



Analyses of Allele Expression and DNA Methylation Status of Yak (*Bos grunniens*) *PMM2* Gene in Tissues

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ABSTRACT

Background: Imprinted genes are closely related to mammalian development, growth and behavior. To date more than 150 imprinted genes have been identified in human and mice but only a very limited number of imprinted genes have been reported in bovine. A novel gene *PMM2* was identified as paternally imprinted in cattle (*Bos taurus*), but little is known about its expression pattern in yak (*Bos grunniens*). The aim of the present study was to investigate the imprinting status as well as expression levels of *PMM2* gene in yak tissues and to determine whether DNA methylation would play a role in its expression.

Methods: The exonic SNPs of yak *PMM2* gene were discovered by direct sequencing of PCR amplicons from genomic DNA, by which the allelic expression was confirmed with reverse transcription PCR. Moreover, RT-qPCR was used to derive the expression levels of this gene in various tissues, and the DNA methylation status in the promoter was finally analyzed by bisulfite sequencing.

Result: A total of four SNPs were screened out through direct sequencing of PCR amplicons in exon 9, based on these SNPs, we found that only one allele of the *PMM2* gene was expressed in tissues of heart, lung, kidney, testis and muscle. Moreover, RT-qPCR revealed that the *PMM2* gene was highly expressed in testis but at extremely low levels in lung. Finally, the DNA methylation status of a CpG enriched region in promoter was analyzed by using bisulfite sequencing, and a hypermethylated status was found in all the tissues in 18 CpG sites, indicating that the DNA methylation modification of promoter region may not be involved in the regulation of *PMM2* expression in yak. This study provides a reference for further exploring the molecular mechanisms of *PMM2* gene function and imprinting in yak.

Key words: DNA methylation, Imprinting, *PMM2* gene, Polymorphism, Yak.

INTRODUCTION

In mammals, about 1% of alleles produce different epigenetic modifications between their parents, leading to the phenomenon of monoallelic expression in their offspring, known as genomic imprinting (Bjornsson *et al.*, 2008). Currently, no more than one percent of the total genes was identified as imprinted genes (Babak *et al.*, 2015). Evidences showed that imprinted genes play critical roles in embryonic development, postnatal growth (Ferguson-Smith and Surani, 2001), as well as brain development (Ho-Shing and Dulac, 2019). Abnormal expression of imprinted genes was found to be associated with many diseases in human, such as nephroblastoma, diabetes, and tumors (Blunk *et al.*, 2020; Fuke *et al.*, 2021; Pignata *et al.*, 2020). However, the number of imprinted genes identified is currently limited, specially, no more than 50 imprinted genes have been found in yak (Li *et al.*, 2021). There are many types of epigenetic modifications in mammals, and the methylation of CpG sites in DNA is the most typical one. Exactly, DNA Methylation is the conversion of cytosine to 5-methylcytosine by methyl addition with the participation of methyltransferases and complex proteins (Magee *et al.*, 2014). For imprinted genes that exist alone in the genome, there are usually differential methylation regions (DMR) near the promoter and the first exon (Bartolomei *et al.*, 2020). DNA methylation modification in the promoter region of imprinted genes is closely related to cell signal transduction, cell growth and apoptosis,

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and immune function (Livernois *et al.*, 2021). Analysis of the methylation status of specific sites can quickly and accurately diagnose and distinguish imprinted genetic diseases (Ribeiro Ferreira *et al.*, 2019).

As a domestic animal species endemic to the Tibetan Plateau, yaks have physiological characteristics that are adapted to high-altitude and low-oxygen environments (Gu *et al.*, 2021; Zhang *et al.*, 2022). It has been found that yaks have strong foraging ability and vigorous energy metabolism, as well as rapid growth and reproduction, and particularly well-developed cardiorespiratory function (Ding *et al.*, 2020; Ma *et al.*, 2021).

Currently, studies have confirmed the importance of individual genes in regulating functional adaptations in yak (Wan *et al.*, 2018; Gera *et al.*, 2025). For example, *VEGFR2* gene is associated with apoptosis of pulmonary vascular endothelial cells which has a very strong link with lungs under hypoxic conditions (Hansen, 2014) and it is mainly distributed in the epithelial cells of bronchial tubes and their branches and smooth muscle cells of the tubular wall within the lungs. Kasahara *et al.* (2000) confirmed that the lack of expression of the *VEGFR2* gene caused pulmonary tissue endothelial cell apoptosis and emphysema-like lesions. *LRP6* gene is predominantly distributed in the epithelial cells of bronchial tubes and their branches as well as alveolar type II cells in the lungs (Mao and Niehrs, 2003) and deletion and loss of its expression may lead to lung cancer (Fan *et al.*, 2017). The Phosphomannomutase 2 is an enzyme involved in the initiation of the N-glycosylation process and catalyses the conversion of mannose-6-phosphate to mannose-1-phosphate. The Phosphomannomutase 2 encoding gene, *PMM2* is located on chromosome 16 in human, and maternal uniparental disomy of this locus can cause a lethal developmental lung disease in newborns (Schulze *et al.* 2019). In cattle, the *PMM2* gene is located on chromosome 25 and shows a paternally imprinted state (Dong *et al.*, 2022). However, in yak, there are neither reports on the expression pattern of *PMM2* gene nor its involvement in regulating lung function. In this study, we assessed the imprinting status, expression levels and DNA methylation of *PMM2* gene, aiming to provide basic information for an in-depth exploration of the function of the yak *PMM2* gene.

MATERIALS AND METHODS

All animals (11 healthy adult male yaks aged 4-5 years) in this experiment were obtained from Jiangnan abattoir, Guanghan City, Sichuan Province, China, during the year 2023-2024. The animals were subjected to 220 voltages followed by exsanguination, skinning, evisceration, and washing procedures. Immediately, tissues including heart, kidney, lung, muscle and testicular were collected from each yak. The tissues were divided into two portions, one in an EP tube filled with 75% alcohol (LiuHe, Henan, China) for DNA extraction and the other one filled with RNA preservation solution (Takara, Beijing, China) for RNA extraction.

Genomic DNA was extracted using a DNA Extraction Kit (Tiangen, Beijing, China), while total RNA was extracted using a TRIzol RNA extraction kit (Invitrogen, MA, USA) following the instruments. The RNA was treated with DNaseI (Takara, Beijing, China) at 37°C for 30 min to remove genomic DNA, followed by reverse transcription to synthesize the cDNA. After that, the concentration and purity of the DNA and cDNA were checked by Nanodrop ND1000 Spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA) and gel electrophoresis, respectively.

Based on the reference sequence of yak *PMM2* in NCBI database (GeneBank No. NW_005394849.1), Primer Premier 5.0 was used to design primers for amplification the exons (Table 1), and the primer pairs were synthesized by Beijing Tsingke Biotech Co, Ltd. PCR was performed in a 30 µL volume containing 1.2 µL of forward and reverse primers (10 µmol/L), 3 µL of genomic DNA template (100 ng/µL), 15 µL of 2×Taq Master Mix (Kangwei Century, Beijing, China) and 9.6 µL of ddH₂O. The PCR conditions were as follows: 94°C for 3 min, followed by 32 cycles (at 94°C for 25 s, 30 s at the appropriate annealing temperature (Table 1), 72°C for 10 s) and a final extension at 72°C for 5 min. The amplicons were detected by 2% agarose gel electrophoresis and then purified using a UNIQ-10 column DNA gel extraction kit (Sangon, Shanghai, China) and were sequenced recently using an ABI PRISM 3730 automated sequencer (Applied Biosystems, Massachusetts, MA, USA).

A specific primer pair (7F/7R) targeting exon 9, and the mRNA of each tissue (heart, lung, kidney, muscle and testis) of heterozygous yak were taken for allelic expression analysis. The PCR amplification system and reaction procedure were as above, but the template was replaced by cDNA. The PCR products were sequenced directly and which let us to discover allelic expression patterns based on the SNPs.

With β -actin gene was used as an internal control (Table 1), the RT-qPCR reactions were performed in a 25 µL volume containing 1 µL of forward and reverse primers (10 µmol/L), 1 µL of cDNA template (100 ng/µL), 12.5 µL of 2×Es Taq MasterMix (Kangwei Century, Beijing, China) and 9.5 µL of dd H₂O. The PCR conditions were as follows: 95°C for 15 min, followed by 40 cycles (at 95°C for 10 s, 60°C for 30 s, 72°C for 30 s) and a final extension at 56°C for 3 min. Each sample was run in triplicate. Finally, the relative expression level of the target gene was calculated according to the $2^{-\Delta\Delta CT}$ method.

To analyze the DNA methylation status in each tissue, genomic DNA was modified using the DNA Bisulfite Conversion Kit (Tiangen, Beijing, China). The CpG island was predicted by the MethPrimer online tool (<https://www.urogene.org/methprimer/index.html>), and the primer information was listed in Table 1. Similarly, the PCR conditions were as described above, but the template was replaced by bisulfite treated DNA.

RESULTS AND DISCUSSION

Mutation analysis of *PMM2* gene

Yak *PMM2* gene contains 9 exons (Fig 1a). In order to identify the SNPs of *PMM2* in different tissues, five primer pairs targeting the exon regions were designed. Among which, primer 5F/R targeting exon 9 amplified a product length of 864 bp and four SNPs (g.30100T>G; g.30111T>C; g.30118C>T; g.30138G>A) were screened out (Fig 1b). However, no SNPs were detected in exon 1, exon2, exon6, or exon7. In humans, Phosphomannomutase 2 was

encoded by *PMM2* gene and the phosphomannomutase 2 is responsible for catalysing the isomerisation of mannose 6-phosphate to mannose 1-phosphate, a precursor that is essential for the synthesis of dolichol-poligosaccharides. Mutations in the *PMM2* gene have been clearly shown to be the cause of inborn errors of glycosylation and hyperinsulinemia-hypoglycaemia (Demirbilek *et al.*, 2017). Despite these important functions in human, mutations in the *PMM2* gene have not been previously reported in yak. The current study identified four SNPs, it is not clear whether the mutations are involved in the glycosylation and hyperinsulinaemic hypoglycaemic pathways. Moreover, we amplified only part of the exon regions and failed to amplify all exons. Further studies should focus on whether mutations of *PMM2* gene causes alterations in this pathway in yak.

Monoallelic expression of *PMM2* gene

Based on the heterozygosity of the DNA, only one of the heterozygous bases expressed in the mRNA indicating a single allele expression (Li *et al.*, 2021). To analyze the allelic expression of *PMM2* in heterozygous yak tissues, primer pairs 6F/R located within exon 9 (Fig 2a) were designed for amplification the cDNA from each tissue. Based on the comparison of DNA heterozygotes, only one allele was found at the cDNA level in each tissue (Fig 2b), indicating that the gene was imprinted. Altered expression of imprinted genes can lead to abnormal growth and development and related diseases in mammals through various physiological responses and signaling pathways. It has been found that many imprinted genes are important regulators of embryonic and postnatal growth and development and these genes are usually involved in behavior and brain functions (Scagliotti *et al.*, 2021), even induced cancer (Lambertini *et al.*, 2012). Moreover, Yak is an economically important livestock species and serve as a potential model species for studying human

preimplantation embryonic development and for determining the genetic etiology of sporadic human diseases (Bourneuf *et al.*, 2017; Hansen, 2014). Currently, the cattle *PMM2* gene showed paternally imprinted in heart, liver, kidney, lung and brain tissues (Dong *et al.*, 2022). In this study, we found that the yak *PMM2* gene was monoallelically expressed in heart, lung, kidney, testis, and muscle, indicating *PMM2* is an imprinted gene in all of these tissues. However, we did not identify its parentally imprinted pattern. In addition, we only conducted in male animals, so future experiments could pay attention to these issues in yak.

Expression level of *PMM2* gene in different tissues

The RT-qPCR amplification was performed by primer pairs 7F/R using cDNA as template from all the five tissues. As shown in (Fig 3a), the *T_m* values of the *PMM2* and β -actin gene PCR primer pairs are relatively homogeneous and there is a single peak of proliferation on the proliferation curve. This indicates that there is no dimer and non-specific amplification products for both the target and internal reference genes, and that the fluorescence intensity is derived from the amplification products. The results of RT-qPCR indicating that *PMM2* was expressed in all tissues of heart, kidney, lung, testis and muscle although the expression levels were varied. For instance, the testis expressed highest level of *PMM2* but very low level in lung, with similar levels in other tissues (Fig 3b). Of note, it has been reported in other literature to be associated with lung function (Cai *et al.*, 2020), so we hypothesised that it may play a negative regulatory role in yak lungs. On the other hand, high expression level in the testis may be involved in the reproductive pathway in yaks or even mammals, however, no correlation between *PMM2* and testicular function has been reported. Future studies could also focus on *PMM2* and yak reproductive performance.

Table 1: Information of the primers used for PCR reactions.

Primer	Primer sequence (5'-3')	Products length/bp	<i>T_m</i> /°C	Application
1F	CTGTAGTCCAGGGCACATCC	418	57	Amplification of exon1
1R	TCTTGAGATCTGGCTAGGGCTTCC			
2F	ATCTGAGCCACCAGGGAC	426	59	Amplification of exon2
2R	ATCGAGGAAGCAGGAAGG			
3F	GCCTGCTGTCTACTGCTCAG	333	54	Amplification of exon6
3R	TGCTTTCCCTCTTATGG			
4F	GGGGCTCCAGTTACAGAC	244	52	Amplification of exon7
4R	CTGCTAACACCCTTCCAT			
5F	ACGCTACTCGCTGAGGAC	864	53	Amplification of exon9
5R	GAACCACCCAGGGACAAA			
6F	ATCGTGCGTGACATCAAAGAC	168	60	Reference gene
6R	AAGAAGGAAGGCTGGAAAA			
7F	ACAAAAGTTCTGTTGAGGAGA	157	60	RT-qPCR
7R	AATAGATGGTCTTATAGCC			
8F	TTGTTTTTATTGTATTGTTGGATTTA	268	53	Methylation
8R	CCCTTCAACTCCTTATACCTAAC			

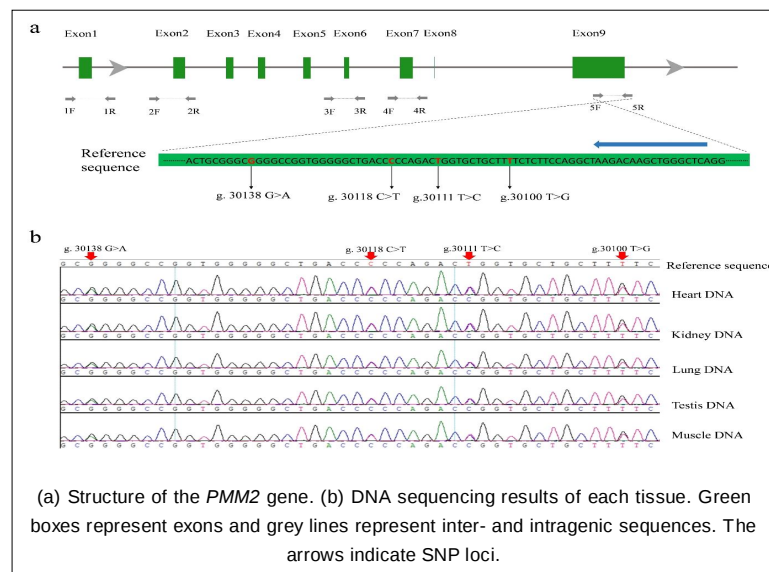


Fig 1: *PMM2* structure and DNA sequencing results.

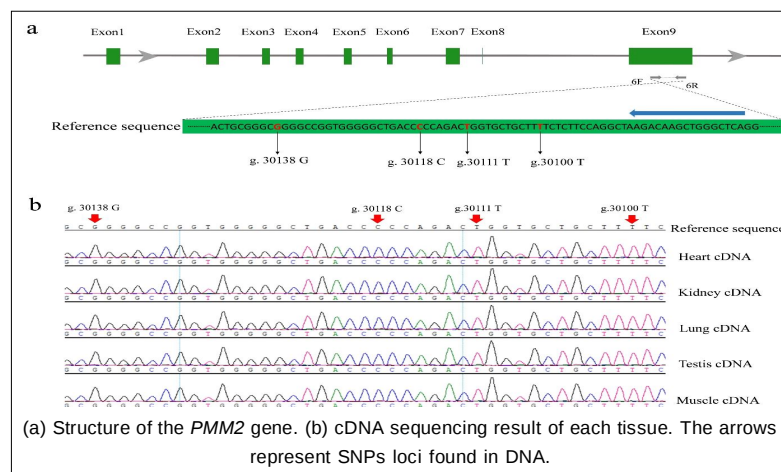


Fig 2: *PMM2* structure and cDNA sequencing result of exon 9.

Methylation status of the promoter region of *PMM2*

In order to further explore the regulatory mechanisms of imprinting expression of *PMM2* gene in yak, a 300-bp CpG-rich fragment including 18CpGs in the promoter region of this gene was selected to analyze methylation status (Fig 4a). We found that *PMM2* was hypermethylated in all the studied tissues (Fig 4b). DNA methylation, a widely studied and stably inheritable epigenetic modification, is an important molecular mechanism in the establishment and maintenance of genomic imprinting (Li *et al.*, 1993; Shiraishi *et al.*, 2002). DNA methylation mainly occurs in CpG dinucleotide-rich regions, also known as CpG islands (Schmitz *et al.*, 2019). CpG islands are usually located in the promoters of genes, and they are an important marker for imprinted genes (Xu *et al.*, 2017). Imprinted genes usually form differentially methylated regions (DMRs) in the CpG island region to regulate their expression (Barlow,

2011; Li and Sasaki, 2011). It is generally believed that the DNA methylation of the promoter has an inhibitory effect on gene expression, especially the methylation of the CpG island in the promoter region (Huang *et al.*, 2014). However, our results showed that the gene was hypermethylated in all tissues, no association between the methylation and expression level was found, which suggested that the expression of *PMM2* may not be regulated by methylation and imprinting status. Dong *et al.* (2022) investigated the methylation status of the 28-CpG fragment against the promoter region of the *PMM2* gene in cattle and found that it was hypomethylated in the liver, brain and placenta, but hypermethylated in the spleen. The reason for the existence of this phenomenon may be that the species studied are different or the gene fragments studied for methylation are too short and fail to fully reflect the methylation status of the entire promoter region.

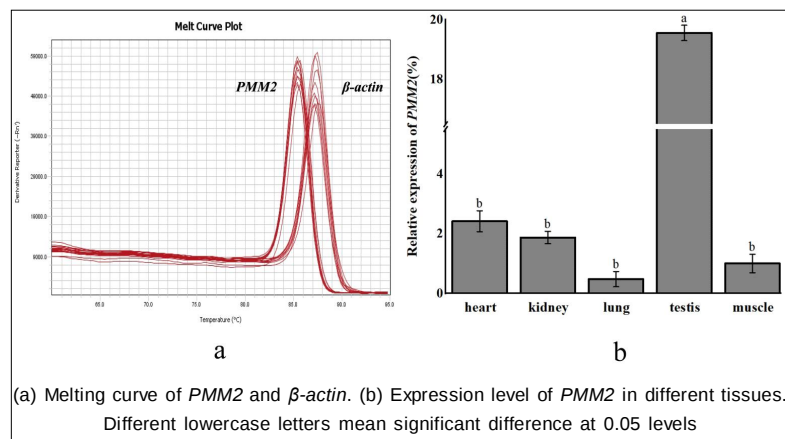


Fig 3: The expression of *PMM2* in yak tissues.

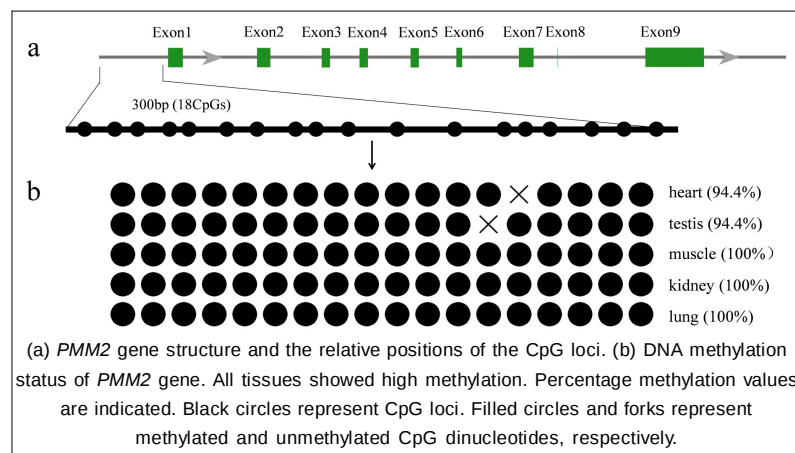


Fig 4: The structure, CpG location and methylation status of the yak *PMM2* gene.

CONCLUSION

In this study, we determined the allelic expression status, expression levels and promoter DNA methylation status of *PMM2* gene in heart, lung, kidney, testis and muscle of yak. The results showed that *PMM2* gene was monoallelically expressed in these tissues. Further, the expression levels varied significantly among tissues but no differentially methylated regions were detected in the promoter region, indicating that the allele expression of the *PMM2* gene may not be regulated by DNA methylation. This study enriched the number of imprinted genes in yak and provided a reference basis for an in-depth exploration of the molecular mechanisms of *PMM2* function and imprinting.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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