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ORIGINAL PAPER

An imprinted long noncoding RNA located between genes *Meg8* and *Meg9* in the cattle *Dlk1-Dio3* domain

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Abstract The *Dlk1-Dio3* imprinted domain is located on the cattle chromosome 21 and contains three paternally expressed protein-coding genes and a number of maternally expressed short or long noncoding RNA genes. We have previously obtained two maternally expressed long noncoding RNA genes, *Meg8* and *Meg9*, from the cattle. In this study, we identified a novel noncoding RNA located between *Meg8* and *Meg9* known as *LINC24061* according to the GENCODE annotated bibliography. Two alternatively spliced transcripts (*LINC24061-v1* and *LINC24061-v2*) were obtained using RT-PCR and RACE, and the expression pattern of *LINC24061-v1* and *LINC24061-v2* was shown to be tissue-specific. The *LINC24061-v1* splice variant was expressed in only three types of tissues: heart, kidney and muscle; in contrast, *LINC24061-v2* was expressed in all eight tissues examined, including heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain of adult cattle. The allele-specific expression of *LINC24061* was identified based on a single nucleotide polymorphism (SNP) in exon 2 of *LINC24061*. The results showed that *LINC24061* exhibited monoallelic expression in all the examined cattle tissues.

Keywords Cows · *LINC24061* · Splice variants · lncRNA · Allele expression

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Introduction

In mammals, nearly 1% of protein-coding genes show parent-of-origin monoallelic expression due to the different epigenetic modifications inherited by the zygote from the sperm and the egg; these parent-dependent epigenetic marks are defined as genomic imprinting (Barlow and Bartolomei 2014). To date, there are more than 300 imprinted genes identified in mouse and human (http://igc.otago.ac.nz), which often occur in clusters. The *Dlk1-Dio3* domain is a large imprinted cluster located on mouse chromosome 12q and human chromosome 14q32. This domain is a 1 Mb region flanked by the paternally expressed protein-coding gene *Dlk1* (delta-like homolog 1) and *Dio3* (the type III iodothyronine deiodinase), with an interior containing maternally expressed long noncoding RNAs (lncRNAs): *Meg3/Gtl2*, *Meg8/Rain*, *Meg9/Mirg*, *Peg11as*, *Irm*, and numerous microRNAs and small nuclear RNAs (snoRNAs) (Charlier et al. 2001) (Fig. 1). The expression of the maternally expressed noncoding RNAs in the *Dlk1-Dio3* locus has been associated with the development potential of induced pluripotent cells (iPSCs) (Liu et al. 2010; Stadtfeld et al. 2010).

In recent years, the international ENCODE (Encyclopedia of DNA Elements) and GENCODE project has uncovered over 98% of the human genome does not encode protein sequences, whilst at least 80% of genomic DNA is transcribed as noncoding RNAs (Harrow et al. 2012; Pennisi 2012). LncRNAs are non-protein-coding transcripts greater than 200 bp in length, or molecules longer than 2 kb with a coding potential of less than 100 amino acids. LncRNAs can be classified into antisense

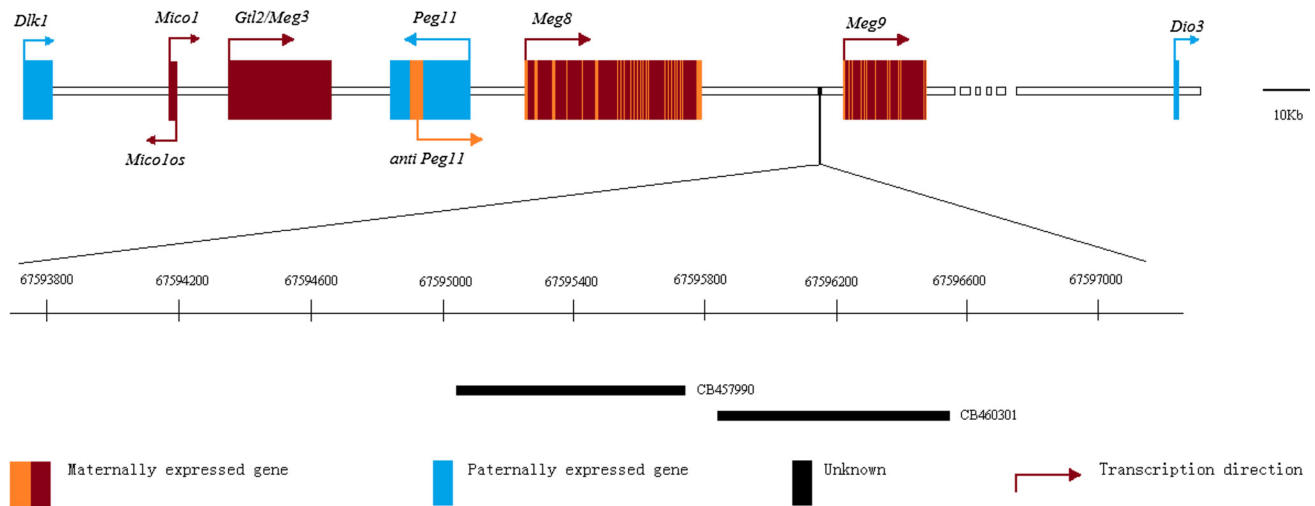

Fig. 1 Genomic organization of the cattle *Dlk1-Dio3* imprinted domain and genomic location of CB457990 and CB460301. The CB457990 and CB460301 are located between *Meg8* and *Meg9*

lncRNAs, intergenic lncRNAs, intronic lncRNAs, and enhancer lncRNAs. By chromatin signature analysis, over a thousand highly conserved large intergenic noncoding RNAs (lincRNAs) have been identified in the mouse (Guttman et al. 2009). Using public expressed sequence tag (EST) data from many developmental stages and tissues of the cattle, 23,060 cattle ncRNAs were predicted, with the majority (57%) of these ncRNAs being intergenic transcripts (Qu and Adelson 2012).

Recently, two novel intergenic long noncoding RNAs (*AK044800* and *B830012L14Rik*) were identified between *Meg8* and *Meg9* genes in mouse *Dlk1-Dio3* imprinted region (Han et al. 2013; Zhang et al. 2011). Previously, we have analyzed the gene structure and alternative splicing patterns of the cattle *Meg8* and *Meg9* genes (Hou et al. 2011; Zhang et al. 2014). Searching candidate imprinted genes in the cattle *Dlk1-Dio3* domain in the NCBI database and UCSC Genome Browser revealed two EST sequences, *CB457990* and *CB460301*, located 115 bp apart in the region between *Meg8* and *Meg9* (Fig. 1). The two EST sequences were determined to be part of the same transcript, and named *LINC24061* according to the GENCODE annotated bibliography. The aim of the present work was to first obtain the full-length cDNA sequence of *LINC24061*, then analyze the expression of alternative splicing variants and determine the imprinting status of *LINC24061*.

Materials and methods

Animals and tissues

Samples from heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain from 32 dairy cattle (Holstein) were collected from a local abattoir and frozen at −70 °C for further analysis. Protocols involving the use of animals were approved by the Agriculture Research Animal Care Committee of the Agricultural University of Hebei.

RNA isolation and cDNA synthesis

Total RNA was extracted from tissue samples of heterozygous individuals stored at −70 °C using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. First-strand cDNA was synthesized using 20 µL reaction volume, it containing 1 µL total RNA, 1 µL oligo $(dT)_{18}$ (0.5 µg/µL, Sangon, Shanghai, China), 1 µL dNTP (10 mM each), 4 µL 5 × M-MLV buffer, 0.5 µL (200 units) M-MLV reverse transcriptase (Promega, Madison, WI, USA), 0.5 µL RNase inhibitor (40 units/µL) and 12 µL RNase-free H_2O, with incubation at 42 °C for 60 min.

Cloning of *LINC24061*

A 1050-bp *LINC24061* cDNA sequence was first obtained by RT-PCR with primers *LINC24061*-F1 (5′-TCTAAATACTTGCCCGAG-3′) and *LINC24061*-R1 (5′-AGAGTTACAGAACCCGTG-3′) designed according to the EST sequences CB457990 and CB460301, respectively. RT-PCR was performed in a 25 µL volume containing 1 µL of first-strand cDNA (50 ng/µL), 12.5 µL 2 × Es Taq MasterMix (CWBIO, Beijing, China), 0.5 µL of forward and 0.5 µL of reverse primers (10 mM), 10.5 µL of ddH_2O and using the following temperature cycle: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 10 min. The PCR product was

separated on 1.5% agarose gel, purified with an E.Z.N.A.GelR Extraction Kit (Omega, Monsanto, USA), and sequenced (BGI, Beijing, China).

The 5′ RACE (rapid amplification of cDNA ends) and 3′ RACE reaction was performed to obtain the 5′ and 3′ ends of the *LINC24061* cDNA sequence, respectively, using the SMARTer^{TM} RACE cDNA Amplification Kit. The RACE reaction was performed by nested PCR according to the manufacturer's protocol. Gene-specific primers were as follows: 5′ GSP1 (5′-ATGGAGCAGCATCTACAAAGTT CCGAGG-3′) and 5′ GSP2 (5′-GGTAGGACTTCCAGGT CAACAGATACGATG-3′), 3′ GSP1 (5′-TGCTTCTGGG GATTCTGGCTTTTCTAA-3′) and 3′ GSP2 (5′-CATGCT GGCAAAGTCACGGTGGGGGAAC-3′). The amplified products were purified using an E.Z.N.A.Gel^{R} Extraction Kit (Omega, USA), cloned into PMD18-T (TaKaRa, Shanghai, China), and sequenced (BGI, China).

RT-PCR

RT-PCR was performed to detect the expression patterns of the two transcripts of *LINC24061* in different tissues, including heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain. Two forward primers and one reverse primer were as follows: *LINC24061*-F2 (5′-TGGC ATCCGTGTCACT-3′) on exon 1, *LINC24061*-F3 (5′-CTC CAGGGAAGACACACT-3′) on exon 2 and *LINC24061*-R2 (5′-GCCCTGATGGTTACTTCTGGG-3′). An amplified fragment of the *GAPDH* (GenBank accession no.BTU 85042) gene was used as an internal control with primers *GAPDH*-F (5′-GCACAGTCAAGGCAGAGAAC-3′) and *GAPDH*-R (5′-GGTGGCAGTGATGGCGTGGA-3′).

Strand-specific RT-PCR (ssRT-PCR) of LINC24061

To detected the *LINC24061* mRNA is transcribed in sense or in antisense, samples of eight tissues (heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain) were performed strand-specific reverse transcription using using primer *LINC24061*-F4 (5′-AAGTAACCAT-CAGGGCT-3′) or *LINC24061*-R1, which were used to detect antisense and sense strand transcripts, respectively. A 721 bp PCR product was amplified using *LINC24061*-F4 and *LINC24061*-R3 (5′-GCATCTACAAAGTCCGA-3′) in a 25 μL volume.

DNA extraction and SNP identification

Genomic DNA was extracted from liver tissue of 32 adult Holstein cattle with a DNA Extraction kit (Sangon, Shanghai,China) according to the manufacturer's instruction. The SNP site was identified by sequencing the PCR products directly. A 1050 bp fragment was amplified using the primers *LINC24061*-F1 and *LINC24061*-R1, which are located on a commonly shared exon 2 sequence of both transcripts. PCR was performed in a 25 μL volume containing: 1 μL Genomic DNA (100 ng/μL), 0.5 μL forward and reverse primers (10 μM/μL), 2 μL dNTP (2.5 nm/μL), 2.5 μL 10 × Taq Buffer, 0.3 μL Taq DNA polymerase (5 U/μL) and 18.2 μL ddH$_2$O. The PCR procedure was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and final extension at 72 °C for 10 min. The amplified products were purified using an E.Z.N.A.Gel^{R} Extraction Kit (Omega, monsanto, USA) and sequenced (BGI, China). The SNP site was identified by observing the double peaks in the sequencing chromatogram.

Allele-specific expression of LINC24061

The heterozygous animals with the identified SNP site were used to analyze the allele-specific expression of *LINC24061*. Total RNA prepared from the tissues of heterozygous animals was reverse transcribed into cDNA. RT-PCR was then performed using the primers *LINC24061*-F1 and *LINC24061*-R1, and the reaction volume and cycling conditions were the same as that of the SNP identification. The amplified products were purified and sequenced directly.

Results

Cloning and structure analysis of LINC24061 gene

The RT-PCR and RACE were used to determine the full-length cDNA sequences of *LINC24061*. A 1050 bp product containing partial sequence of both CB457990 and CB460301 was first obtained by RT-PCR using primers *LINC24061*-F1 and *LINC24061*-R1, indicating that the two EST sequences belong to the same transcript. The sequencing results of RACE indicated that two transcripts exist in the 5′ end and one in the 3′ end. The two splice variants of *LINC24061* have been submitted to GenBank (KU870638 and KU870639). The *LINC24061* molecular structure and two splice variants are shown in Fig. 2a. *LINC24061-v1* has 2 exons, but *LINC24061-v2* lacks exon 1 and only has a shorter exon 2 with 181 bp missing at the 5′ end. The open reading frames (ORFs) were detected using the Open Reading Frame Finder (www.ncbi.nlm.nih.gov/gorf.html), and multiple small ORFs were found in the two transcripts of *LINC24061*. However, none of the ATG start codons were consistent with the Kozak consensus sequence (ACCAUGG), suggesting that *LINC24061* is a noncoding RNA gene (Fig. 2b). The results of ssRT-PCR

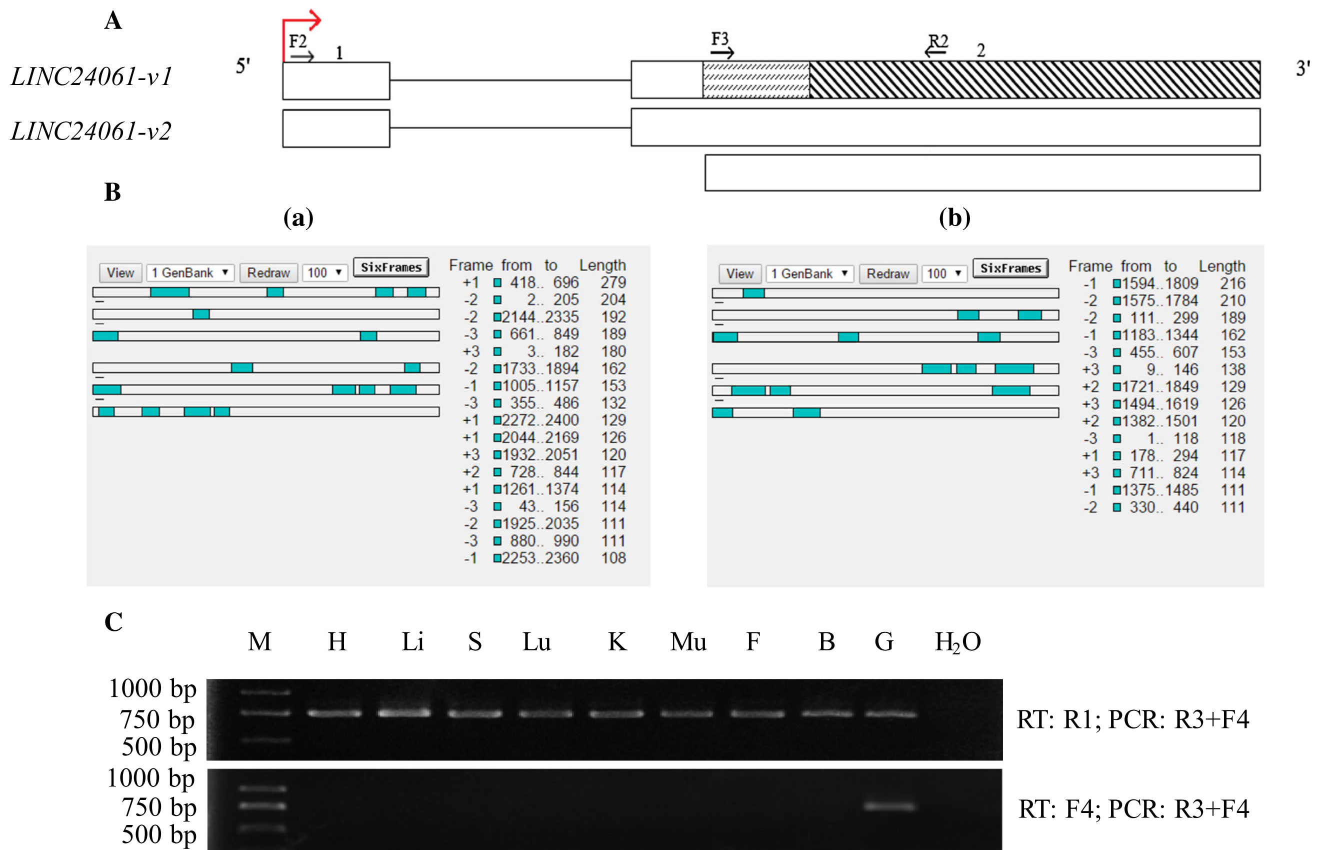

Fig. 2 **a** Gene structure, splice variants of the cattle *LINC24061*. Exons are indicted by *white boxes* and *straight lines* between exons indicated introns. *Boxes* with *different patterns* stand for alternative exons. The *red arrow* indicates that the *LINC24061* is transcribed in sense orientation, and black ones stand for the orientation of tissue-specific PCR primers. **b** Sequence analysis of *LINC24061*-v1 (*a*) and *LINC24061*-v2 (*b*) by ORF Finder in both sense and anti-sense showed it lacked long (>100 amino acids) ORFS. **c** Strand transcripts analysis by Strand-specific RT-PCR in eight tissues, only the transcripts in sense were detected. A 721 bp fragments of *LINC24061* amplified were both from cDNA and genomic DNA. Marker (M), Heart (H), Live (Li), Spleen (S), Lung (Lu), Kidney (K), Muscle (Mu), Fat (F), Brain (B), Genomic (G)

assay indicated that *LINC24061* is only transcribed in senses in the cattle tissues (Fig. 2c).

Expression profile of LINC24061

The expression patterns of the two splice variants of *LINC24061* in eight tissues were determined using RT-PCR, including heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain (Fig. 3). Two forward primers (*LINC24061*-F2 and *LINC24061*-F3) and one reverse primer *LINC24061*-R2 were designed according to the two splice variants of the cattle *LINC24061*. The locations of the primers were shown in Fig. 2a. A 1123 bp fragment of *LINC24061-v1* and a 598 bp fragment of *LINC24061-v2* were obtained using RT-PCR. The *LINC24061-v1* splice variant was expressed in only three types of tissues: heart, kidney, and muscle, whereas *LINC24061-v2* was expressed in all eight tissues examined.

Identification of SNP

The SNP site was identified by direct sequencing of the PCR products. A 1050 bp fragment of *LINC24061* was amplified from genomic DNA using the primers *LINC24061*-F1 and *LINC24061*-R1 (Fig. 4b). An A/C SNP was determined at nucleotide 1512 of *LINC24061-v1* (accession number: KU870638). The sequencing results of three genotypes, heterozygous (A/C) and homozygous (A/A) and (C/C), were shown in Fig. 4b. In 32 cattle, six individuals were the A/C heterozygous genotype, three were the A/A homozygous genotype, and 23 were the C/C homozygous genotype.

Allele-specific expression analysis of LINC24061 in the cattle

The allele-specific expression analysis of *LINC24061* was investigated by comparing the base of the heterozygosis

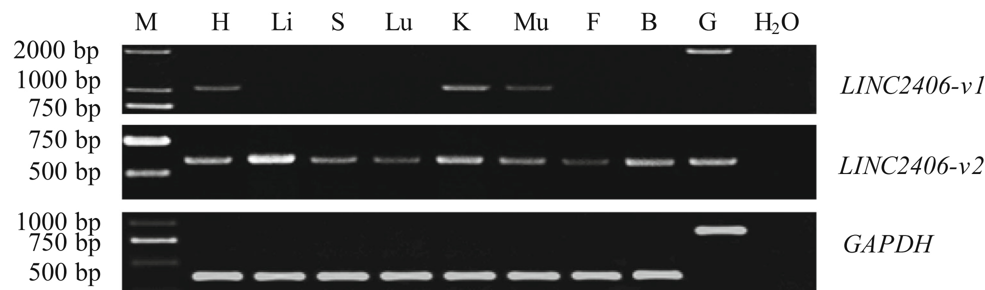

Fig. 3 Expression patterns of *LINC24061-v1* and *LINC24061-v2* in eight tissues analyzed by RT-PCR. The cDNA amplified fragment size of *LINC24061-v1*, *LINC24061-v2* and *GAPDH* were 1123, 598 and 375 bp. And the amplified sizes of genomic DNA were 1945, 598 and 873 bp. Marker (M), Heart (H), Live (Li), Spleen (S), Lung (Lu), Kidney (K), Muscle (Mu), Fat (F), Brain (B), Genomic DNA (G)

site from the sequencing chromatograms between products of the genomic DNA PCR and RT-PCR from the same heterozygous animal. The sequencing results of RT-PCR products obtained from heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain tissues revealed that only one parental allele (C) was expressed at the A/C SNP locus (Fig. 4c), suggesting that *LINC24061* is imprinted in cattle.

Discussion

Long noncoding RNAs (lncRNAs) are transcribed across most of the mammalian genome (Carninci et al. 2005), and the field of lncRNA research has been rapidly advancing in recent years. The ultra RNA deep sequencing analysis revealed that the number of lncRNA expressed genes is more than double of protein-coding genes in human (Iyer et al. 2015). LncRNAs can be classified into intergenic lncRNAs, antisense lncRNAs, intronic lncRNAs, and enhancer lncRNAs based on their location. Most lncRNAs play a functional role in genomic imprinting, with the exception of intronic lncRNAs (Kanduri 2016). In this study, we identified *LINC24061* as a novel imprinted lncRNA in the *Dlk1-Dio3* domain.

Alternative splicing is a normal phenomenon in eukaryotes, and can greatly add to the biodiversity without increasing the number of genes encoded by a genome (Black 2003). Approximately 95% of multi-exonic genes are alternatively spliced in humans (Pan et al. 2008). Previously, we obtained the alternative splices of three lncRNAs in the cattle *Dlk1-Dio3* domain: six splice variants of *Gtl2* gene (Su et al. 2011), 12 splice variants of *Meg8* (Hou et al. 2011), and three splice variants of *Meg9* (Zhang et al. 2014). In our analysis of *LINC24061*, two splice variants (*LINC24061-v1* and *LINC24061-v2*) were obtained using RT-PCR and RACE method. Although multiple small ORFs were encoded by the two splice variants, neither contained a Kozak sequence for translation initiation, suggesting that *LINC24061* plays a role as a noncoding RNA.

In this study, *LINC24061* was identified a ncRNA with monoallelic expression in tissues of adult cattle. LncRNAs often show tissue- and cell-specific expression (Dinger et al. 2008; Mercer et al. 2008; Ravasi et al. 2006). In mouse, the intergenic lncRNA *B830012L14Rik* was primarily expressed in brain, heart, lung, and liver at embryonic day 15.5 (Zhang et al. 2011). In this study, the expression of an *LINC24061-v1* variant was detected in three tissues, heart, kidney, and muscle, whereas *LINC24061-v2* was detected in all eight tissues examined, including heart, liver, spleen, lung, kidney, skeletal muscle, subcutaneous fat, and brain. These expression patterns were similar to that of *Meg9*, with three splice variants showing tissue-specific expression (Zhang et al. 2014).

The function of *LINC24061* is unknown, but several lines of evidence provide hints to its potential role. The potential promoter of *LINC24061* was predicted using PromoterScan software. Analysis of the potential promoter region of *LINC24061* revealed that two DNA elements, Bov-A2 and La2, were observed in the 5 kb upstream sequence of *LINC24061*. The Bov-A2 is a retroposon, that is one of the most common short interspersed nucleotide elements (SINEs) among the genomes of ruminants, and is generally present in the noncoding regions of several genes preferentially expressed during the cellular response to environmental stress or activation signals (Damiani et al. 2008). The DNA element, L2a, contains binding sites for two MAR (matrix-associated region)-interacting proteins (SATB1 and CDP), and functions as a silencer of CD8 gene encoding an important T cell co-receptor in mouse (Yao et al. 2010). Therefore, *LINC24061* may play a role in the cell growth and differentiation.

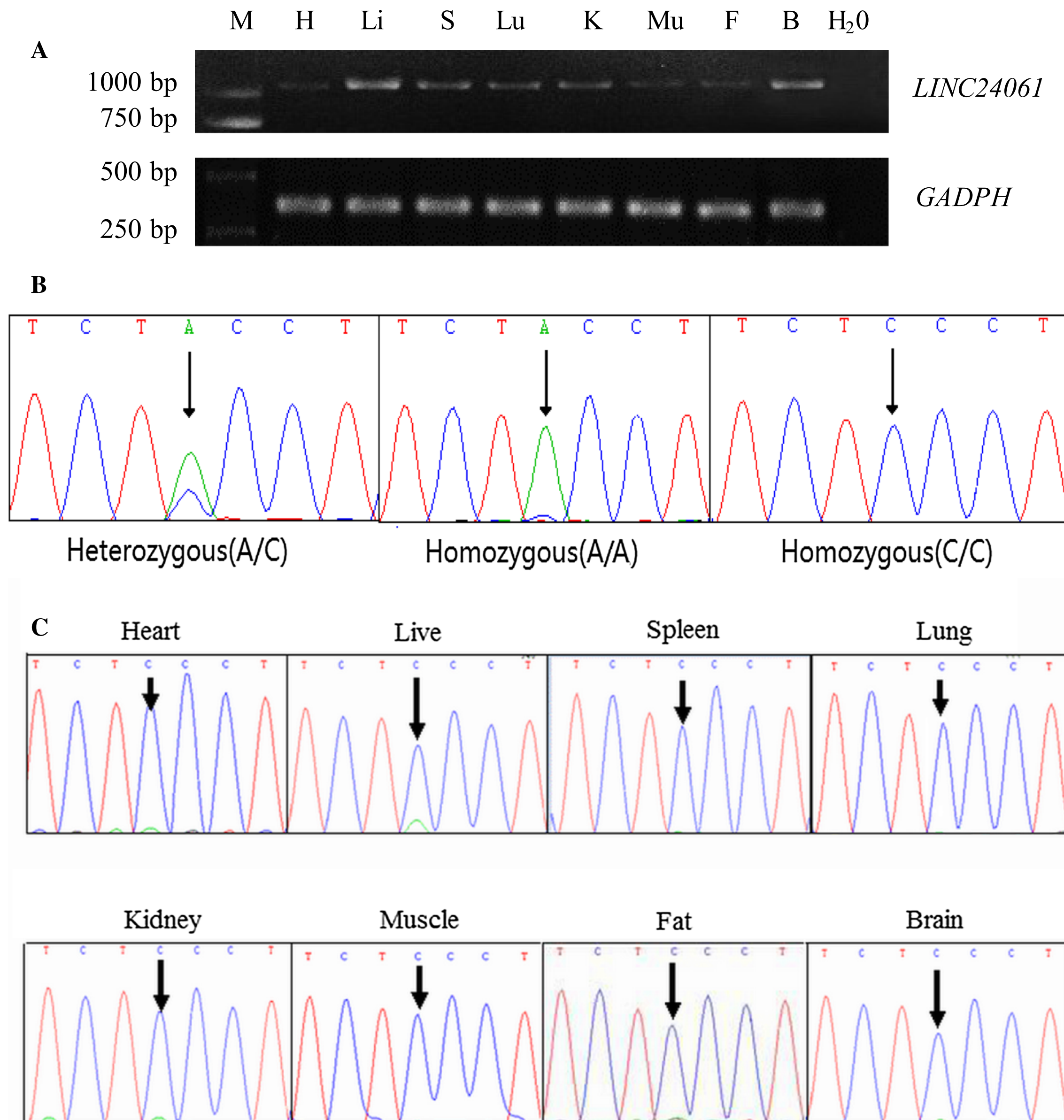

Fig. 4 Allele-specific expression analysis of *LINC24061* by direct sequencing of RT-PCR products. **a** Relative expression of *LINC24061* and *GAPGH* in eight tissues by RT-PCR. **b** Sequence chromatograms of gDNA obtained from the heterozygous cattle with A/C, homozygous cattle with C/C and with A/A. The *arrow* points to the c. 1512 A > C SNP. **c** Representative sequence chromatograms of cDNA obtained from eight tissues of heterozygous cattle. *Arrows* point to the c. 1512 A > C SNP. Compared with the sequence chromatograms of DNA at the c. 1512 A > C SNP, only 1 parental allele (C) was expressed. Marker (M), Heart (H), Live (Li), Spleen (S), Lung (Lu), Kidney (K), Muscle (Mu), Fat (F), Brain (B), Genomic (G)

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