Methylation differences and expression profiles of the caprine *DIO3* gene

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ABSTRACT. *DIO3* gene encoding type 3 iodothyronine deiodinase is an imprinted gene, located in the *DLK1-DIO3* (delta-like 1 homolog-type 3 iodothyronine deiodinase) imprinted domain, and is potentially involved in degrading excessive amounts of thyroid hormone to protect embryogenesis. However, the underlying regulatory mechanism of the imprinted *DIO3* gene expression during fetal and neonatal development in goats has not been elucidated. In this study, we explored the DNA methylation patterns of the caprine *DIO3* intragenic CpG island and quantified gene expression level in six tissues from Chinese Nanjiang Yellow 3-day old kids. The expression of the *DIO3* gene was determined using quantitative reverse transcription-polymerase chain reactions (qRT-PCRs), while the identification of methylation patterns was determined using bisulfite-sequencing PCRs. Modest, and non-significant ($P > 0.05$), methylation patterns were noted for

the *DIO3* CpG island methylation in the brain, heart, liver, kidney, lung, and longissimus dorsi tissues (ranging from 26.48 to 34.92%). The expression level of the *DIO3* mRNA was significantly higher ($P < 0.05$) in the liver tissue than in the other five tissues. Pearson's correlation analysis revealed that there was no significant relationship between methylation and gene expression ($P > 0.05$), which indicated that the expression of the caprine *DIO3* gene was likely modified by other regulatory elements. This study identified DNA methylation and expression patterns of the *DIO3* gene in goats and provided insights into further regulatory mechanisms of expression and imprinting in the *DLK1-DIO3* domain.

Key words: *DIO3*; Chinese Nanjiang Yellow goat; Methylation; Gene expression; Correlation

INTRODUCTION

Genomic imprinting is an epigenetic phenomenon that causes asymmetric expression of specific parental-origin genes in their offspring, which is conferred by modifications in transcriptional and post-transcriptional processes in the germ line and embryo, such as DNA/ RNA methylation, histone modifications, and chromatin structural changes (Ferguson-Smith and Surani, 2001; Reik and Walter, 2001). Specifically, spatial and temporal expressions of imprinted genes (at appropriate dosages) have a dominant effect on embryonic growth, nutrient transfer between mother and fetus, the postnatal neurodevelopmental process, and maintenance of a normal metabolic profile (Charalambous et al., 2007; Wilkinson et al., 2007; Renfree et al., 2008; Monk, 2015). Epigenetic modifications are established, propagated, and gradually changed during gametogenesis. These epigenetic mechanisms result in the complementary functions of the maternal and paternal genomes, which play essential roles in embryogenesis (Reik and Walter, 2001).

DNA methylation, one of several epigenetic modifications, is involved in many cellular processes and plays a crucial role in genomic imprinting for dynamic regulation of gene expression, in particular, gene silencing (possibly mediated by methyl-CpG binding proteins) in the processes of dosage compensation, mono-allelic expression, and transcriptional silencing of retroviruses and retrotransposons (Bourc'his and Bestor, 2004; Bestor et al., 2015). DNA methylation usually occurs in CpG dinucleotides in mammals, and methylation patterns are established and maintained by DNA methyltransferases and associated proteins through cell division (Yoder and Bestor, 1998; Okano et al., 1999; Bourc'his et al., 2001). In addition, methylation can be removed, which is dependent on the active demethylation by the ten-eleven translocation family in vertebrates (Tahiliani et al., 2009). DNA methylation is predominantly localized in tandem repetitive genomic regions (especially transposons), exhibiting heterogeneous methylation in single-copy sequences (such as exons, introns, and intergenic regions), promoter CpG islands (CGIs), and enhancer sequences (Bestor et al., 2015). To date, explorations of the potential relationship between gene expression and cytosine methylation levels have been mainly focused on CGIs in promoters of genes, and there have been few studies on genes in intragenic CGIs.

The *DLK1-DIO3* (delta-like 1 homolog-type 3 iodothyronine deiodinase) domain is

an imprinted gene cluster containing three protein-coding imprinted genes (*DLK1*, *RTL1*, and *DIO3*) expressed in the paternally inherited chromosome and a series of noncoding RNA genes, including many microRNA genes and small nucleolar RNA genes that are expressed in the maternally inherited copy (da Rocha et al., 2008). This domain is implicated in different epigenetic modifications, and can be employed as a paradigmatic model to research on the epigenetic regulation of genomic function. The *DIO3* gene is on the distal region of the *DLK1-DIO3* gene cluster, situated at approximately 800 kb downstream of *DLK*1 in mice and humans. To date, the homologs of *DIO3* have been identified in ruminants, which are located in bovine chromosome 21 (Connor et al., 2005), ovine chromosome 18 (Colosimo et al., 2009), and caprine chromosome 21 (Dong et al., 2013). The *DIO3* gene encoding Type 3 iodothyronine deiodinase (D3) and catalyzing the clearance of 3,5,3',5'-tetraiodothyronine (T4) and 3,5,3'-triiodothyronine (T3), is highly expressed at the fetal stage in vertebrate development, and plays a vital role in many pathophysiological processes, such as maturation and function of the thyroid axis, sensory functions, insulin secretion, and protection of the fetus from premature exposure to high levels of maternal thyroid hormones (Galton et al., 1999; Hernandez et al., 2006; Ng et al., 2010; Medina et al., 2011).

There have been many studies focusing on the *DIO3* gene methylation profile and expression patterns in mammals, but none has focused on ruminants. In this study, we investigated the methylation profile of the intragenic CGI of the *DIO3* gene in the Chinese Nanjiang Yellow goat and assessed the relationship between methylation and gene expression.

MATERIAL AND METHODS

Animals and sampling

Three genetically unrelated Chinese Nanjiang Yellow goat kids (3-days old) were randomly selected from the Nanjiang Yellow Goat Breeding Institute (Bazhong, Sichuan, China). The herd was raised under standard conditions. Six tissues - brain, heart, liver, lung, kidney, and longissimus dorsi - were sampled and immediately stored at -80°C. All experimental procedures in this study were approved by the Sichuan Agricultural University's College of Animal Science and Technology's Institutional Animal Care and Use Committee.

DNA preparation and methylation detection

Genomic DNA was isolated from the six tissues using a standard phenol-chloroform extraction protocol. Polymerase chain reaction (PCR) amplification of caprine *DIO3* gene was performed on a C1000™ Thermal Cycler (Bio-Rad, Richmond, CA, USA) with nested primers designed by Primer Premier 5.0 (DIO3_1F/1R and DIO3_2F/2R; Table 1). A PCR was conducted in a 40-μL volume containing 20 μL 2X Taq PCR Master Mix (Tiangen, Beijing, China), 1 μM of each primer, and 50 ng DNA in the first round or 0.5 μL PCR products in the second round. The two-round PCRs were initiated at 95°C for 5 min, then 35 cycles at 95°C for 30 s, melting temperature for 30 s, and 72°C for 1 min, followed by the final extension at 72°C for 7 min. The second-round PCR products were purified and cloned into the pMD19 (Simple) T-vector (Takara, Dalian, China), and 10 bacterial clones were cultivated and sequenced on an ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, USA). DNA (500 ng) was subsequently processed with a bisulfite conversion reaction using the EZ DNA Methylation™

Kit (Zymo Research, Orange, CA, USA). The methylation of the *DIO3* gene was then detected using bisulfite-sequencing PCRs (BSP). Another set of nested primer pairs (DIO3_BSP_1F/1R and DIO3_BSP_2F/2R; Table 1) was designed using the Methprimer program (http://www.urogene.org/methprimer/). PCRs were run in a 50-µL volume containing 25 µL ZymoTaq™ PreMix (the enzyme was at 4 U/50 µL; Zymo Research, Orange, CA, USA), 1 µM of each primer, and 100 ng bisulfite-treated DNA. PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 64°C (second round: 55.5°C) for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 7 min. For bisulfite sequencing, the second-round PCR products were also cloned into the pMD19 (Simple) T-vector (Takara, Dalian, China), and 10 bacterial clones per individual were sequenced. The sequences containing methylated CGI were analyzed using the QUantification tool for Methylation Analysis (QUMA; http://quma.cdb.riken.jp/) for sequence quality control and diagram generation, and only the sequences with bisulfite conversion efficiency exceeding 98% were processed using this tool.

Table 1. Primer pair sequences.

Primer	Sequence (5'-3')	Size (bp)	Tm (°C)
DIO3_1F	GTGATCCCTTCGGGCTCAA	1169	61.6
DIO3_1R	GTTGGCTGAGAGGCAGACT		
DIO3_2F	ATTGATGCCTCGGCTATA	970	61.6
DIO3_2R	CGTGTCCATCGGTTGTTTA		
DIO3_BSP_1F	TTGGTTTTTAGATTTTTTTTGTATT	641	64.0
DIO3_BSP_1R	CCTAATACATAATAATACCACTCTAA		
DIO3_BSP_2F	TTGGTTTTTAGATTTTTTTTGTATT	465	55.5
DIO3_BSP_2R	TAAAAAAAATCTATAATAACCCAACC		
DIO3_QP_F	GTTGGATTTCTTCAAGCAGGC	111	60.1
DIO3_QP_R	CGGTTTCCTCGGGCGTA		
ACTB-F	CCTGCGGCATTCACGAAACTAC	87	59.7
ACTB-F	ACAGCACCGTGTTGGCGTAGAG		

RNA isolation, cDNA synthesis, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted following the manufacturer protocol for TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was measured with a spectrophotometer to confirm the purity ($A_{260}/A_{280} > 1.8$), and each sample was electrophoresed on 1% agarose gel. First-strand cDNA was synthesized from 1 µg total RNA in a 10-µL reaction volume using the PrimeScript™ RT reagent Kit (TaKaRa, Dalian, China). The extracted total RNA and synthesized cDNA were stored at -80° and -20°C, respectively.

PCRs were performed with qRT-PCR primers (ACTB_F/R and DIO3_QP_F/R; Table 1) using the SYBR Premix Ex Taq Kit (Takara, Dalian, China) and CFX96™ Real-Time PCR detection system (Bio-Rad, Richmond, CA). The reactions contained each primer at 0.25 µM, 5 µL 2X SYBR® Premix Ex Taq™ II, and 1 µL diluted cDNA. PCR conditions started with 95°C for 30 s, then 39 cycles at 95°C for 10 s, and 60.1°C for 30 s. The melting curve analysis was employed to confirm the specificity of PCR products. Each sample was analyzed in triplicate for the gene expression quantification analysis. The mRNA gene expression level, relative to the expression level of *ACTB* (a house keeping

gene widely used for gene expression analysis), was calculated in the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). A template-free reaction (negative control) was also performed to evaluate the veracity of the detection.

Data analysis

The methylation profiles of *DIO3* were determined using QUMA. The levels of methylation between tissues were corrected for using the Bonferroni test and comparisons were performed using one-way ANOVA in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). The comparison of expression levels for the different tissues was also conducted using one-way ANOVA and are reported as means ± SE. In addition, the association analysis between *DIO3* gene expression and methylation level was performed using Pearson's correlation procedure (SAS 9.2). The P ≤ 0.05 represented the significant level. N represented the numbers of clones used in BSP analysis per tissue.

RESULTS

### Bisulfite sequencing of the *DIO3* gene in goats

The methylation levels of the *DIO3* gene were measured for the six tissues (brain, heart, liver, kidney lung, and longissimus dorsi) from the Nanjiang Yellow kids. The 465-bp fragment was identified with 44 CpGs in the *DIO3* exon at the positions 168 to 632 bp (Figure 1A). The CGI that covered all 44 CpGs was amplified and sequenced by the BSP method. Methylation profiles were generally consistent with the methylation dot diagrams among the six tissues. DNA methylation levels of the brain, heart, liver, kidney, lung, and longissimus dorsi ranged from 26.48 to 34.92% (Figure 1B). Lower average methylation was noted for CpGs in the 5' distal region than for those in the 3' distal region, which indicated that CpGs were more likely to be methylated in the 5' distal region of the *DIO3* gene coding sequence than in the 3' region. There were no significant differences in methylation levels among the six tissues (P > 0.05).

Tissue expression of the *DIO3* gene

Quantitative real-time PCR was performed to determine the *DIO3* mRNA expression in tissues from 3-day old goat kids. *DIO3* gene expression values ranged from 0.19 ± 0.04 (lung) to 8.57 ± 1.97 (liver). The qRT-PCR results indicated that gene expression was significantly higher in the liver tissue than in the brain, heart, lung, kidney, and longissimus dorsi tissues (P < 0.05; Table 2).

Correlation analysis of DNA methylation and gene expression

Liver tissue had the highest methylation rate (34.92%). There were no significant correlations between methylation and gene expression for any of the six tissues (Table 2). The highest absolute value of Pearson's correlation coefficient (*r*) was 0.983 for the longissimus dorsi tissue, but it was not statistically significant (P = 0.116).
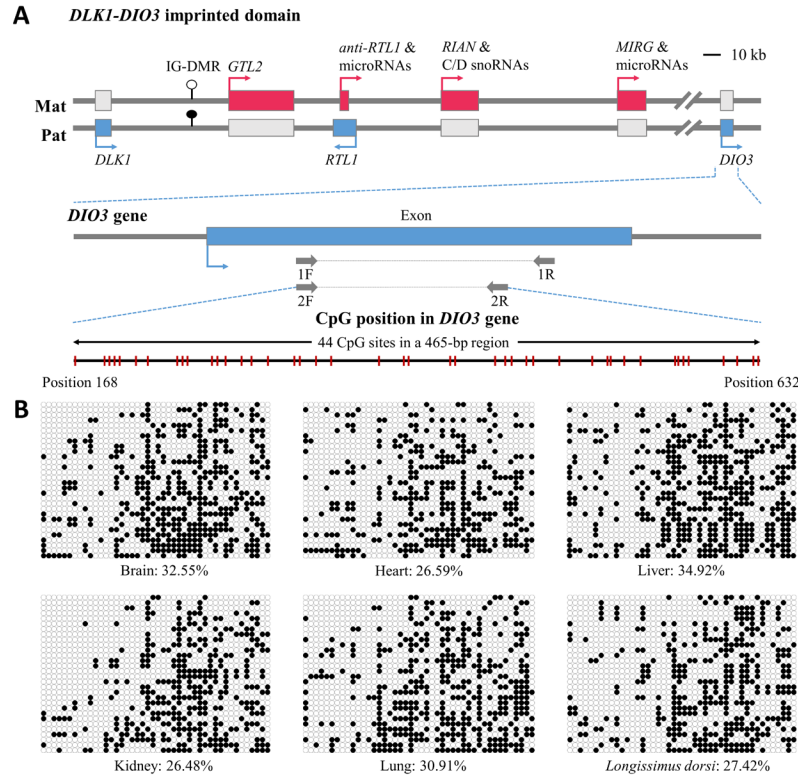
Figure 1. Overview of *DLK1-DIO3* imprinted regions, distribution of intragenic CpG sites, and methylation status of the coding sequence of the *DIO3* gene in six tissues (brain, heart, liver, kidney, lung, and longissimus dorsi) from Chinese Nanjiang Yellow goat kids. **A.** Schematic representation of the *DLK1-DIO3* imprinted domain and intragenic CpG dinucleotides in a 465-bp region of the caprine *DIO3* gene coding sequence. IG-DMR refers to intergenic differentially methylated region of the *DLK1-DIO3* domain. *GTL2*, *anti-RTL1*, *RIAN*, *MIRG*, related microRNA and small nucleolar RNA genes are expressed in the maternally inherited copy. *DLK1*, *RTL1* and *DIO3* are expressed from paternally inherited chromosome. Differential methylation at the IG-DMR is illustrated with filled (methylated) and unfilled (unmethylated) circles. Red vertical lines correspond to the CpG sites. **B.** Methylation status of the caprine *DIO3* gene. Methylation data of all individual clones from the same tissue are represented in one diagram. Percentages of methylation were calculated by counting the number of methylated sites of the total number of CpG sites in this region. Each horizontal line represents a sequence from each clone, while each vertical bar corresponds to a specific CpG site. Filled and unfilled circles indicate methylated and unmethylated cytosine, respectively, and the "x" marks designate missing values.

Table 2. *DIO3* gene expression levels in six goat tissues and analyses of their correlations with methylation patterns.

Tissue	Methylation		Expression level	Correlation analysis	
	CpGs	Rate		r	P value
Brain	429/1318	32.55 ± 3.25	0.85 ± 0.11	-0.021	0.987
Heart	351/1320	26.59 ± 2.55	0.53 ± 0.12	0.438	0.711
Liver	461/1320	34.92 ± 3.46	8.57 ± 1.97*	0.904	0.282
Kidney	349/1318	26.48 ± 2.95	0.62 ± 0.06	0.428	0.718
Lung	408/1320	30.91 ± 3.22	0.19 ± 0.04	0.762	0.448
Longissimus dorsi	362/1320	27.42 ± 3.16	1.66 ± 0.23	-0.983	0.116

Gene expression data are reported as means ± SE. *P < 0.05.

DISCUSSION

To ensure normal development in vertebrates, thyroid hormones, functioning in the hypothalamic-pituitary-thyroid axis, cause pleiotropic effects on diverse processes responsible for the regulation of metabolic rate, neural maturation, and cell division (Hernandez et al., 2006; McAninch and Bianco, 2014). The iodothyronine deiodinases, comprising types 1, 2, and 3 (D1, D2, and D3), have key roles to play in the activation and inactivation of thyroid hormones, injury response, tissue repair, energy homeostasis, and hypothalamic function (Barca-Mayo et al., 2011; Fonseca et al., 2014). D3 has an inactivating role in converting T4 into T3 and T3 into diiodothyronine (T2) by removing iodine moieties from T4 and T3 inner rings, respectively (Bianco et al., 2002). There is evidence that D3 provides an adverse effect to D2, but cooperates faithfully with D2 as a physiological response of the central nervous system to iodine deficiency, in which D2 and D3 contribute to dynamic maintenance of T3 in the brain (Peeters et al., 2001; Forrest et al., 2002). Therefore, D3 is a major inactivating enzyme that controls the availability of thyroid hormones. D3 is abundantly expressed in placenta and several fetal tissues, including skeletal muscle, liver, intestine, and cerebellum in rats, and skin in adult rats (St Germain, 1994). Qiao et al. (2012) investigated porcine *DIO3* gene expression patterns in ten different tissues, and high levels of gene expression were detected in backfat, liver, and uterus tissues, whereas there was low expression in heart, stomach, kidney, small intestine, and muscle tissues, which highlights the key role of *DIO3* in controlling adipose differentiation. D3 gene expression has been detected in cattle and the highest levels of *DIO3* transcripts were noted in mammary glands and kidney tissue, which suggests that there are low levels of *DIO3* mRNA expression in most adult tissues (Connor et al., 2005). In this study, the caprine *DIO3* gene expression patterns noted for the different tissues were unclear, and therefore we subsequently defined the expression profile. Consistent with previous studies, the highest expression level was found in the liver, followed by the longissimus dorsi, brain, kidney, heart, and lung tissues (Zhong et al., 2016).

Different mechanisms of epigenetic modifications inherited from parental chromosomes lead to differential expression of imprinted genes. DNA methylation has a vital role in gene expression as an essential epigenetic signature. Aberrant methylation profiles can initiate abnormal gene expression and are responsible for clinical mastitis in dairy cows (Wang et al., 2013), developmental failure in swine (Wang et al., 2014), and abnormal thyroid hormone levels in mice embryos (Tsai et al., 2002). In a previous study, DNA methylation, in general, was negatively correlated with gene expression in the endometrium of cattle during early pregnancy (Walker et al., 2013). Recently, Wang et al. (2016) revealed that the different methylation CGIs in 5'-untranslated and first exon regions negatively regulated the expression levels of the *GPR120* gene in pigs.

DNA methylation has been studied more than other epigenetic mechanisms that regulate imprinting in the *DLK1-DIO3* imprinted domain. Analyses of the methylation status of available CpGs in five ovine imprinted genes (including the promoter region of the *DIO3* gene) determined that there were few methylated CpGs within the *DIO3* CpG-rich region in mature spermatozoa, female oocytes at different stages, and *in vitro* matured oocytes (Colosimo et al., 2009), which was concordant with findings for mouse embryo tissue (completely unmethylated in the CGI promoter; Tsai et al., 2002). These results suggest that the necessary cis-acting regulatory determinants for imprinting are not contained in the *DIO3* CGI promoter.

Previous studies have noted variations in tissue-specific patterns for parental allelic contributions to gene expression in the *DIO3* imprinted gene. Researchers have noted the

preferential expression from the paternal inherited allele (Hernandez et al., 2002; Tsai et al., 2002), but without the complete repression of the maternal allele (e.g., considerable number of transcripts derived from the maternal allele in the placenta; Hernandez et al., 2002; Lin et al., 2007). *DIO3* gene expression patterns in different mouse tissues exhibited the phenomena of paternal allelic and biallelic expression. In different mouse tissues, methylation and correlation analyses in two regulatory regions of the *DIO3* imprinted domain revealed that *DIO3* gene imprinting varies between tissues, but there was no correlation with tissue-specific expression patterns (Martinez et al., 2014). Generally, negative correlations of gene expression with CGI methylation of sequences exist at the transcriptional start sites harboring a minimum methylation, but this relationship cannot be expanded to other genomic contexts located in the gene body (e.g., intragenic CGI of the *DIO3* gene). In this study, *DIO3* methylation patterns were determined in six tissues from Chinese Nanjiang Yellow goat kids. *DIO3* gene expression was significantly higher in the liver tissue than in the other five tissues, but there was no correlation between intragenic CpGs methylation levels and gene expression for any of the tissues. Converging lines of evidence suggest that *DIO3* gene expression is regulated by tissue-specific epigenetic information. Due to the limited sample size in this study, more investigation and research is required.

Conflicts of interest

The authors declare no conflict of interest.

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