Journal of Animal &Plant Sciences, 2013. Vol.18, Issue 1: 2668-2681
Publication date 31/5/2013, http://www.m.elewa.org/JAPS*; ISSN 2071-7024*

Cloning, sequence identification and tissue transcription profile analysis of novel inwardly rectifying potassium channel *KCNJ12* gene from the Chinese water buffalo (*Bubalus bubalis*)

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Key words: Water Buffalo; *KCNJ12* gene; tissue transcription profile; bioinformatics analysis

1 SUMMARY

Potassium inwardly-rectifying channel, subfamily J, member 12 (KCNJ12) plays a critical role in central ventilator chemosensitivity, and this role could possibly exhibit developmental changes. In the present study, water buffalo *KCNJ12* cDNA was cloned and characterized. This novel gene was then deposited into NCBI database and assigned to accession number KC011846 (amino acids accession number AGC91909). The complete open reading frame of water buffalo *KCNJ12* gene was 1281 bp encoding a KCNJ12 protein of 427 amino acids with a molecular weight of 48.44 kDa and a pI of 5.55. The putative proteins of KCNJ12, which are located in the cytoplasm (94.1%), contain a conserved domain of Ion_trans_2 superfamily and three potential transmembrane regions without N-terminal signal peptide, which indicates that KCNJ12 was non-secretory and membrane-embedded proteins. Similarity comparisons for amino acid sequences reveal that the water buffalo KCNJ12 protein shares 99.8%, 99.8%, 96.5%, 96.3%, 94.4% and 94.1% identity with that of *Bos taurus*, *Ovis aries*, *Pan troglodytes*, *Homo sapiens*, *Rattus norvegicus* and *Mus musculus*. The phylogenetic tree analysis revealed that the buffalo *KCNJ12* gene has a closer genetic relationship with the *KCNJ12* genes of *Bos taurus* and *Ovis*

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aries **than with those of** ***Pan troglodytes*****,** ***Homo sapiens*****,** ***Rattus norvegicus*** **and** ***Mus musculus*****. Real-time PCR analysis shows that water buffalo** ***KCNJ12*** **gene is expressed in various tissues, but at different levels. The expression levels of this gene are high in longissimus dorsi, fat, cerebrum, and heart, moderate in uterine wall, skin, lung, duodenum, oviduct, placenta, spleen, and mammary gland, non-expressed in liver, stomach, pancreas, and ovary. These data provide a foundation for further insight into this buffalo gene.**

2 INTRODUCTION

The inwardly rectifying potassium current I_{K1} in cardiac myocytes appears to play an important role in stabilizing the resting membrane potential (RMP) and forming the late repolarization phase of the action potential (Shimoni *et al.*, 1992; Lopatin & Nichols, 2001; Miake *et al.*, 2003). The Kir2 (IRK) subfamily is expressed in many cell types including cardiac myocytes and neurons, and controls the excitability of these cells (Nichols and Lopatin, 1997). It is not surprising that there is functional evidence from transcriptional analysis in combination with comparisons of the biophysical characteristics of cloned Kir channels that members of the Kir2 subfamily provide the major component of cardiac I_{K1} (Dixon & McKinnon, 1994; Barry *et al.*, 1995; Brahmajothi *et al.,* 1996). In the rat brain, three different subunits of the Kir2 channel subfamily have been found and identified: Kir2.1 (Kubo *et al.*, 1993), Kir2.2 (Takahashi *et al.*, 1994), and Kir2.3 (Morishige *et al.*, 1994). In guinea pig heart, Kir2.2 (potassium inwardly-rectifying channel, subfamily J, member 12, KCNJ12) have been discovered contribute to I_{K1} (Liu *et al.*, 2001). Recent studies displayed that, in the rat brainstem, a group of chemosensitive nuclei (e.g., locus coeruleus, raphe nucleus) expressed Kir2.2 in an age-dependent way, whereas other subtypes of Kir2: Kir2.1 and Kir2.3 were not (Karschin *et al.*, 1996). This finding indicates that Kir2.2 may play a significant part in central ventilatory chemosensitivity; furthermore, the role could be exhibited in developmental changes in a large part. In Kaibar's study, they have identified human Kir2.2 gene in normal individuals that contained R285 (Arginine residue at position 285) in the deduced amino-acid sequence (Kaibara *et al.*, 2002). Water buffalo are the important domestic animals in subtropical and tropical areas, in this study we firstly cloned the full-length coding sequence of the water buffalo *KCNJ12* gene, and subsequently did the bioinformatics analysis based on the data obtained, and finally examined their expression in 16 tissues by quantitative real-time PCR. These will provide a primary foundation for further research on this water buffalo gene.

3 MATERIALS AND METHODS

3.1 Animals and samples collection: Sixteen kinds of tissue samples, including the cerebrum, skin, heart, liver, spleen, lung, stomach, pancreas, duodenum, longissimus dorsi, fat, mammary gland, ovary, oviduct, uterine wall and placenta were collected from six adult female Binlangjiang buffalo and five adult female Dehong buffalo in Yunnan province, China. The samples were snap-frozen immediately in liquid nitrogen after buffalo had been slaughtered.

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3.2 RNA isolation, cDNA synthesis: The total RNA was extracted using the RNAiso Plus (TaKaRa, Dalian) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested with RNase-free DNase I (Huo *et al*., 2012; Deng *et al*., 2013). Three micrograms of RNA were reverse transcribed with oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase (Invitrogen, USA). The efficiency of reverse transcription was checked on 2% agarose gel electrophoresis stained with ethidium bromide.

3.3 Isolation of the water buffalo *KCNJ12* gene: The *KCNJ12* sequences for cattle (accession no. NM_001024690), rat (accession no. NM_053981) and human (accession no. NM_021012) were used to design a primer pair to amplify the complete coding sequence of *KCNJ12* gene by using Primer Premier 5.0 software. The primer set was: 5'-TGCCCCCACCTCCTGGATGAC -3' (forward) and 5'- CTCAGATCTCAGACTCCCGT -3' (reverse). Reverse transcription-polymerase chain reaction (RT-PCR) was performed to isolate the water buffalo *KCNJ12* using the pooled cDNAs from different tissues mentioned above. The 25 μl reaction system was: 1 μl (25 ng/μl) cDNA, 4 μl 2.5 mM mixed dNTPs (TaKaRa, Dalian), 12.5 μl 2×GC buffer I (TaKaRa, Dalian), 0.5 μl 10 μM forward primer, 0.5 μl 10 μM reverse primer, 0.25 μl 5 U/μl Ex Taq HS DNA polymerase (5 U/μl, TaKaRa, Dalian), and 6.25 μl sterile water. The PCR program of *KCNJ12* initially started with a 95 °C denaturation for 5 min, followed by 34 cycles of 94 °C /30 s, 58 °C /45 s, 72 °C /2 min, then 72 °C extension for 5 min, finally 4 °C to terminate the reaction. The PCR products from water buffalo *KCNJ12* cDNA were then cloned into pMD18-T vector (TaKaRa, Dalian) and sequenced bidirectionally with the commercial fluorometric method. At least ten independent clones were sequenced for each PCR product. The complete coding sequence of the water buffalo *KCNJ12* gene has been deposited in the NCBI database and was assigned accession no. KC011846.

3.4 Bioinformatics analysis: Sequences were examined and edited by using the DNASTAR software. Sequence alignments were performed using online software in National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The position and number of SNPs as well as corresponding haplotypes were exported with DNASTAR, Clustal X 1.83, EditPlus and MEGA 4.0 softwares. The base composition analysis was done by employing Mega version 4.0. Number of amino acids, theoretical pI, molecular weight, amino acid composition, total number of negatively charged residues, total number of positively charged residues, atomic composition, formula, total number of atoms, extinction coefficients, half-life, the N-terminal of the sequence, instability index, aliphatic index were predicted using ProtParam tool (http://web.expasy.org/protparam/). Signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Protein sorting signals and localization sites were predicted using PSort II (http://psort.hgc.jp/). protein domains, families and functional sites was predicted using Prosite (http://prosite.expasy.org/). The protein conserved domains and alignment were analyzed using the Conserved Domain Architecture Retrieval Tool of BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST).

Transmembrane helices in proteins were also predicted by TMHMM Server version 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Secondary structures of deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/). Protein hydrophobicity structure was predicted using ProtScale (http://us.expasy.org/cgi-bin/protscale.pl). Sequence similarity comparison and

the neighbor-joining phylogenetic trees were constructed based on KCNJ12 protein sequences by employing the Clustal X 1.83 and MEGA 4.0 and DNAMAN, which subsequently were edited manually. Statistical significance of groups within phylogenetic trees was evaluated using the bootstrap method with 10,000 replications. Web-based microRNA (miRNA) predicting programs were used to locate conserved potential miRNA targets: miRBase (http://www.mirbase.org/).

3.5 Expression profile analysis by quantitative real-time PCR: Quantitative real-time PCR was performed with Mastercycler ep realplex[4] (Eppendorf) using GoTaq® qPCR Master Mix (Promega) according to the manufacturer's instructions. We selected the housekeeping gene *18S* as the endogenous control. The control primers used were: 5'- GGACATCTAAGGGCATCACAG -3' (forward) and 5'- AATTCCGATAACGAACGAGACT -3' (reverse) with a predicted amplicon size of 145 base pairs. The primers of KCNJ12 were: 5'- GGCAACCTACGCAAGAGC -3' (forward), 5'- CAGGATGGTGATGGGAGACA -3' (reverse) with a predicted amplicon size of 101 base pairs. Relative transcript quantification was performed using standard curves generated for *18S* and *KCNJ12* gene from a 2 fold serial dilution of cDNA. Heart sample cDNA was used to generate the standard curves. The amplification conditions were used the default setting. Optical data were collected at the end of each extension step, and relative expression of PCR products was determined by the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001).

4 RESULTS

4.1 PCR result of water buffalo *KCNJ12* gene: The PCR products amplified from different tissue cDNAs for water buffalo *KCNJ12* gene were 1301 bp, (Fig. 1). This cDNA nucleotide sequence analysis using the BLAST software at NCBI server revealed that BMI *KCNJ12* gene was not homologous to any of the known water buffalo genes and it was then deposited into the GenBank database under accession No. KC011846.

4.2 Physical and chemical characteristics of KCNJ12: The coding region for the *KCNJ12* was 1284 bp encoded 427 amino acids. The *KCNJ12* CDS had an overall base composition of A 19.39% (249), G 30.84% (396), T 18.77% (241) and C 31% (398). The complete CDS and the encoded amino acids were presented in Fig.2 The theoretical pI and the molecular weight of KCNJ12 are 5.55 and 48.44 kDa respectively. The protein contains twenty kinds of amino acids: 32 Ala (A) (7.5%), 30 Arg (R) (7.0%), 14 Asn (N) (3.3%), 24 Asp (D) (5.6%), 10 Cys(C) (2.3%), 15 Gln (Q) (3.5%), 34 Glu (E) (8.0%), 26 Gly (G) (6.1%), 12 His (H) (2.8%), 28 Ile (I) (6.6%), 37 Leu (L) (8.7%), 16 Lys (K) (3.7%), 13 Met (M) (3.0%), 25 Phe (F) (5.9%), 16 Pro (P) (3.7%), 26 Ser (S) (6.1%), 22 Thr (T) (5.2%), 5 Trp (W) (1.2%), 11 Tyr (Y) (2.6%), 31 Val (V) (7.3%).

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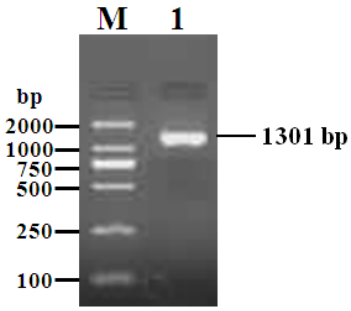

Figure 1: RT-PCR result for BMI *KCNJ12* gene.
M, DL2000 DNA marker; 1, PCR product.

Total number of negatively charged residues (Asp + Glu) is 58 and total number of positively charged residues (Arg + Lys) is 46. The protein contains 2158 Carbon (C), 3369 Hydrogen (H), 591 Nitrogen (N), 632 Oxygen (O), 23 Sulfur (S), formula is $C_{2158}H_{3369}N_{591}O_{632}S_{23}$, total number of atoms is 6773, Ext. coefficient is 44515 (extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water), estimated half-life is 30 hours (mammalian reticulocytes, in vitro), the N-terminal of the sequence considered is M (Met), the instability index (II) is computed to be 43.84 and aliphatic index is 87.92. Submitting the KCNJ12 protein sequence to SignalP, the KCNJ12 protein has no N-terminal signal peptide and which is a non-secretory protein (Petersen *et al.*, 2011). For subcellular localization analysis, the amino acid sequence was submitted to the PSORT II program, and Reinhardt's method showed water buffalo KCNJ12 was probably located in the cytoplasm with up to 94.1% probability (Nakai and Horton, 1999). Then putative protein was analyzed using Prosite (http://prosite.expasy.org/), five kinds sites were found, which were Protein kinase C phosphorylation sites (4-SgR-6, 38-TrR-40, 64-SqR-66, 354-TpR-356, 358-SaK-360), Casein kinase II phosphorylation sites (14-SseE-17, 15-SeeD-18, 74-TcvD-77, 284-SrqD-287, 358-SakD-361, 385-SrdE-388, 414-TalE-417), N-myristoylation sites (178-GAimAK-183, 301-GMveAT-306); Tyrosine kinase phosphorylation site (236-RvteEge.Y-243) and cAMP- and cGMP-dependent protein kinase phosphorylation site (422-RReS-425). Examined using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) indicated that KCNJ12 contains two separated conserved domain-inward rectifier potassium channel N-terminal (from 2 to 46 amino acid residues) and inward rectifier potassium channel (from 47 to 383 amino acid residues), (Fig.2 and Fig.3). Transmembrane topology prediction made by TMHMM program (Moller *et al.*, 2001) indicated that there were three transmembrane sequences found in the KCNJ12 (84-106AA, 126-148AA and 155-177AA) (Fig. 4). The prediction of secondary structure by SOPMA indicates that the deduced water buffalo KCNJ12 contains 147 alpha helices (34.43%), 85 extended strands (19.91%), 17 beta turns (3.98%) and 178 random coils (41.69%) (Fig. 5). The hydrophobic structure prediction of buffalo KCNJ12 by ProtScale indicated that KCNJ12 had the hydrophobic maximum 3.011 at 104th amino acids and the minimum -3.078 at 422th and 421th amino acids (Fig. 6)

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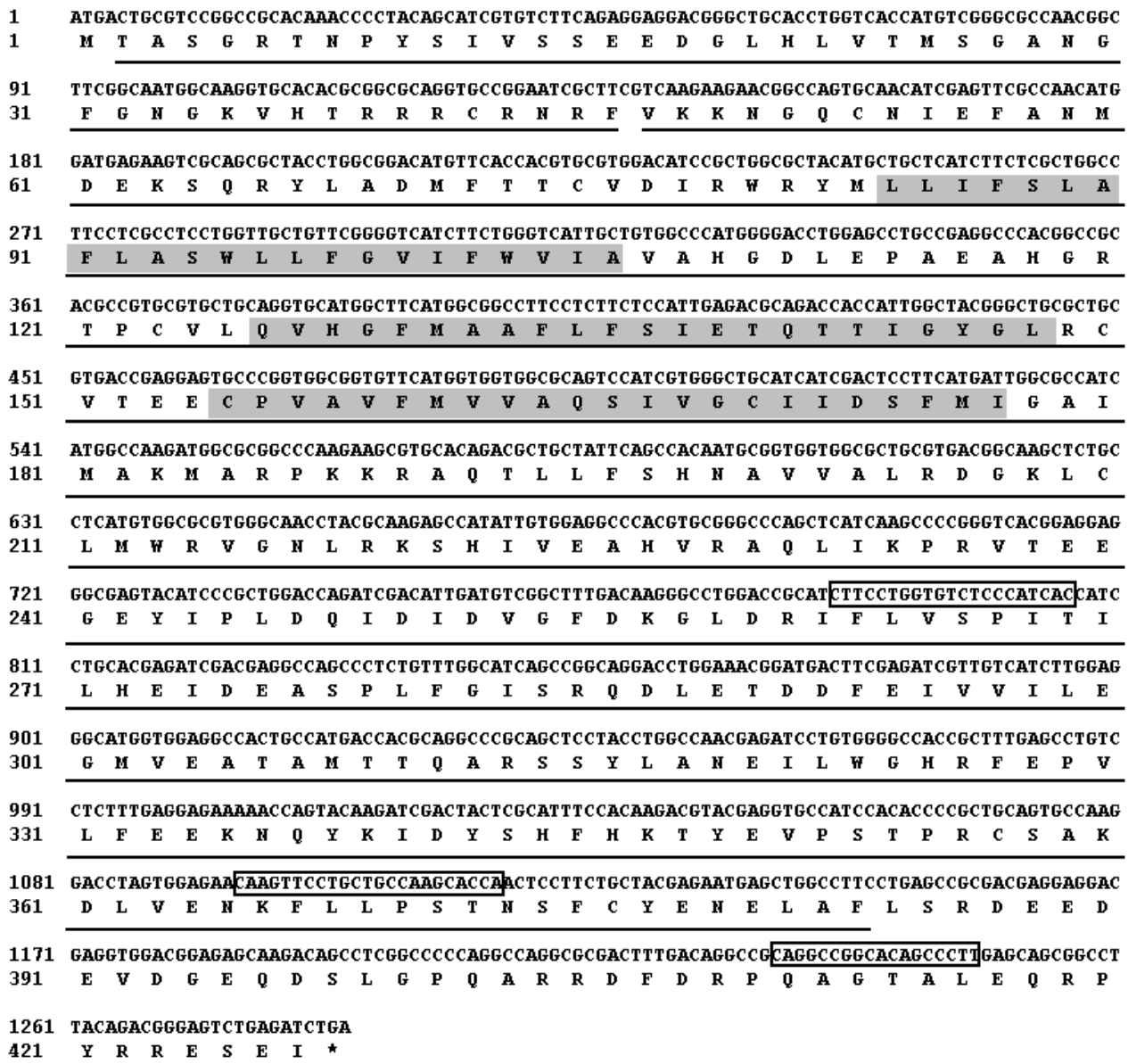

Figure 2: The complete CDS and its deduced amino acids of buffalo *KCNJ12* gene (GenBank accession number: KC011846). Asterisk denotes the stop codon. Two conserved domain sequences of inward rectifier potassium channel are underlined. Transmembrane sequences are shaded. Predicted microRNA target sites are boxed.

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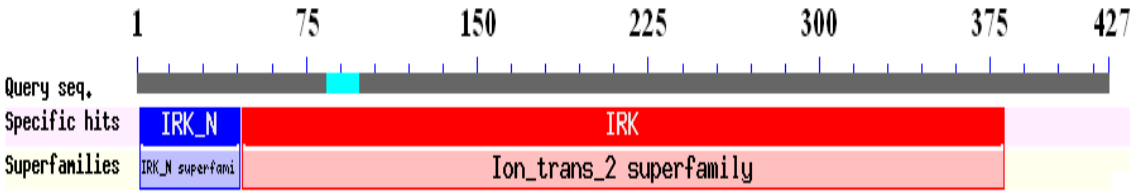

Figure 3: The putative domains of the protein encoded by *KCNJ12*

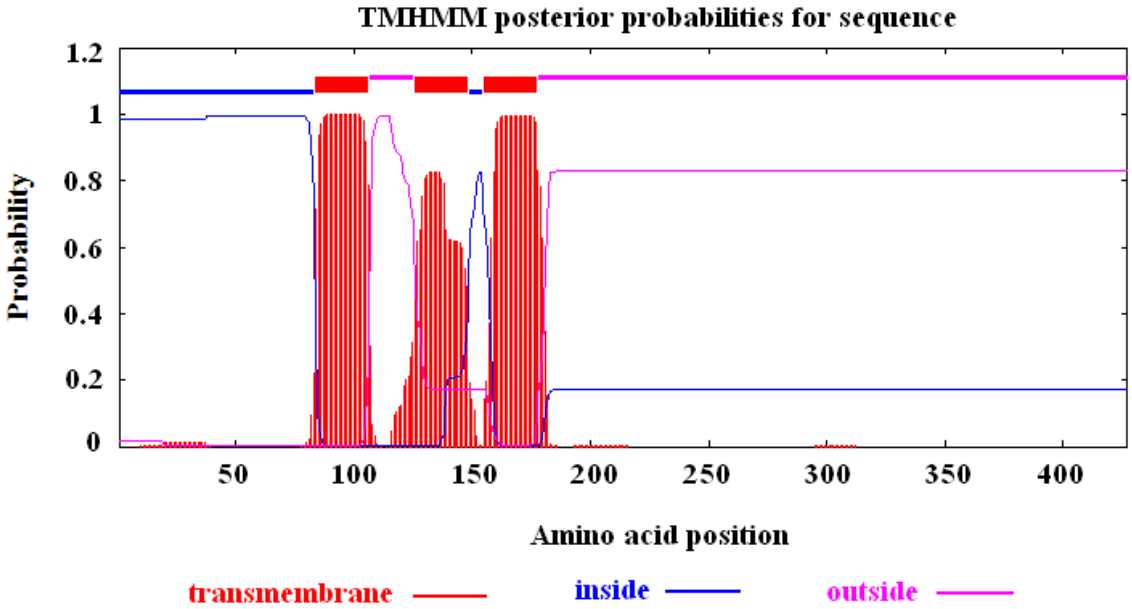

Figure 4: Prediction of transmembrane regions of buffalo KCNJ12 by TMHMM. abscissa axis, Amino acid position; vertical axis, probability; Red shows transmembrane, blue shows inside, pink shows outside of membrane.

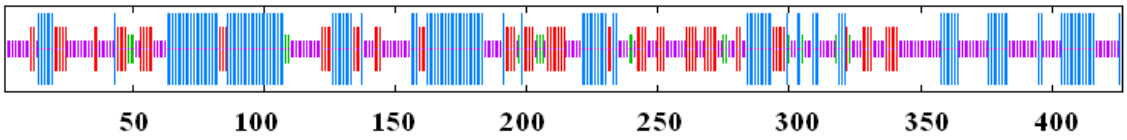

Figure 5: The predicted secondary structure of the KCNJ12 protein by SOPMA. Alpha helices, extended strands, beta turns and random coils are indicated, respectively, with the longest, the second longest, the third longest and the shortest vertical lines.

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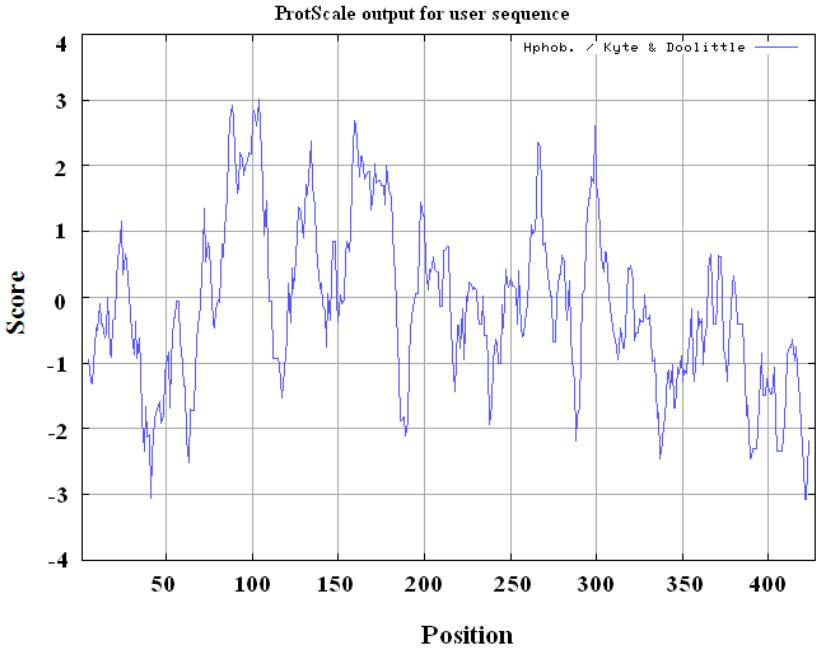

Figure 6: The hydrophobicity structure prediction of buffalo KCNJ12 by ProtScale. Score>0, hydrophobic; score<0, hydrophilic

4.3 Sequence polymorphism analysis: Further sequence alignment in Bovidae family revealed that buffalo *KCNJ12* CDS in this study had 99.3% and 98.0% identity with that of *Bos taurus* and *Ovis aries*. There was no polymorphism found in the coding region of the *KCNJ12* within buffalo. However, there were three nucleotide differences for the *KCNJ12* gene between buffalo and other bovine species, which were synonymous. The c.354C, c.895T and c.918T in the *KCNJ12* CDS are unique in buffalo. The sequence variations of the *KCNJ12* haplotypes in buffalo and other bovine species were shown in Fig. 7.

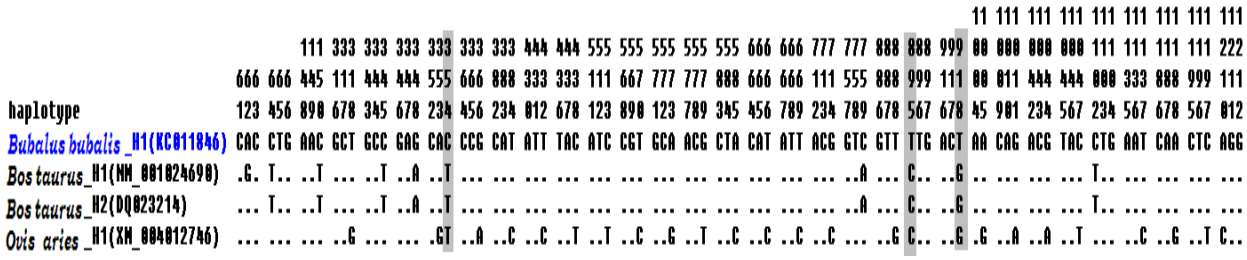

Figure 7: Alignment of the coding region of the *KCNJ12* gene of Bovidae family. Numbering is scored relative to first nucleotide of coding sequence of *KCNJ12* in buffalo. Dots (·) denote identity with the buffalo reference sequence. The nucleotide variable sites between buffalo and other bovine species are shaded.

4.4 Analysis of sequence identity and evolutionary relationships of buffalo KCNJ12: The deduced protein sequence of buffalo KCNJ12 was submitted to generate BLAST reciprocal best hits, and similarity comparison revealed that buffalo KCNJ12 protein has high homology with the KCNJ12 proteins

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of six other species: *Bos taurus* (99.8%), *Ovis aries* (99.8%), *Pan troglodytes* (96.5%), *Homo sapiens* (96.3%), *Rattus norvegicus* (94.4%) and *Mus musculus* (94.1%) (Fig. 8 and Fig. 9). To evaluate the evolutionary relationships of buffalo KCNJ12 with other species, then we constructed a phylogenetic tree using DNAstar, Cluster, MEGA and DNAMAN softwares based on the KCNJ12 amino acid sequences. The phylogenetic tree analysis revealed that the buffalo *KCNJ12* gene has a closer genetic relationship with the *KCNJ12* genes of *Bos taurus* and *Ovis aries* than with those of *Pan troglodytes*, *Homo sapiens*, *Rattus norvegicus* and *Mus musculus* (Fig. 10).

Percent Identity

	1	2	3	4	5	6	7		
1		99.8	99.8	96.5	96.3	94.4	94.1	1	Bubalus bubalis
2	0.2		99.5	96.3	96.0	94.1	93.9	2	Bos taurus
3	0.2	0.5		96.7	96.5	94.6	94.4	3	Ovis aries
4	3.6	3.8	3.4		99.8	96.5	95.6	4	Pan troglodytes
5	3.8	4.1	3.6	0.2		96.7	95.8	5	Homo sapiens
6	5.9	6.1	5.6	3.6	3.4		98.4	6	Rattus norvegicus
7	6.1	6.4	5.9	4.6	4.3	1.7		7	Mus musculus
	1	2	3	4	5	6	7		

Divergence

Figure 8: Percent identify and divergence result of comparison of the deduced amino acid sequence from buffalo *KCNJ12* gene with those from *Bos taurus* (NP_001019861), *Ovis aries* (XP_004012795), *Pan troglodytes* (JAA00661), *Homo sapiens* (NP_066292), *Rattus norvegicus* (NP_446433) and *Mus musculus* (CAA56622). Upper matrix, percent identity; lower matrix, divergence.

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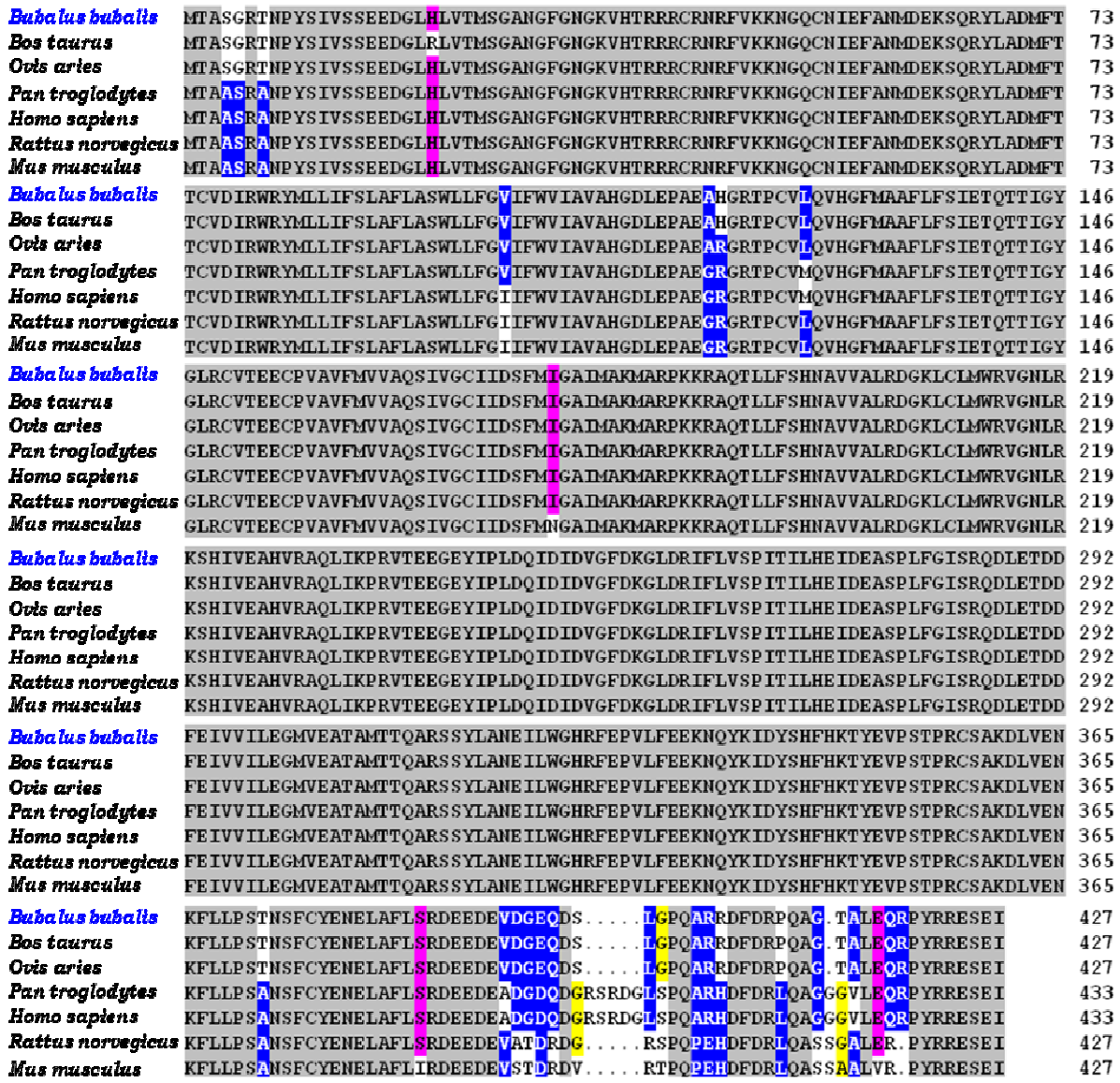

Figure 9: The alignment of the protein encoded by the buffalo KCNJ12 and other six kinds of KCNJ12 from *Bos taurus* (NP_001019861), *Ovis aries* (XP_004012795), *Pan troglodytes* (JAA00661), *Homo sapiens* (NP_066292), *Rattus norvegicus* (NP_446433) and *Mus musculus* (CAA56622).

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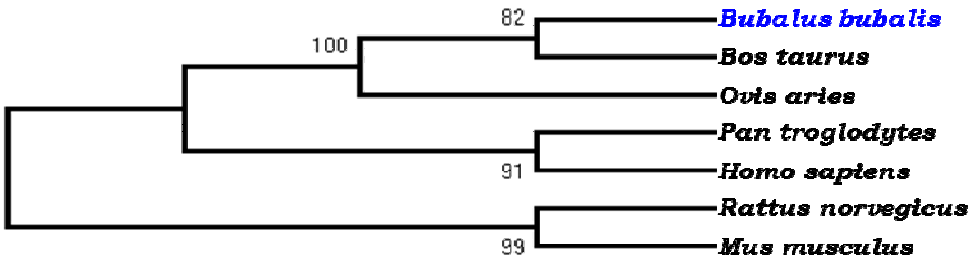

Figure 10: The phylogenetic tree for several kinds of KCNJ12 protein from *Bubalus bubalis* (AGC91909), *Bos taurus* (NP_001019861), *Ovis aries* (XP_004012795), *Pan troglodytes* (JAA00661), *Homo sapiens* (NP_066292), *Rattus norvegicus* (NP_446433) and *Mus musculus* (CAA56622)

4.5 Location of potential miRNA targets: In this study, three *Bos taurus* microRNAs (bta-miR-2382-3p, bta-miR-248, bta-miR-2382-3p and bta-miR-873) were found to have the target sites in the buffalo *KCNJ12* CDS. They were 786-cuuccuggugucucccaucac-806, 1231-caggccggcacagcccuu- 1248 and 1095-caaguuccugcugccaagcacca -1117.

4.6 Tissue transcription profile: In order to examine the differential distributions of *KCNJ12* in tissues of water buffalo, the relative mRNA expression levels of *KCNJ12* were evaluated by qPCR. *KCNJ12* mRNA was widely expressed in the tissues examined, being high in longissimus dorsi, fat, cerebrum and heart, moderate in uterine wall, skin, lung, duodenum, oviduct, placenta, spleen and mammary gland, non-expressed in liver, stomach, pancreas and ovary (Fig. 11).

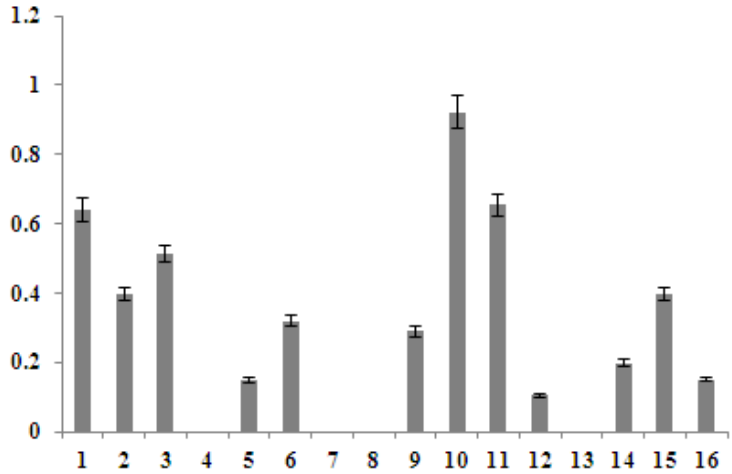

Figure 11: Tissue transcription profile of buffalo *KCNJ12* gene. The *18S* expression level is used for the internal control. M, DL2000 DNA marker; 1, cerebrum; 2, skin; 3, heart; 4, liver; 5, spleen; 6, lung; 7, stomach; 8, pancreas; 9, duodenum; 10, longissimus dorsi; 11, fat ; 12, mammary gland; 13, ovary; 14, oviduct; 15, uterine wall; 16, placenta

5 DISCUSSION

In this study, the full-length CDS of the *KCNJ12* were obtained from buffalo cDNAs. The *KCNJ12* CDS has 1284 nucleotides encoding a protein of 427 residues with a molecular weight of 48.44 kDa and a pI of 5.55. The KCNJ12 protein has two conserved domain of Inward rectifier potassium channel. Most protein functions are regulated by the modification of some amino acids in polypeptide chain, such as phosphorylation, acetylation, glycosylation and myristoylation, and so on. There were five kinds of putative functional sites (protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, N-myristoylation sites, tyrosine kinase phosphorylation site and cGMP-dependent protein kinase phosphorylation site) found here in buffalo KCNJ12 protein, which suggests that the KCNJ12 protein may exert critical functional effects through these sites and their corresponding domains. The Sequence alignment revealed that *KCNJ12* CDS was highly similarly among different bovine species, indicating functional conservation of the KCNJ12 within the Bovidae family. The evolutionary relationship based on the KCNJ12 amino acid sequences revealed that buffalo had closer genetic relationships with the Bovidae species. This implied that the buffalo KCNJ12 has minor divergence functionally with that of other Bovidae species and may have large function differences with other mammals. Therefore, the studying about buffalo KCNJ12 can be used as a reference for understanding possible function of the KCNJ12 in other Bovidae species. MicroRNAs are small noncoding single-stranded RNA molecules of 17 to 24 nucleotides that can regulate gene expression by binding to or regulating the translation of their target mRNAs (Bartel 2004; Zeng *et al.* 2003). In the present study, three *Bos taurus* microRNAs have been found to have their corresponding target sites in the CDS of buffalo *KCNJ12* gene by theoretical prediction. Further investigation is needed to confirm whether corresponding miRNA molecules can regulate the *KCNJ12* gene expressions in buffalo. Tissue transcription profile analysis showed that the *KCNJ12* was widely expressed in tissues examined, but at different levels. It was expressed remarkably high in longissimus dorsi, fat, cerebrum and heart, moderate in uterine wall, skin, lung, duodenum, oviduct, placenta, spleen and mammary gland, non-expressed in liver, stomach, pancreas and ovary. From the tissue transcription profile analysis in our experiment, it can be seen that *KCNJ12* gene was obviously differently expressed in some tissues. The suitable explanation for this is that at the same time those biological activities associated with the gene function was presented diversely in different tissues. In conclusion, we firstly isolated the buffalo *KCNJ12* gene and performed necessary sequence analysis and tissue transcription profile analysis. This established the primary foundation for further insight into the buffalo gene.

6 ACKNOWLEDGMENTS

This study was financially supported by the Natural Science Foundation Key Project of Yunnan Province, China (No. 2007C0003Z), the National Natural Science Foundation of China (No.30660024), the Applied and Basic Research Foundation of Yunnan Province, China (No. 2006C0034M and 2010ZC243) and the Foundation of Yunnan Department of Finance, China (Study on the germplasm characteristics of Binglangjiang water buffalo).

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