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Original study

Isolation, bioinformatic analysis and tissue expression profile of a novel water buffalo gene-*MFG-E8*

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Abstract

Milk fat globule-epidermal growth factor 8 (*MFG-E8*) is a milk membrane-associated glycoprotein, which plays a critical role in phagocytic clearance of apoptotic cells and mammary gland development, involution and remodelling. In the present study, the complete CDS of water buffalo *MFG-E8* was obtained and characterized. The genetic variations of nine water buffalo were investigated. Further, the tissue expression profile was carried out using quantitative real-time PCR method. The full-length coding region of *MFG-E8* from water buffalo tissues consists of 1296 nucleotides, which encodes 431 amino acids with a molecular weight of 47.85 kDa and an isoelectric point (pI) of 7.02. Bioinformatic prediction indicates that the *MFG-E8* protein contains one signal peptide, two repeated EGF-like domains in its N-terminal side and two repeated discoidinlike Factor 5/Factor 8 domains in its amino acids. The sequence homology analysis in Bovidae family revealed that the coding region of water buffalo *MFG-E8* had 98.5, 98.3, 93.9, and 88.0 % identity with that of cattle, yak, goat and sheep. There was no polymorphism found in water buffalo, but there existed five nucleotide differences between water buffalo and other bovine species. The phylogenetic tree based on the amino acid sequences of *MFG-E8* from thirteen species revealed that buffalo had a closer genetic relationship with the species of Bovidae family. Real-time PCR analysis showed that water buffalo *MFG-E8* gene was expressed in various tissues, but at different levels. The expression levels of this gene were higher in the mammary gland than in other tissues, suggesting that the *MFG-E8* protein plays a role in mammary gland functions.

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Abbreviations: CDS: , *MFG-E8*: Milk fat globule-epidermal growth factor 8; pI: isoelectric point; SNPs: single nucleotide polymorphisms

Introduction

Milk fat globule-epidermal growth factor-factor 8 (*MFG-E8*), also named lactadherin (Taylor *et al.* 1997) or SED1 (Raymond *et al.* 2009), is a major milk membrane-associated glycoprotein secreted from mammary epithelial cells (Stubbs *et al.* 1990), activated macrophages (Hanayama *et al.* 2002) and immature dendritic cells (Miyasaka *et al.* 2004). *MFG-E8* had a signal peptide sequence in its N-terminus, which can guides *MFG-E8* to the extracellular space (Aziz *et al.* 2011). It was known to facilitate the clearance of apoptotic cells by binding to phosphatidylserine (PS) exposed on apoptotic cells via the C-terminal Factor 5/Factor 8 like domains (Mather *et al.* 1993), while its highly conserved arginine-glycine-aspartate (RGD) motif of the second EGF-repeat recognizes $a_v ß_{3/5}$ integrin of phagocytic cells (Hanayama et al 2004). Owing to eliminating the dying cells from a variety of organs, *MFG-E8* has been shown to participate in controlling the process of various inflammatory diseases and immune diseases, including colitis (Chogle *et al.* 2011, Zhao *et al.* 2012), haemorrhagic shock (Zhang *et al.* 2012), sepsis (Komura *et al.* 2009, Shah *et al.* 2012, Wu *et al.* 2010), cerebral ischemic injury (Cheyuo *et al.* 2012) and systemic lupus erythematosus (Yamaguchi *et al.* 2010).

Although *MFG-E8* was initially identified as a most abundant constituent of the milk fat globules and speculated to be involved in milk fat secretion (Cavaletto *et al.* 1999, Stubbs *et al.* 1990), its specific function in the milk or mammary gland is still obscure. Nevertheless, *MFG-E8* has also been detected in various other tissues, including brain, lung, heart, kidney and spleen in some mammals, such as mouse (Aoki *et al.* 1997) and cow (Andersen *et al.* 1997), but its expression levels are lower than that of lactating mammary glands. The goat *MFG-E8* gene showed two single nucleotide polymorphisms (SNPs) associated with total solid and milk fat yield (Qu *et al.* 2011). Even more, increasing number of evidence suggests that *MFG-E8* plays a pivotal role in mammary gland development, involution and remodelling (Atabai *et al.* 2005, Ensslin & Shur 2007, Nakatani *et al.* 2006).

As an important domestic animal in subtropical and tropical areas, the water buffalo is vital to the agricultural economy of many countries worldwide. However, the report of water buffalo's *MFG-E8* is still rare. In this study, based on the abundant bioinformatics resources and software tools, we firstly isolated the full-length coding sequence of the water buffalo *MFG-E8* gene, subsequently conducted polymorphism detection and bioinformatics analysis based on the data obtained. Finally we examined its expression patterns in thirteen tissues by quantitative real-time PCR. This study establishes the primary foundation for understanding the mechanisms of mammary gland development, involution and remodelling in water buffalo.

Materials and methods

Animals and sample collection

The fresh tissue samples were obtained from nine female water buffalo without blood relationship (including three Binlangjiang buffalo, two Dehong buffalo, three Diandongnan buffalo and one Yanjin buffalo). Thirteen tissues, i.e. adipose tissue, abomasum, kidney, heart, liver, brain, lung, mammary gland, pituitary, intestine, spleen, skin and muscle, were immediately dissected after slaughter. The samples were frozen in liquid nitrogen until RNA extraction.

RNA isolation, cDNA synthesis

The total RNA was extracted using the RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. To remove genomic DNA contamination, total RNA was digested with RNase-free DNase I (TaKaRa). Three micrograms of RNA were reverse-transcribed with oligo $(dT)_{18}$ primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The efficiency of reverse transcription was checked on 2 % agarose gel electrophoresis contained ethidium bromide and the cDNA concentrations of different tissues were measured using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Isolation and polymorphism detection of MFG-E8 gene

The *MFG-E8* sequences for cattle (acc. no. BC102354) and goat (acc. no. GU593981) were used to design a primer pair by using Primer Premier 5.0 software (PREMIER Biosoft, Palo Alto, CA, USA). The primer set was: 5'-CAC ACC ATG CCG TGT CCC-3' (forward) and 5'-GAG GCA AAG GTC ACC CAC GC-3' (reverse).

The RT-PCR with the primer set was performed to isolate the complete coding sequence of *MFG-E8* gene and detect its polymorphisms by employing the cDNAs obtained from different tissues of nine water buffalo mentioned above. The 25 µl reaction system consisted of: 2.5 µl cDNA (50 ng/µl), 1.25 µl 10 mM mixed dNTPs (TaKaRa), 12.5 µl 2×GC buffer I (TaKaRa), 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.25 µl EX Taq DNA polymerase (5 U/µl, TaKaRa) and 7.5 µl sterile water. The PCR program initially started with 95 °C denaturation for 2 min, followed by 34 cycles of 94 °C/45 s, 55 °C/45 s, 72 °C/1 min 45 s, then 72 °C extension for 10 min and finally 4 °C to terminate the reaction. The PCR products from water buffalo *MFG-E8* cDNAs were then sequenced bi-directionally with the commercial fluorometric method.

Sequence analysis

Sequences were examined and edited by using the DNASTAR software (DNASTAR, Inc., Madison, WI, USA). The complete coding sequence of the water buffalo *MFG-E8* gene has been deposited in the National Centre for Biotechnology Information (NCBI) database and was assigned acc. no. KC329460. Sequence alignments were performed using online software in NCBI (http://www.ncbi.nlm.nih.gov). The base and the amino acid composition analyses were done by employing MEGA 4.0 program (Centre for Evolutionary Medicine and Informatics, Tempe, AZ, USA) (Kumar *et al.* 2008). The protein prediction and analysis were conducted

using the Conserved Domain Architecture Retrieval Tool of BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The molecular weight and theoretical pI were calculated by Compute pI/Mw (http://us.expasy.org/tools/pi_tool.html). Signal peptides were predicted using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). PSort II (http://psort.hgc.jp/) was used to predict protein sorting signals and intracellular localization. Protein hydrophobic analysis was conducted by online software ProtScale (http://web.expasy.org/protscale/). Secondary structures of deduced amino acid sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/). The protein domains and functional sites were analysed using SMART (http://smart.embl-heidelberg.de/). Homology analysis based on the *MFG-E8* amino acid sequences in some species was done using DNAMAN software (http://www.lynnon.com). The position and number of SNPs as well as corresponding haplotypes were exported with Mega version 4.0 (Kumar *et al.* 2008).

Phylogenetic analysis

The neighbour-joining phylogenetic tree was constructed based on *MFG-E8* amino acid sequences by employing the CLUSTAL X 2.0 and MEGA 4.0 programs, which subsequently were edited manually. Statistical significance of groups within phylogenetic trees was evaluated using the bootstrap method with 10 000 replications.

Expression profile analysis by quantitative real-time PCR

Quantitative real-time PCR was performed with ABI 7500 Fast System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. We selected the housekeeping gene GAPDH as the endogenous control. The control gene primers used were: 5′-ATC AAG AAG GTG GTG AAG CAG -3′ (forward) and 5′-GGT AGA AGA GTG AGT GTC GCT G-3′ (reverse). The primers of *MFG-E8* were: 5′-CCC CTT GGA GAC GCA GTA TGT-3′ (forward), 5′-TGC CCT GAT TAT CCA GTC GTG-3′ (reverse). Relative transcript quantification was performed using standard curves generated for *MFG-E8* and GAPDH gene from a 5-fold serial dilution of the cDNA. All the tissue cDNAs were used to generate the standard curves. In the present study, the amplification efficiency is in an ideal range from 90 % to 105 % for each tissue. 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 & Schmittgen 2001).

Results

Sequence and polymorphism analysis

The sequence alignment revealed that the *MFG-E8* gene sequence obtained in this study was not homologous to any of the known water buffalo genes. The coding region for *MFG-E8* was 1296 bp, which encoded 431 amino acids. The coding region sequence of *MFG-E8* had an overall base composition of 21.14 % A, 27.62 % G, 21.14 % T and 30.09% C.

A comparative analysis was conducted by pooling the sequences in this study with all the published data of *MFG-E8* gene on some bovine species together. The coding sequences of water buffalo *MFG-E8* in this study had 98.5, 98.3, 93.9 and 88.0% identity with that of cattle (NM_176610), yak (JH883919), goat (GQ344829) and sheep (XM_004018049). The gene sequence differences between buffalo and cattle and yak were smaller than those between buffalo and goat and sheep. There were no polymorphisms found in the coding region of *MFG-E8* gene within water buffalo, but there were five nucleotide differences identified in the sequences of *MFG-E8* gene between water buffalo and other bovine species. Among them, only the difference at c.400 (A>G) was nonsynonymous and this difference leads to the corresponding deduced amino acid change at p.134 from methionine (M) in other bovine species to valine (V) in water buffalo. The c.195G, c.348A, c.400G, c.687C and c.1017G differences in *MFG-E8* coding sequence are unique in water buffalo. It is noteworthy that nucleotide differences at c.688 and c.689 in the coding region of *MFG-E8* gene caused the deduced amino acid change at p.230 from bovine species.

The pI of water buffalo *MFG-E8* was 7.02 and the molecular weight was 47.85 kD. Bioinformatic analysis indicated that *MFG-E8* is a hydrophilic protein located in the cytoplasm, which has four high probably domains, namely two repeated EGF-like domains (27-63 AA and 68-110 AA) and two repeated discoidinlike Factor 5/Factor 8 domains (112-269 AA and 273-431 AA) (Fig.1) in its amino acid sequence. The second EGF-like domain and two repeated discoidinlike Factor 5/Factor 8 domains were highly conserved. The prediction of secondary structure by SOPMA indicated that the deduced *MFG-E8* of water buffalo contained 77 AA alpha helices, 97 AA extended strands, 29 AA beta turns and 228 AA random coils.

Figure 1
Predicted functional domains of water buffalo *MFG-E8* by SMART. The red region: signal peptide, EGF: Epidermal growth factor-like domain, FA58C: Coagulation Factor 5/Factor 8 C-terminal domain, discoidin domain.

Evolutionary relationships of MFG-E8

To evaluate the evolutionary relationships of water buffalo *MFG-E8* with other species, we constructed the unrooted phylogenetic trees on the basis of the *MFG-E8* amino acid sequences using the neighbour-joining method (Figure 2). The phylogenetic tree showed that water buffalo had a closer genetic relationship with the species of Bovidae family.

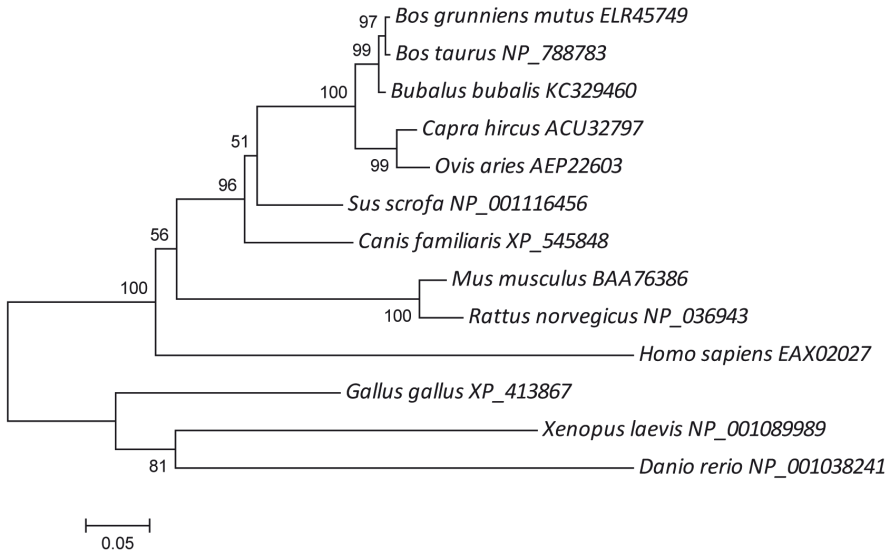

Figure 2
Phylogenetic tree based on the amino acid sequences of *MFG-E8* among thirteen species. The tree was constructed with the neighbour-joining method, the numbers on the branches represent bootstrap values for 10 000 replications.

Tissue expression profile analysis of the MFG-E8

In order to examine the differential distributions of *MFG-E8* in tissues of water buffalo, the relative mRNA expression levels of *MFG-E8* were evaluated by qPCR (Figure 3). *MFG-E8* mRNA was widely expressed in the tissues examined, being high in adipose tissue, skin, intestine, pituitary, brain, mammary gland, heart, lung, kidney and muscle, moderate in abomasum, and low in liver and spleen.

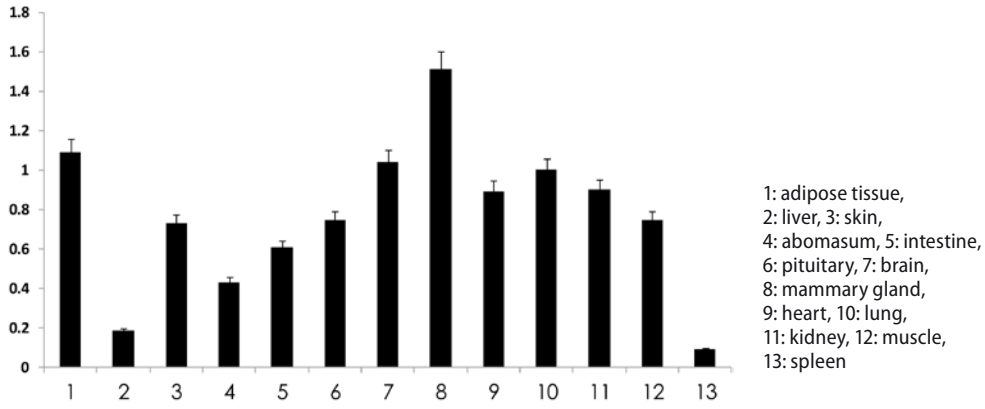

Figure 3
Tissue expression profile of water buffalo *MFG-E8* gene. The horizontal axis and vertical axis indicate different tissues and $2^{-\Delta\Delta}$Ct value (mean±SE), respectively. The brain was chosen as the calibrator. Each sample was repeated three times.

Discussion

In this study, the full-length coding sequence of the *MFG-E8* was isolated from water buffalo. The coding sequence of *MFG-E8* has 1296 nucleotides encoding a protein of 431 residues with a molecular weight of 47.85 kD and a pI of 7.02. *MFG-E8* has been reported to play a critical role in phagocytic clearance of apoptotic cells and mammary gland development, involution and remodelling (Mather *et al.* 1993, Hanayama *et al.* 2004, Atabai *et al.* 2005, Nakatani *et al.* 2006, Ensslin & Shur 2007). Thus, this study will provide a molecular basis for unfolding the genetic variation characteristics about *MFG-E8* and the primary foundation for understanding the mechanisms of mammary gland development, involution and remodelling in water buffalo.

The coding region of water buffalo *MFG-E8* had 98.5, 98.3, 93.9, and 88.0 % identity with the homologous sequences of cattle, yak, goat and sheep. There were five nucleotide differences in *MFG-E8* gene between water buffalo and other bovine species. But only one of these nucleotide difference sites leads to nonsynonymous substitution (c.400A>G). It is a same property substitution corresponding deduced amino acid change (p.134M>V), which indicate the functional conservation of *MFG-E8* within the Bovidae family. The variable sites found between water buffalo and other bovine species could be used as molecular markers to distinguish water buffalo from other Bovidae species.

Bioinformatic analysis indicated that the water buffalo *MFG-E8* protein contains one signal peptide in its N-terminal side, two repeated EGF-like domains and two repeated discoidinlike Factor 5/Factor 8 domains. This is consistent with the former studies (Aziz *et al.* 2011, Hanayama *et al.* 2004, Mather *et al.* 1993). So it can be speculated that the water buffalo *MFG-E8* also can be directed to the extracellular space by the signal peptide and serve as a bridge between phagocyte cells and apoptotic cells by binding to $a_v ß_{3/5}$ integrin of phagocytic cells via its second EGF-repeat and to phosphatidylserine (PS) exposed to apoptotic cells via the Factor 5/Factor 8 domains.

The evolutionary relationship based on *MFG-E8* amino acid sequences revealed that water buffalo had closer genetic relationship with the species of bovidae. This implied that the *MFG-E8* protein of water buffalo has minor functional divergence with bovine species and may have large functional differences with other mammal species. Therefore, the results about water buffalo *MFG-E8* studying can be used as a reference for understanding possible function of the *MFG-E8* in other bovine species.

The expression patterns of water buffalo *MFG-E8* in thirteen tissues were analysed by quantitative real-time PCR and the results showed that this gene was abundantly expressed in adipose tissue, skin, intestine, pituitary, brain, mammary gland, heart, lung, kidney, muscle and low expressed in liver and spleen. The expression level was the highest in the mammary gland, which implied that the *MFG-E8* gene may be important for the regulation of milk fat synthesis or mammary gland functions in water buffalo. In fact, it was observed that *MFG-E8* mutant would cause abnormal development of mammary gland in mice and cow (Atabai *et al.* 2005, Nørgaard *et al.* 2008). At the same time, we also noticed that cattle *MFG-E8* gene is expressed in most tissues (http://www.ncbi.nlm.nih.gov/UniGene). Compared with the tissue distribution in cattle, the gene was obviously expressed in different patterns in some tissues in water buffalo. For example, there was no expression in the liver of cattle, but low expression in water buffalo. Furthermore, there was the highest expression level for *MFG-E8*

gene in adipose tissue of cattle, whereas the highest expression level in water buffalo was in the mammary gland. To explain these differential expressions of the gene, further research based on these primary results is needed.

In summary, we have firstly isolated water buffalo *MFG-E8* gene and performed necessary polymorphism detection, bioinformatic analyses and tissue expression profile analysis. This provides the primary foundation for further insights into the water buffalo *MFG-E8* gene.

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