Short communication: A mutation in the 3' untranslated region diminishes microRNA binding and alters expression of the *OLR1* gene

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ABSTRACT

The oxidized low-density lipoprotein (lectin-like) receptor 1 (*OLR1*) gene plays an important role in the degradation of the oxidized low-density lipoprotein, which causes damage to the arterial endothelium. Previous studies have shown that a single nucleotide polymorphism (SNP) in the 3' untranslated region (UTR) of *OLR1* was associated with milk production and health traits in dairy cattle and with loin eye area and marbling depth in Qinchuan beef cattle. However, the mechanisms by which this SNP affects these traits are not well understood. MicroRNA (miRNA or mir) are small noncoding RNA that regulate gene expression by binding to target mRNA at their UTR to degrade or to repress translation of the target transcript. We hypothesized that miRNA bind to the 3' UTR of *OLR1* to cause expression changes of the gene. To test this hypothesis, the *Bos taurus* autosome (bta)-mir-370 miRNA was selected for this study based on bioinformatics prediction analysis. Two vectors that included A or C nucleotides of the 3' UTR SNP and 1 control vector were co-transfected with the vector of bta-miR-370 into human embryonic kidney 293 (HEK293) cells. Results of the dual-luciferase reporter assay showed that the activity of luciferase was significantly lower in cells transfected with the A nucleotide vector than that of the C nucleotide and control vectors. The assay also indicated that activity of miRNA bta-mir-370 was associated with a differential allelic regulation of *OLR1* expression. These results imply that the 3' UTR SNP of the *OLR1* gene is a strong candidate marker for selection in cattle breeding programs.

Key words: oxidized low-density lipoprotein receptor 1 (*OLR1*) gene, *Bos taurus* autosome microRNA 370 (bta-mir-370), single nucleotide polymorphism

Short Communication

Oxidized low-density lipoprotein (lectin-like) receptor 1 (**OLR1**) is a protein that can bind and degrade oxidized low-density lipoprotein. Several studies have reported QTL affecting milk production traits such as milk fat percentage (Heyen et al., 1999), milk fat yield (Olsen et al., 2002), and milk yield (de Koning et al., 2001) that were located in the *OLR1* region within bovine chromosome 5. Subsequent studies have shown that among several SNP identified in different regions of *OLR1*, only 1 SNP (C/A; University of Maryland genome assembly UMD3.1 position 100,254,823; http://bovinegenome.org/cgi-bin/gbrowse/bovine_UMD31/) located in the 3' untranslated region (**UTR**) was significantly associated with milk production and health traits in different cattle populations (previously reported as g.8232C>A). The 3' UTR SNP was found to be associated with milk production traits in the US Holstein bull population, US Holstein cows, Italian Brown Swiss cows, Israeli Holstein cows, and a Dutch Holstein-Friesian population (Khatib et al., 2006; Khatib et al., 2007; Schennink et al., 2009; Wang et al., 2012). Recently, we found the C/A SNP to be associated with beef cattle traits. Individuals with the CC genotype had superior loin eye and marbling depth than those with the AA genotype (our unpublished data).

Given that CC individuals had higher expression of *OLR1* than AA individuals and that this SNP solely demonstrated significant association with production traits across cattle populations and breeds (Khatib et al., 2006), we hypothesized that the C/A SNP is a candidate causative mutation. To test this hypothesis, we sought to investigate whether the C/A mutation affects binding of microRNA (**miRNA** or **mir**) and subsequent regulation of *OLR1* expression.

MicroRNA are a class of single-stranded, endogenous, and noncoding small RNA molecules (18–24 nucleotides) that regulate gene expression. Action of miRNA occurs posttranscriptionally either by binding to partially complementary sequences in the 3' UTR to inhibit the translation of the target mRNA or by

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Figure 1. Relative expression of the oxidized low-density lipoprotein (lectin-like) receptor 1 (*OLR1*) gene in muscle tissue from Qinchuan cattle individuals. The bars represent the means $\pm$ SEM from 6 AA- and 8 CC-genotype individuals. The relative expression was analyzed using the $2^{-\Delta\Delta C_t}$ method (where C_t = cycle threshold; Livak and Schmittgen, 2001) and analyzed by F-test. ** $P < 0.01$.

accelerating its degradation (Lindberg et al., 2010). Differential expression of miRNA was reported to be associated with different production and reproduction traits in livestock, such as adipogenesis (Romao et al., 2012), muscle development (Miretti et al., 2011), and sperm motility in pigs (Curry et al., 2011). Interestingly, Zhang et al. (2011) reported that the C/A SNP in the 3' UTR of *OLR1* is located in the target complementary region of *Bos taurus* autosome (bta)-mir-370 using miRanda 1.0 software (Enright et al., 2003). This discovery prompted us to examine the potential of bta-mir-370 binding to the sequence within the 3' UTR of *OLR1* and its effects on gene expression.

First, we sought to test if expression level of *OLR1* CC animals is different than that of AA animals. Genotyping of 23 Qinchuan beef cattle individuals using PCR-RFLP (Khatib et al., 2006) revealed 6 homozygotes for AA and 8 homozygotes for CC genotypes. For the selected animals with AA and CC genotypes, the loin eye scores (cm^2 ; mean $\pm$ SE) were 53.89 ± 15.88

and 66.748 ± 23.635 (t -test $P < 0.05$), respectively. The marbling depth scores (cm) of AA and CC genotypes were 4.095 ± 1.239 and 5.216 ± 2.180 (t -test $P < 0.05$), respectively.

Total RNA was extracted from skeletal muscle tissues of the homozygous animals using an RNAPrep Pure Tissue Kit (Tiangen Biotech Co. Ltd., Beijing, China) and reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Tiangen Biotech Co. Ltd.). The skeletal muscle tissue was chosen for this study because of the role of *OLR1* in lipid metabolism in muscle tissue (Chui et al., 2005) and because of the association of CC and AA genotypes of this gene with loin eye and marbling depth traits in the Qinchuan beef cattle population. Then, a real-time quantitative PCR (qPCR) analysis was performed to assess the relative expression of CC versus AA individuals. The qPCR reactions included the forward (5' CCCAGGGAAATGCCTGCTAC 3') and reverse (5' GCTGTGACCTTGAGTTAGGCA 3') primers, SYBR Premix (X2), 100 ng of cDNA, and RNase-free water. The reaction conditions were as follows: 95°C for 30 s, followed by 39 cycles of 95°C for 5 s, and 54°C annealing for 30 s, with a dissociation curve analysis at the end of amplification. The *GAPDH* gene served as an internal control using the following primers: forward (5' ATG GTG AAG GTC GGA GTG AAC 3') and reverse (5' TTG CCG TGG GTG GAA TCA TAC 3'). The qPCR reactions were done in triplicate. The data were analyzed using one-way ANOVA by SPSS software (http://www.spss.com/statistics/ez_rfm). The expression level of *OLR1* was significantly higher in CC ($P < 0.01$) than AA individuals (Figure 1).

The differential expression between CC and AA individuals prompted us to test whether the miRNA bta-mir-370 shows differential binding to 3' UTR SNP of CC and AA animals using the dual-luciferase reported assay system in human embryonic kidney 293 (HEK293) cells. This assay included constructing vectors with and without 3' UTR CC or AA fragments, co-transfection of these constructs along with bta-mir-370 constructs into HEK293 cells, and measurement of luciferase activity as an indication of miRNA binding. To construct 3' UTR-A-pGL3 and 3' UTR-C-pGL3 vectors from AA and CC individuals, respectively (Figure 2), the primers OLR-3UTR-f (GCTCTAGAAAGGCGAATCTATTGAGAGC; with an *Xba*I restriction site) and OLR-3UTR-r (GGAC-TAGTCCTAGAAGAAAGCATAGGAC; with an *Spe*I restriction site) encompassing the C/A mutation were designed to amplify a 360-bp fragment in the 3' UTR (Figure 2). The PCR conditions were as follows: 95°C for 4 min, followed by 32 cycles of 94°C for 45s, 55°C for 45s, 72°C for 45s, and a final extension at 72°C for

5 min. After the AA and CC fragments were digested by *Xba*I and *Spe*I enzymes and purified, they were inserted into the *Xba*I site downstream of the luciferase (*luc*+) gene in the pGL3 vector (Figure 2) according to the manufacturer's instructions (Promega Biotech Co. Ltd., Beijing, China). To verify correct insertion, the constructs 3' UTR-A-pGL3 and 3' UTR-C-pGL3 were digested by *Xba*I and *Bam*HI restriction enzymes and sequenced. For constructing the miRNA vector, the primers (forward: 5' CCAAGCTTAATGCCTGCTACTGGTTGAG 3'; reverse: 5' CCGGAATTCGAAAGCATAGGACAAAAGTGAG 3') with *Eco*RI/*Hind*III recognition sites were designed according to the sequence of bta-mir-370 from the miRBase website (<http://www.mirbase.org/search.shtml>). A 227-bp fragment of the bta-mir-370 gene was amplified from Qinchuan beef cattle, using touchdown PCR conditions. The PCR temperature cycles were as follows: 95°C for 5 min, followed by 32 cycles of 94°C for 30 s, touchdown annealing from 65 to 55°C for 30 s (−1°C/cycle), 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were purified and digested by *Eco*RI/*Hind*III and directionally cloned in the pcDNA3.1 (+) vector (Invitrogen Corp., Carlsbad, CA).

For transfection, HEK293 cells were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transfection was performed using the Sofast transfection system (Xiamen Sunma Biotechnology Co. Ltd., Xiamen, China) in 24-well plates with a density of 8×10^4 cells/well. A total of 200 ng of each of the 3 vectors shown in Figure 2 (pGL3-control, 3' UTR-A-pGL3, and 3' UTR-C-pGL3) was co-transfected with

200 ng of pRL-TK *Renilla* luciferase construct (Promega Biotech Co. Ltd.) and 200 ng of miR-370-pcDNA3.1 construct. Each transfection experiment was repeated 4 times. Cells were harvested 24 h after transfection and treated in 1× PLB lysis buffer (Promega Biotech Co. Ltd.). Firefly and *Renilla* luciferase activities assays were performed using the Dual-Luciferase Reporter Assay System (Promega Biotech Co. Ltd.) by automatic microplate reader (PerkinElmer Inc., Waltham, MA) following the manufacturer's instructions. Statistical analysis was performed by SPSS software (version 17.0).

Figure 3 shows that the luciferase reporter gene activity was significantly lower for the A nucleotide construct ($P < 0.01$) compared with that of the C nucleotide and control constructs. Therefore, we concluded that the activity of miRNA bta-mir-370 leads to degradation of the A allele to prohibit subsequent mRNA translation. Indeed, significant decrease in expression of *OLR1* was shown in AA compared with CC individuals (Figure 1).

Thus, results of this study provide evidence of differential allelic regulation of *OLR1* expression that appears to be influenced by the presence of bta-miR-370 miRNA. However, other assays such as the anti-miRNA assay could be performed in future studies to provide further support for the binding of bta-miR-370. Given that the CC genotype was associated with increased milk fat and fat percentage in previous studies, the results of the miRNA activity provide further support to the involvement of the 3' UTR SNP in these traits as a causative mutation. Recent studies in different species have shown that miRNA can bind target mRNA at their 3' UTR to maintain or degrade these mRNA, which in turn leads to phenotypic changes. For

Figure 2. Schematic representation of the constructs pGL3-control, 3'UTR-A-pGL3, and 3'UTR-C-pGL3 (where UTR = untranslated region) used in the transfection of human embryonic kidney 293 (HEK293) cells. *luc* + = luciferase; *OLR1* = oxidized low-density lipoprotein (lectin-like) receptor 1. Color version available in the online PDF.

Figure 3. Testing the interaction between *Bos taurus* autosome microRNA 370 (bta-mir-370) and the oxidized low-density lipoprotein (lectin-like) receptor 1 (*OLR1*) 3' untranslated region (UTR). The ratios of firefly luminescence/*Renilla* indicated that activity of the luciferase reporter gene in the wild-type encompassing 3'UTR-A site was significantly lower than that in the control group. The bars represent means $\pm$ SEM. $**P < 0.01$.

example, it was reported that a G/A mutation in the 3' UTR of growth differentiation factor 8 (*GDF8*) creates target sites of mir-1 and mir-206, which leads to decreased expression levels of *GDF8* and to subsequent musculature phenotypic changes (Cloup et al., 2006). Also, a C to T base change in the 3' UTR of the bone morphogenetic protein (*BMP*) swine gene was associated with differential binding of the miRNA let-7c and mir-184 and differential expression of the gene (Shao et al., 2011). Taken together, results of this study and the aforementioned studies clearly demonstrate that mutations in 3' UTR have significant effects on phenotypic variation, which may be regulated by miRNA.

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