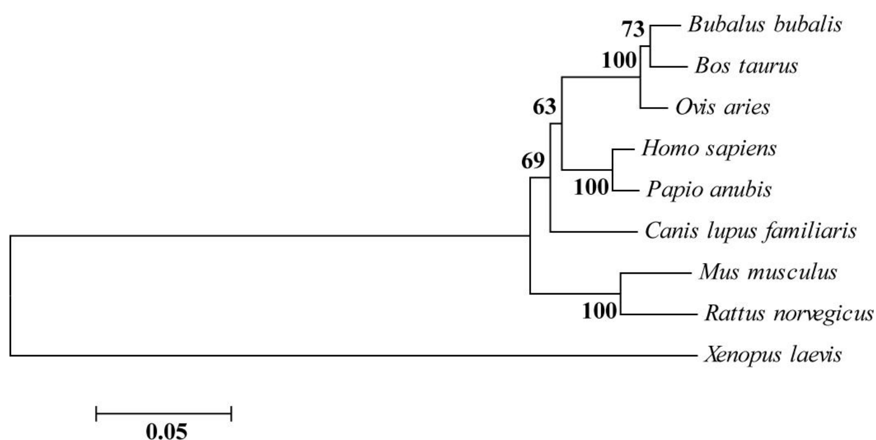

Figure 3
Phylogram based on *ATP1B2* gene sequences using the neighbor-joining method. The nucleotide sequence of water buffalo *ATP1B2* and the *ATP1B2* of other species were used in this analysis. *Bos taurus* (cow) NM_174677, *Ovis aries* (sheep) XM_004012670, *Homo sapiens* (human) NM_001678, *Papio anubis* (baboon) XM_003912271, *Mus musculus* (mouse) NM_013415, *Rattus norvegicus* (rat) NM_012507 and *Xenopus laevis* (African clawed frog) NM_001086893.

Figure 4
Transmembrane regions (A) and hydrophobicity profile (B) of water buffalo ATP1B2 generated with the ProtScale. Score>0 means hydrophobic; score<0 means hydrophilic.

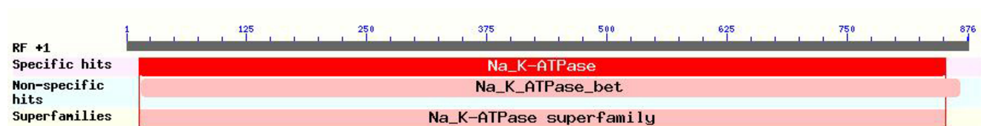

Figure 5
The putative conserved domain of the protein encoded by water buffalo *ATP1B2* gene.

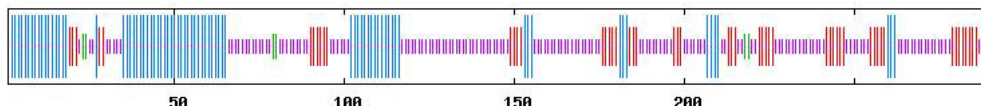

Figure 6
Predicted secondary structure of the water buffalo ATP1B2 protein by SOPMA. Alpha helices, extended strands, beta turn, random coils are indicated with the longest, second longest, third longest and shortest vertical lines, respectively.

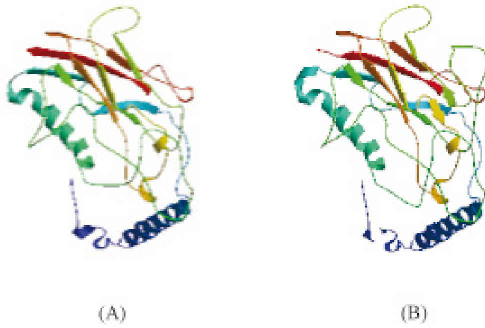

Figure 7
The tertiary structure of buffalo ATP1B2 predicted by SWISS-MODEL. (A) Putative tertiary structure of buffalo ATP1B2. (B) Tertiary structure of pig ATP1A1

Tissue expression profile analysis of water buffalo ATP1B2 gene

The differential expression analysis showed the *ATP1B2* gene was widely expressed in the examined tissues of non-lactating water buffalo (Figure 8A), being high in the pituitary gland and brain, moderate in the muscle, spleen, liver, mammary gland, kidney and rumen, weak in the heart, small intestines and skin, and not expressed in the lung and adipose tissue. As can be seen in figure 8B, the *ATP1B2* gene displayed differential expression in the mammary tissues of non-lactating and lactating stages. The expression level of water buffalo *ATP1B2* gene in the mammary glands at lactating stage is higher than at non-lactating stage.

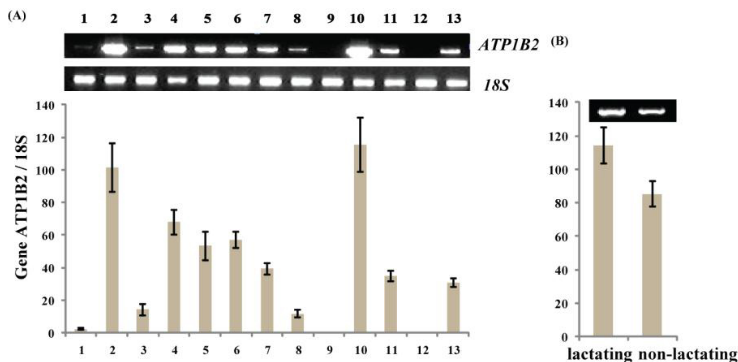

Figure 8
(A) The expression levels of *ATP1B2* gene in 13 water buffalo tissues. (B) The differential expressions of water buffalo *ATP1B2* gene in the mammary gland between lactating and non-lactating stage.
Note: (A) 1-heart, 2-pituitary gland, 3-small intestine, 4-muscle, 5-spleen, 6-liver, 7-mammary gland, 8-skin, 9-lung, 10-brain, 11-kidney, 12-adipose tissue, 13-rumen, M, DNA marker (DL2000).

Discussion

The Na^+, K^+-ATPase is an ubiquitous membrane enzyme which plays important roles in preserving the ionic gradients across the cell membrane and thus the membrane potential and osmotic equilibrium of the cell (Köksoy 2002, Vague *et al.* 2004). In the present study, the complete coding sequence of water buffalo Na^+, K^+-ATPase β_2-subunit (*ATP1B2*) was amplified and identified using the RT-PCR based on *in silico* cloning. The data confirmed that

the cloned sequence in this study encoded a new water buffalo gene. The full-length coding sequence of the *ATP1B2* gene contains 873 nucleotides encoding a putative protein of 290 AA, 33.4 kDa in size, with a pI of 8.37. The results of protein analysis indicated that water buffalo *ATP1B2* contains a N-terminal signal-anchor sequence, a transmembrane domain and a Na^+, K^+-ATPase β domain, and it functions in the plasma membrane with high reliability. In addition, hydrophobicity structure prediction showed that there is a strong hydrophobicity structure in water buffalo *ATP1B2*, which was consistent with the result of transmembrane structure prediction. The above results suggest that the water buffalo *ATP1B2* is a membrane protein, and it possesses the characteristics shared by all the Na^+, K^+-ATPase superfamily members, which supports that it involves in transporting Na^+ ions out of and K^+ ions into the cell as a transmembrane carrier protein (Köksoy 2002).

N-glyscans can stabilize the tertiary structure of glycoproyeins and comprised the binding sites for the endoplasmic reticulum-resident lectins. Also, they assist a correct folding of newly synthesized glycoproteins and they are crucial for maturation of the Na^+, K^+-ATPase β_2 subunit (Tokhtaeva *et al*. 2010). Previous study in human showed that there are 8 N-Glycosylation sites in the *ATP1B2*, and two N-Glycosylation sites (Asn118 and Asn238) are essential for calnexin-mediated folding and quality control of the former Na^+, K^+-ATPase β_2 subunit (Tokhtaeva *et al*. 2010). But in the present study, only five N-Glycosylation sites (Asn 96, Asn 118, Asn 197, Asn 238 and Asn 288) were predicted in water buffalo *ATP1B2*. All of these five N-Glycosylation sites were located in the Na^+, K^+ ATPase β chain domain. Alignment analysis showed that two N-Glycosylation sites of *ATP1B2* are quite conserved in the species of cattle, sheep, human, baboon, mouse, rat and African clawed frog. This implies water buffalo *ATP1B2* probably have similar functions as the *ATP1B2* of human or other species. However, the differences in N-Glycosylation site number between humans and water buffalo may be implied they have some functional differences.

Homology analysis showed that water buffalo *ATP1B2* has high identity with other mammal species at the nucleotide level, indicating that the *ATP1B2* is functionally conserved among different mammal species. Compared to other species, water buffalo *ATP1B2* has higher identity with cattle and sheep. This implies that the *ATP1B2* gene in water buffalo is more similar functionally to cattle and sheep.

The subunit composition of the Na^+, K^+-ATPase is tissue-specific. In order to investigate the distribution of *ATP1B2* in water buffalo tissues, we have examined the expression profile of the water buffalo *ATP1B2* gene by employing semi-quantitative RT-PCR. The *ATP1B2* gene is widely expressed in the diverse tissues of adult buffalo at different levels within an individual organism. The results in this study showed that water buffalo *ATP1B2* gene was expressed in all tested tissues except the lung and adipose tissue. Gene expression in which parts of animals was related to its corresponding gene functions. So, we speculate that water buffalo *ATP1B2* gene may play roles in the diverse tissues, especially in the pituitary gland, brain, muscle, spleen, liver, mammary gland, kidney and rumen. The *ATP1B2* was extremely abundant in the brain, which was consistent with the result from adult rat (Martin-Vasallo *et al*. 1989). The tissue expression profiles from six cattle cDNA libraries in NCBI database (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Bt.91787) show that the cattle *ATP1B2* gene is only expressed in the brain, adrenal gland, kidney, intestine and muscle. Compared with the tissue distribution in cattle, water buffalo *ATP1B2* gene showed obviously

different expression patterns. The reason for expression differences between cattle and water buffalo needs further investigation.

In the present study, gene expression analysis showed that water buffalo *ATP1B2* gene was moderately expressed in the mammary gland, and the expression level of the *ATP1B2* gene was found to be higher in lactating than in non-lactating stage. This phenomenon may be interpreted as that Na^+, K^+-ATPase has a rapid turnover and resynthesis in lactating mammary gland as mentioned in a previous study (Martin-Vasallo *et al.* 1989).

Genetic association studies have revealed that the *ATP1B2* gene has an important influence on milking performance in cattle. A prior study on Chinese-Holstein cattle showed that two novel single polymorphisms, G2258A and C2833T, in the second and fourth introns of *ATP1B2* gene have a significant impact on the milk traits (Wang *et al*. 2011). G2258A significantly affected milk fat content and 305-day milk yield, and C2833T significantly affected milk protein content and 305-day milk yield. Also, the single nucleotide polymorphism of C2833T of *ATP1B2* gene is a genetic marker of heat-tolerance traits (Wang *et al*. 2011). However, there have been no genetic association studies of polymorphisms in the *ATP1B2* gene with milk traits in water buffalo. This study provides a molecular basis for detecting the polymorphisms of water buffalo *ATP1B2* gene in population samples, and further unfolding the genetic association between the polymorphisms and the physiological process controlled by this protein.

In conclusion, we firstly identified water buffalo *ATP1B2* gene in this study. By conducting necessary bioinformatics analysis and tissue expression profile analysis, we clarified some characteristics and potential functions of water buffalo *ATP1B2*. These informations will provide the primary foundation for further insights into the role and functional mechanism of water buffalo *ATP1B2* gene.

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