

# Association of BMPR-1B Gene 3'-UTR Region Polymorphism with Litter Size in Tibetan Sheep

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## Abstract

Bone Morphogenetic Protein Receptor-1B (BMPR-1B) is considered as the primary gene in sheep for follicular development and litter size trait. It has been defined as the most major candidate gene for genetic markers of sheep reproductive performance. In our study, polymorphisms in the BMPR-1B gene 3'-UTR region were investigated in 363 Tibetan sheep (119 Plateau-type Tibetan sheep, 141 Valley-type Tibetan sheep, and 103 Oula-type Tibetan sheep) by DNA sequencing analysis. Two single nucleotide polymorphisms (SNPs) were identified, which were G1339A and A1354G. The frequencies of SNPs were in Hardy-Weinberg equilibrium (Chi-square test,  $P > 0.05$ ). AA and AG genotypes were found in the A1354G variant of the 3'-UTR region, that AA and A were the preponderant genotype and allele, respectively. The  $\chi^2$  independence test analyses indicated that the A1354G variant of BMPR-1B gene 3'-UTR region polymorphisms was significantly correlated with litter size in all-types Tibetan sheep ( $P < 0.05$ ). These results demonstrate that the BMPR-1B gene 3'-UTR region might be a potential candidate gene for marker-assisted selection (MAS).

**Keywords:** Tibetan sheep, BMPR-1B gene, 3'-UTR region, Polymorphism, Litter size

## Tibet Koyunlarında BMPR-1B Geni 3'-UTR Bölgesi Polimorfizmi İle Batın Genişliği İlişkisi

### Öz

Kemik Morfogenetik Protein Reseptörü-1B (BMPR-1B), koyunlarda foliküler gelişim ve batın genişliği özelliği için birincil gen olarak kabul edilir. Bu gen, koyunlarda üreme performansının genetik belirteçleri için en önemli aday gen olarak tanımlanmıştır. Çalışmamızda, 363 Tibet koyununda (119 plato tipi Tibet koyunu, 141 vadi tipi Tibet koyunu ve 103 Oula tipi Tibet koyunu) BMPR-1B geninin 3'-UTR bölgesindeki polimorfizmler DNA dizi analizi ile araştırıldı. G1339A ve A1354G adlı iki single nükleotid polimorfizm (SNP) saptandı. SNP'lerin frekansları Hardy-Weinberg dengesi içerisindeydi (Ki-kare testi,  $P > 0.05$ ). 3'-UTR bölgesinin A1354G varyantında AA ve AG genotipleri saptandı ve AA ve A sırasıyla baskın genotip ve aleldi.  $\chi^2$  bağımsızlık testi analizleri, BMPR-1B geni 3'-UTR bölgesi polimorfizmlerinden A1354G varyantının, tüm Tibet koyun türlerinde batın genişliği ile yakından ilişkili olduğunu gösterdi ( $P < 0.05$ ). Bu sonuçlar, BMPR-1B geni 3'-UTR bölgesinin, markör destekli seleksiyon (MAS) için potansiyel bir aday gen olabileceğini göstermektedir.

**Anahtar sözcükler:** Tibet koyunu, BMPR-1B geni, 3'-UTR bölgesi, Polimorfizm, Batın genişliği

## INTRODUCTION

Bone Morphogenetic Protein Receptor-1B (BMPR-1B) is a member of transforming growth factor- $\beta$  family, which plays an imperative role in sheep follicular development and reproductive traits and is called as fecundity (Fec) gene for its function in additive effect on sheep litter size and ovulation rate<sup>[1]</sup>. BMPR-1B is upon binding with BMP

ligands, BMPR-2B transphosphorylates the GS domain of the BMPR-1B, which leads to the activation of downstream cascades and the inactivation of the partial receptor, then Smads state of expression and phosphorylation is changed, the synthesis of estradiol by FSH induction is promoted, the synthesis and secretion of progesterone is inhibited, granular cell differentiation of A746G mutation ewes and follicular maturation are accelerated, and ovulation is increased<sup>[2,3]</sup>.

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Several studies confirm that A746G mutation led to the loss-of-function in BMPR-1B gene, and changes protein conformation that promotes steroid production, increases ovulation rate and litter size in sheep. BMPR-1B had been proved for additive effects on ewe ovulation rate, and it could inhibit granulosa cell apoptosis, prevented follicular atresia, and promote litter size<sup>[4]</sup>. This may be a primary physiological mechanism for BMPR1B to affect ewe fecundity<sup>[5]</sup>. The research showed that the higher ovulation rate of Small Tail Han sheep related to mitochondrial oxidation BMPR-1B protein expressions, and it could provide an advanced recognition of the molecular mechanism for sheep high prolificacy<sup>[6]</sup>.

BMPR-1B gene was widely expressed in some tissues such as ovary, testes, ear, hypothalamus and blood, mostly in ovary tissue, which regulated the release of gonadotropin hormones (FSH and LH)<sup>[7]</sup>. Based on the breed and biological properties in ruminant, we hypothesized that BMPR-1B was a major candidate gene for genetically improving reproductive performance in sheep<sup>[8]</sup>. Tibetan sheep was one of the original sheep breeds in China, and known as excellent adaptability in Tibetan plateau, however, Tibetan ewes were a low reproduction rate breed, which twin rate was only 3~5 percent<sup>[9]</sup>. To date, there are few reports on the effects of the association of BMPR-1B gene polymorphisms with litter size trait in Tibetan sheep. In present study, we performed extensive BMPR-1B gene 3'-UTR region screening by DNA sequencing methods to detect polymorphisms, we present 2 genetic polymorphisms found in BMPR-1B gene 3'-UTR region in Tibetan sheep and examined A1354G variant of 3'UTR region association with litter size trait.

## MATERIAL AND METHODS

The study protocol and experimental animals were approved by the National Administration of Qinghai University (China, 2016).

### Animals Collection

Three hundred and sixty three ewes (80 Singletons Plateau-type Tibetan ewes, 39 Twins Plateau-type Tibetan ewes, 70 Singletons Valley-type Tibetan ewes, 71 Twins Valley-type Tibetan ewes with twins, 51 Singletons Oula-type Tibetan ewes and 52 Twins Oula-type Tibetan ewes) were selected under grazing conditions on natural grasslands in Haibei Tibetan Autonomous Prefecture (Qinghai, China).

All experimental ewes were 3-4 years old, which had a clear lambing record, raising conditions (NRC 2007), and the same breeding ewes bodyweight was no significant difference.

### Genomic DNA Extraction and Genotyping

Five mL jugular blood samples of each ewes were collected in single-use evacuated tubes containing heparin sodium

as anticoagulant. Genomic DNA was extracted from whole samples using the Blood DNA Kit (TaKaRa, Dalian, China). The quality and quantity of extracted DNA samples were measured by Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Genomic DNA samples were diluted to 50 ng/μL and stored at -20°C for subsequent analysis.

PCR primers were designed from BMPR-1B gene 3'-UTR region (1145bp-1500bp) sequence available at GenBank (GenBank accession number: NM\_001009431.1) using the Primer Premier Software (Version 3.0). Genomic DNA was amplified using primer sequences (F: 5'-GGAACAGCAG AGGAATG-3' and R: 5'-CACAGTCAGGAAGTAAAT-3'). DNA was pooled from 180 random samples in 6 types Tibetan sheep and amplified by PCR. PCR amplification product was a 20 μL mixture composed of 1 μL of each primer, 1 μL dNTP, 10 μL 2xTaq MIX (TaKaRa, Dalian, China), and 7 μL RNase-free dd H<sub>2</sub>O. The cycling protocol was as follows: an initial denaturation for 3 min at 94°C; 35 cycles of 94°C for 30 s; annealing at 52°C for 30 s, extension at 72°C for 30 s, and the final extension was performed at 72°C for 10 min. All PCR products were sequenced using an ABI 3730 sequencer (ABI, Foster City, CA, USA).

### Data Analysis

Allele and genotype frequencies were estimated after sequence alignment by direct counting. Chi-square test for Hardy-Weinberg equilibrium (HWE) was applied to assess the deviations in the number of observed versus expected genotypes. Population genetic indexes, including heterozygosity (HE) and polymorphism information content (PIC) were calculated according to Nei's methods<sup>[10]</sup>.

The association between A1354G variant of BMPR-1B gene 3'UTR region polymorphism and litter size trait in Tibetan sheep (the phenotype was directly treated as category) was analyzed using the  $\chi^2$  independence test. Tests of hypotheses were done using t-tests at  $P < 0.05$  with  $P < 0.10$  considered a trend.

## RESULTS

### Genomic DNA extraction and Amplification

The extracted genomic DNA samples were detected by 1% agarose gel, which all samples were good integrity. The amplifications resulted in a product size of 355-bp on 2.5% agarose gel electrophoresis when visualized under the UV transilluminator in all ewes. This product was directly used for DNA sequencing.

### SNP Discovery and Genotyping of Selected SNPs

The sheep BMPR1-B gene maps to chromosome 6. In the current study, the constructed DNA pools were used to amplify the 3'UTR of the BMPR1-B cDNA sequence. The PCR product was sequenced, and two SNPs were identified:

g.3004 G>A (G1339A) and g.3019 A>G (A1354G) (Fig. 1).

The  $\chi^2$  independence test analysis showed that A1354G mutation of BMPR-1B gene 3'UTR were significantly correlated with litter size in all-types of Tibetan sheep. The effects of genotypes on mRNA secondary structure of BMPR-1B gene 3'UTR was analyzed via RNA foldWeb Server on-line prediction software (Fig. 2). The A1354G mutation affected the mRNA secondary structure of BMPR-1B 3'UTR between AA and AG genotypes.

### Population Genetic Variability Analyses

The genotype distribution, allelic and genotypic frequencies of A1354G variant of BMPR-1B gene 3'UTR region was calculated in Table 1. The results revealed AA and AG genotypes for PCR products by DNA sequencing analysis, and AA type was the preponderant genotype, A was the preponderant allele in all Tibetan sheep. GG genotype could not be detected among the animals examined.

The heterozygosity ( $H_e$ ), Hard-Weinberg Equilibrium (HWE), and Polymorphism Information Content (PIC) value of A1354G variant of BMPR-1B gene 3'UTR region were given in Table 2. The convention for classifying PIC is that a value less than 0.250 indicates low polymorphism, 0.250 to 0.500 indicates intermediate polymorphism, and greater than 0.500 indicates high polymorphism. In present study, PIC values ranged from 0.03 to 0.21, which showed that the loci had low genetic diversity, but PIC value of twins Tibetan sheep was significantly greater than that of singletons Tibetan sheep ( $P < 0.05$ ). The  $\chi^2$  test showed

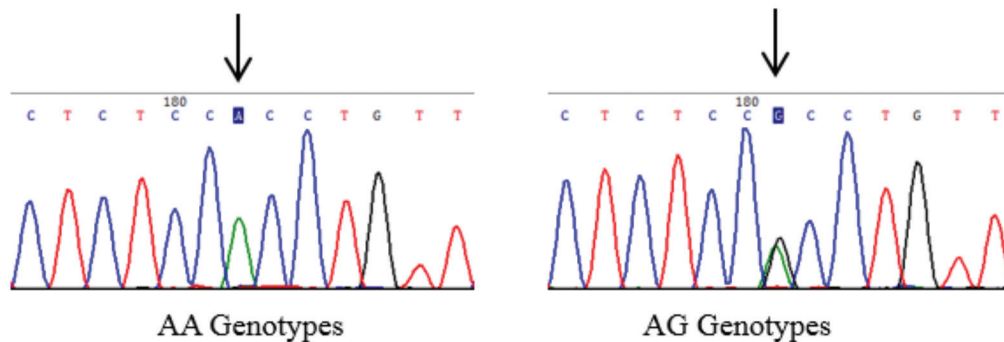
that Tibetan sheep were in HWE ( $P > 0.05$ ), and P value of twins Tibetan sheep was significantly lower than that of singletons Tibetan sheep ( $P < 0.05$ ).

### Genotype Frequencies Analysis and Association Analysis

The  $\chi^2$  independence test was used to analyze the association of genotypic frequency and litter size traits within Tibetan sheep type. For A1354G variant of BMPR-1B gene 3'UTR region, the results showed that differences between the singletons and twins Tibetan sheep in AA and AG genotypic frequency were significant ( $\chi^2 = 29.3788$ ,  $P < 0.0001$ ). There were no significant differences in different Tibetan sheep type between AA genotype and AG genotype ( $\chi^2 = 0.0753$  and  $P = 0.9631$ ). There were statistically significant differences in litter size traits for different genotype  $\times$  types of Tibetan sheep, this meant that G allele frequency promoted the Tibetan sheep litter size ( $\chi^2 = 31.2004$ ,  $P < 0.0001$ ).

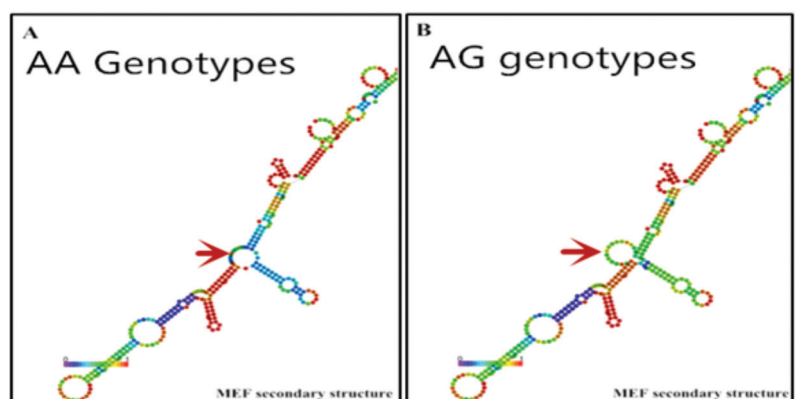
## DISCUSSION

BMPR-1B was a member of TGF- $\beta$  super family and played an important role in ewe follicle development and litter size [11]. The studies identified the BMPR-1B gene was a major gene associated with litter size traits in Australian Merino sheep, Cambridge sheep and Small Tail Han sheep [12]. BMPR-1B gene were reported of 22 single nucleotide polymorphisms (<http://www.ncbi.nlm.nih.gov/gene/443454>) [13]. Previous research on the A746G of BMPR-1B gene found that the additive effect on BMP



**Fig 1.** Sequencing blast of 1354 loci in sheep BMPR-1B gene 3'-UTR

**Fig 2.** Secondary structure prediction of different genotypes of mRNA in BMPR-1B gene 3'-UTR



**Table 1.** Genotype distribution, allelic and genotypic frequencies of 1354 loci of *BMPR-1B* gene 3'UTR

Group	Samples	Genotypic Frequency <sup>a,b</sup>		Allelic Frequency <sup>c</sup>	
		AA	AG	A	G
Singletons Plateau-type Tibetan sheep	80	0.95 (76)	0.05 (4)	0.975	0.025
Twins Plateau-type Tibetan sheep	39	0.72 (28)	0.28 (11)	0.86	0.14
Singletons Valley-type Tibetan sheep	70	0.97 (68)	0.03 (2)	0.985	0.015
Twins Valley-type Tibetan sheep	71	0.77 (55)	0.23 (16)	0.885	0.115
Singletons Oula-type Tibetan sheep	51	0.96 (49)	0.04 (2)	0.98	0.02
Twins Oula-type Tibetan sheep	52	0.81 (42)	0.19 (10)	0.905	0.095

**Note:** a- AA was wild-type of 1354 loci of *BMPR-1B* gene 3'UTR, AG was mutation-type of 1354 loci of *BMPR-1B* gene 3'UTR; b- Numbers in parentheses are numbers of individuals that belong to the respective genotypes; c- Mutant allele for 1354 loci of *BMPR-1B* gene 3'UTR (G), wild type allele for 1354 loci of *BMPR-1B* gene 3'UTR (A)

**Table 2.** Genetic diversity index of 1354 loci of *BMPR-1B* gene 3'UTR

Group	PIC	He	Ne	P-value	$\chi^2$
Singletons Plateau-type Tibetan sheep	0.05	0.28	1.323	0.82	0.06
Twins Plateau-type Tibetan sheep	0.21	0.24	1.451	0.31	1.05
Singletons Valley-type Tibetan sheep	0.03	0.36	1.278	0.91	0.02
Twins Valley-type Tibetan sheep	0.18	0.29	1.454	0.29	1.14
Singletons Oula-type Tibetan sheep	0.04	0.23	1.413	0.87	0.02
Twins Oula-type Tibetan sheep	0.16	0.31	1.357	0.45	0.59

signal pathway during follicle development led to increase average ovulation <sup>[14]</sup>. However, there are few reports on the effects of the non-coding region SNPs, especially in 3' untranslated region and 5' promoter region. Recent evidence indicated that gene UTR region could regulate mRNA location and protein abundance by affecting the mRNA stability or translation <sup>[15]</sup>. The genetic information which stored in gene 3'UTR could be transferred to proteins through protein-protein interaction to affect protein expression <sup>[16]</sup>. In Dorset, Mongolian, and Small Tail Han sheep reported synonymous mutation could activate the native splicing donor site, which resulted the premature stop codon or yielding a shorter mRNA, and affected sheep ovarian specific expressions of follicular oocyte cells and granulosa cells under the unusual condition <sup>[17,18]</sup>. Here, we detected 2 SNPs (g.3004 G>A and g.3019 A>G) in 3'UTR of *BMPR-1B* gene. Data analysis revealed that A1354G variant of *BMPR-1B* gene 3'UTR region (g.3019 A>G) was associated with litter size traits in Tibetan sheep.

As a low heritability trait, litter size was controlled by a major gene and some minor polygenes, then, screening the SNPs of target genes was very important to sheep breeding <sup>[19]</sup>. Sequence alignment demonstrated that A1354G variant located in the 3'UTR did not result in changes of amino acid sequence, but the genotype distribution of different Tibetan sheep type and litter size traits ewes (singletons ewes and twins ewes) had significant differences. There was a significantly association between a C/T polymorphism in the *BMPR-1B* gene 3'UTR and litter size traits in Small

Tail Han sheep, Gansu Alpine Merino sheep <sup>[20]</sup>. There were only two genotypes (AA and AG) without GG genotype in Tibetan sheep, and the causes of this situation could be that i) the allelic frequency of G was too low to be detected in this study as the sample sizes were too small. ii) allele G was a recessive lethal gene and GG genotype Tibetan sheep could not survive.

The twins lambs rate in Tibetan sheep was very low, which was solely 3~5 percent. However, since sheep numbers were particularly important in pasture safe grazing capacity, to explore those traits would greatly promote the development of sheep industry <sup>[21]</sup>. *BMPR-1B* gene plays an important role in sheep reproductive endocrine, ovary development, litter size, organ development and body mass. *BMPR-1B* promoted steroid production, changed the Smad state of expression and phosphorylation, thereby expediting follicular maturation and increasing sheep litter size <sup>[22]</sup>. Many studies demonstrated that *BMPR-1B* gene levels of transcripts had an additive effect on litter size and ovulation rate, but has negative effects on fetal growth and development and body mass during gestation <sup>[23,24]</sup>. Based on these results, *BMPR-1B* appears to be litter size regulatory factor, especially in ruminant species. Combined with the results of our study, we suggest that A1354G variant of *BMPR-1B* gene 3'UTR region may directly or indirectly mediate Tibetan sheep litter size trait.

In this paper, the polymorphisms of *BMPR-1B* gene 3'UTR in Tibetan sheep were analyzed. Genotype association

analysis with litter size trait in Tibetan sheep were performed, which will provide information for the early MAS in Tibetan sheep. Further research with a larger sample size and additional expression analyses of this gene are necessary to support our results.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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## AUTHOR CONTRIBUTIONS

J. JIA and D. XIE Conceptualization; Y. ZHANG and H. ZHANG Data curation; J. JIA, Y. ZHANG, and L. ZHANG Formal analysis; S. HOU Funding acquisition; Q. CHEN Investigation; S. HOU Methodology; H. ZHANG and Q. CHEN Project administration; S. HOU Resources; D. XIE Software; Q. CHEN Supervision; Y. ZHANG Validation; H. ZHANG, and L. ZHANG Visualization; J. JIA Writing - original draft; D. XIE, L. ZHANG and Q. CHEN Writing - review & editing.

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