Article

Molecular Characterization, Expression Profiles of SMAD4, SMAD5 and SMAD7 Genes and Lack of Association with Litter Size in Tibetan Sheep

Ruizhe Sun, Mingming Li, Na He, Xiaocheng Wen and Junxia Zhang *

College of Agriculture and Animal Husbandry, Qinghai University, Xining 810016, China
* Correspondence: 200490009@qhu.edu.cn; Tel.: +86-0971-5313465

Simple Summary: Selection of molecular markers used in the marker-assisted selection of the litter size of sheep is critically essential. In this study, we identified single nucleotide polymorphisms (SNPs), g.51537A>G in SMAD5 and g.319C>T in SMAD7, from Tibetan sheep, and they were genotyped. The expression patterns of SMAD4, SMAD5 and SMAD7 in the tissues of Tibetan sheep were determined. Furthermore, we also performed an association analysis of the SNPs with litter size. The results indicated that SMAD4, SMAD5 and SMAD7 were extensively expressed in the various tissues of Tibetan sheep. Sequence analysis showed that SMAD4 contained 1662 bp encoding 533 amino acids, SMAD5 contained 1667 bp encoding 465 amino acids and SMAD7 contained 1408 bp encoding 427 amino acids. Alignment analysis showed that the amino acid sequence of the three proteins shared high similarity with sheep, yak, cattle, dog, human, pig, chimpanzee, rhesus monkey and house mouse. Furthermore, association analysis showed that both g.51537A>G in SMAD5 and g.319C>T in SMAD7 were not significantly associated with litter size of Tibetan sheep.

Abstract: SMAD4, SMAD5 and SMAD7 belonging to the transforming growth factor β (TGF-β) superfamily are indispensable for oocyte formation and development, ovarian organogenesis and folliculogenesis. However, only a few studies have investigated the characteristics of SMAD4, SMAD5 and SMAD7 in Tibetan sheep and the effect of their polymorphism on litter size. In this study, we examined the expression of SMAD4, SMAD5 and SMAD7 in 13 tissues of Tibetan sheep by reverse transcription-quantitative polymerase chain reaction. Further, cDNA of these genes was cloned, sequenced and subjected to bioinformatics analysis. DNA sequencing was also used to detect single nucleotide polymorphisms (SNPs). However, iM-LDR™ technology was used for SNP genotyping. Associations between polymorphisms and litter size were analyzed using data from genotyping of 433 Tibetan sheep. The results showed that the expression of SMAD4, SMAD5 and SMAD7 genes was ubiquitous in the tissues of Tibetan sheep, such as the ovary, uterus and oviduct, hypothalamus, hypophysis, heart, liver, spleen, lung, kidney, rumen, duodenum and longissimus dorsi. However, the expression was unbalanced and upregulated in the spleen, lung, ovary and uterus and downregulated in the longissimus dorsi. The bioinformatics analysis showed that SMAD4, SMAD5 and SMAD7 in Tibetan sheep encoded proteins of 533, 465 and 427 amino acids, respectively. Sequence homology analysis of the three proteins among other animals showed that the sequences of SMAD4, SMAD5 and SMAD7 of Tibetan sheep were similar to those in sheep, yak, cattle, dog, human, pig, chimpanzee, rhesus monkey and house mouse. Two synonymous mutations, g.51537A>G and g.319C>T, were detected in SMAD5 and SMAD7, respectively. The associations of these SNPs and litter size were determined, and it was found that both g.51537A>G and g.319C>T have no significant effect on the litter size of Tibetan sheep. The results provided novel insights into the molecular characterization, expression profiles and polymorphisms of SMAD4, SMAD5 and SMAD7 in Tibetan sheep, but our results do not support associations between these genes and the litter size of Tibetan sheep.

Keywords: SMAD4; SMAD5; SMAD7; gene expression; litter size; Tibetan sheep
1. Introduction

The Qinghai–Tibet Plateau (Tibetan Plateau) in China is the largest and highest plateau in the world [1]. It is located in northwest China and is characterized by extreme environmental conditions and a unique ecological environment involving high altitude, low oxygen levels, strong ultraviolet rays, low temperature, less rainfall and complex and variable terrain [2–5] inhabited by the numerous domestic animals. These animals play an essential role in the ecosystem and the livelihoods of the plateau residents. Many domestic animals have lived for generations and have stable genetic adaptability to harsh living conditions [3]. Tibetan sheep (Ovis aries) is one of the domestic animal breeds distributed on the plateau. After long-term natural selection, Tibetan sheep are well-adapted to the high-altitude region. They are the most numerous livestock in the Qinghai–Tibet Plateau [1]; however, they have low reproductive and production rates.

The transforming growth factor-β (TGF-β) superfamily of cytokines participates in cellular proliferation, survival and differentiation [5]. The members of TGF-β include TGF-β activins, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), glial-derived neurotrophic factors (GDNFs), nodal, lefty and anti-Müllerian hormones (AMH) [6–9]. TGF-β signaling is mediated by TGF-β ligands that transduce the signals to the nucleus via the action of SMADs [10]. The different TGF-β superfamily members signal via different SMAD proteins. Thus far, eight mammalian SMAD proteins have been isolated and designated from SMAD1 to SMAD8 [11]. Based on their function, SMADs are classified into the following three groups that exert their effects: receptor-activated SMADs (R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8), common mediator SMADs (Co-SMADs: SMAD4) and inhibitory SMADs (I-SMADs: SMAD6 and SMAD7) [10]. The SMAD proteins transduce signals in a dynamic process: they continually shuttle between the cytoplasm and nucleus, and ligand stimulation tunes this process. SMAD4 is the only co-mediated SMAD (Co-SMAD) in mammals. SMAD4 cannot be phosphorylated by the receptors; therefore, they are oligomerized with activated R-SMADs and form complexes via the regulation of p21 and other downstream genes for cell cycle arrest in the G1 phase and inhibition of cell proliferation [12]. SMAD5, which belongs to R-SMAD, is activated by BMP ligands and is phosphorylated by type I receptors [13], which are highly homologous in structure, but their functions are not the same [12]. SMAD7 is induced by TGF-β family members and acts as negative feedback by interfering with R-SMADs for receptor interaction and marking the receptors for degradation [6,14]. It generally antagonizes growth inhibition of the cell, matrix formation, apoptosis induction and embryonic lung formation [12]. The individual SMAD proteins influence proper functioning of the ovary [15]. As reported, SMADs are ubiquitously expressed in nearly all cell types in the body [6]. In the ovary, several TGF-β superfamily members are expressed by the oocyte, granulosa and thecal cells at different stages of folliculogenesis, although it has been demonstrated that SMADs are related to the activity of the ovary [16]. Currently, various candidate genes in the TGF-β signaling pathway have been identified, and several genes, which, to a greater or lesser extent, affect ewe prolificacy, have been identified in studies. FecB mutation (c.746 A>G) in BMPR-IB (bone morphogenetic protein receptor IB) [17] and the mutations in BMP15 (bone morphogenetic protein 15) [18] and GDF9 (growth differentiation factor 9) [19] have been identified as the major genes controlling prolificacy in sheep; however, the association between SMAD4, SMAD5 and SMAD7 genes and litter size of Tibetan sheep remains unclear. Therefore, this study aimed to clone SMAD4, SMAD5 and SMAD7 genes, detect polymorphism and perform association analysis for litter size in Tibetan sheep. Moreover, expression profiles of these genes were measured in different tissues.
2. Materials and Methods

2.1. Animals and Sampling

Tibetan sheep were obtained from Xiangkemeiduo Sheep Industry Co. Ltd. in Qinghai, China, and a total of 433 ewes were selected for the experiment. During the raising period, they were pastured in similar grassland conditions, and environmental conditions were kept consistent for all Tibetan sheep. The health and reproduction records of the ewes were kept by the farmers. Their mean litter size was calculated from 3 consecutive records from the first to the third parity (Table S1). Finally, under the supervision of qualified veterinarians, blood samples (5 mL) were taken from the jugular vein of each sheep in the morning for DNA extraction. DNA was extracted using an EasyPure Blood Genomic DNA Kit (TransGen Biotech, Beijing, China). DNA was dissolved in Elution Buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at −20 °C.

A total of three ewes of Tibetan sheep were selected from the purebred herds of the same farm. The selected 6-month-old ewes were healthy and had similar weight. The ewes were slaughtered, and tissues were obtained from the hypothalamus, hypophysis, heart, liver, spleen, lung, kidney, ovary, oviduct, uterus, rumen, duodenum and longissimus dorsi. The tissue samples were immediately frozen in liquid nitrogen and then stored at −80 °C in a cryogenic freezer. The RNA from tissues was extracted using TransZol (TransGen Biotech, Beijing, China). RNA integrity, concentration and purity were measured by electrophoresis in 1% agarose gel and Nanodrop spectrophotometry (Gel DocTM XR+, Thermo Fisher Scientific Inc., Waltham, MA, USA), respectively. Only complete and clear bands of RNAs representing a 260 nm/280 nm ratio between 1.8 and 2.0 were used in this study. Then, the total RNA for each tissue was reverse-transcribed to cDNA by TransScript One-Step gDNA Removal (TaKaRa, Dalian, China). RNA integrity, concentration and purity were measured by electrophoresis in 1% agarose gels and Nanodrop spectrophotometer (Gel Doc TMXR+, Thermo Fisher Scientific Inc.), respectively. Only complete and clear bands of RNAs representing a 260 nm/280 nm ratio between 1.8 and 2.0 were used in this study.

2.2. cDNA Cloning and Sequence Analysis

PCR amplification was performed using the synthesized cDNA as a template with several pairs of degenerate primers, which were designed using the coding regions of the 3 genes (Table 1). PCR program was as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, Tm °C for 30 s and 72 °C for 30 s, followed by one cycle at 72 °C for 5 min. PCR products were electrophoresed in a 1% (w/v) agarose gel and visualized by staining with Goldview Nucleic Acid Dye (Goldview type I). The DNA fragments were purified using an Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China), further cloned into a pMD19-T vector (volume of 10 µL of 50 ng DNA, 50 ng pMD19-T vector, 5 µL Solution I, incubated at 4 °C overnight). They were finally transformed into Escherichia coli DH5α (Takara) competent cells and grown in Luria–Bertani agar plates with Ampicillin. White colonies were selected. The positive clones were sequenced by Shanghai Sangon Biological Engineering Company (China). Alignments of multiple sequences were obtained by BLAST analyses. DNAMAN software (version 6.0, Lynnon Biosoft, Montreal, QC, Canada) was used to determine the homologies of SMAD4, SMAD5 and SMAD7 proteins from different species, and the phylogenetic trees were constructed using the N-J method of MEGA7. The secondary structure prediction of Tibetan sheep SMAD4, SMAD5 and SMAD7 proteins was performed by the online protein analysis system SOPMA. The specific 3D structures (tertiary structures) were predicted using SWISS-MODEL.
Table 1. Primer information.

| Gene     | Primer Name       | Primer Sequence (5′~3′)                  | Product Length/bp | Tem/◦C |
|----------|-------------------|-----------------------------------------|-------------------|--------|
| SMAD4    | SMAD4-CDS1-F      | CGAATACACACCAAGTAATGATGC                | 648               | 61     |
|          | SMAD4-CDS1-R      | AACACCTCGGTGCTTTTAAACCA                 |                   |        |
| SMAD4    | SMAD4-CDS2-F      | ATCCGACATCCACCAAGTAATGCG                | 641               | 64     |
|          | SMAD4-CDS2-R      | AGTGAGAACAGTTGACGATATGTC                |                   |        |
| SMAD4    | SMAD4-CDS3-F      | CTGGAGGAGATCCTTTTCAAAACCA               | 582               | 65     |
|          | SMAD4-CDS3-R      | TACCGACCCCCAGCCTTTATCTC                 |                   |        |
| SMAD5    | SMAD5-CDS-S       | CACTCTTTTTAAATTCGCGATGC                | 1667              | 55     |
|          | SMAD5-CDS-A       | AATACCTACTCAGCACGTTTC                  |                   |        |
| SMAD7    | SMAD7-CDS-S       | CCCGACTTCTTCTCAGTGTTGT                 | 1408              | 61     |
|          | SMAD7-CDS-A       | TGTCCTGTAACTGACGTTG                    |                   |        |
| SMAD4    | SMAD4-expression-S| AGGGAAGGCTTCAGTGGCTG                   | 71                | 64     |
|          | SMAD4-expression-A| AGGCACCTCAGGCGAGCGG                    |                   |        |
| SMAD5    | SMAD5-expression-S| TCCCCAGCGATGACGGAGCGA                   | 93                | 63     |
|          | SMAD5-expression-A| GCGCTACTACAGGCGACGCG                   |                   |        |
| SMAD7    | SMAD7-expression-S| ATGGTTGCTCTTTCTCAGCT                   | 111               | 64     |
|          | SMAD7-expression-A| CACGGCAGGGTGTCGAGA                     |                   |        |
| GAPDH    | GAPDH-expression-S| GGGATCTCGTGGCAGAACCAGTGA               | 105               | 64     |
|          | GAPDH-expression-A| CCCCTCAGGGAGCAGCGA                     |                   |        |

2.3. Gene Expression Analysis

SMAD4, SMAD5 and SMAD7 primers for real-time quantitative-polymerase chain reaction (qPCR) were designed according to NM_001267886.1, XM_004008815.3 and XM_027961075.1, respectively (Table 1). Real-time PCR was performed at 95 ºC for 15 min, followed by 95 ºC for 10 s, 50 ºC for 32 s for 40 cycles and 72 ºC for 30 s. qPCR was performed using a CFX96 Touch Real-Time PCR (BIO-RAD, Hercules, CA, USA). GAPDH was used as an internal reference to normalize target gene expression. All experiments were performed in triplicate.

2.4. SNP Identification and Genotyping

Using the dbSNP database (http://www.ncbi.nlm.nih.gov/snp, accessed on 4 May 2022), SMAD4, SMAD5 and SMAD7 genes’ SNPs were screened and verified by DNA sequencing. Improved multiplex ligation detection reaction (iMLDR™) was used for genotyping following the instrument operating guidelines.

2.5. Statistical Analysis

The relative gene expression was calculated according to the $2^{-\Delta \Delta Ct}$ method [20], where $\Delta Ct = Ct$ (target gene) − $Ct$ (GAPDH) and $2^{-\Delta \Delta Ct}$ indicates fold change in gene expression of the SMAD4, SMAD5 and SMAD7 genes’ mRNA relative to GAPDH gene. The data were analyzed with statistical software SPSS 19.0. One-way ANOVA was used to calculate differences in expression among different tissues.

The association analysis between genotypes and litter size of ewes was determined according to a general linear model (GLM) program. Based on the characteristics of sheep, the statistical model was as follows:

$$Y_{ij} = \mu + P_i + G_j + I_{PG} + \epsilon_{ij}$$

where $Y_{ij}$ is the phenotypic observation value; $\mu$ is the overall population mean; $P_i$ is the fixed effect of the $i$th parity ($i = 1, 2$ or $3$); $G_j$ is the effect of the $j$th genotype ($j = 1, 2$ or $3$); $I_{PG}$ is the interactive effect of parity and genotype and $\epsilon_{ij}$ represents random error.
3. Results

3.1. Expression Profiles of Genes in Sheep

The RT-qPCR was used to investigate the general tissue distributions of the SMAD4, SMAD5 and SMAD7 genes in Tibetan sheep. The results showed that three genes were widely expressed (Figure 1). They were detected in all thirteen tissues, including heart, liver, spleen, lung, kidney, rumen, duodenum, hypothalamus, pituitary, longissimus dorsi, oviduct, ovary and uterus tissues. SMAD4 gene expression was significantly higher in uterus than in other tissues ($p < 0.05$), and it was significantly higher in spleen and lung than in heart, liver, spleen, lung, kidney, rumen, duodenum, hypothalamus, pituitary, longissimus dorsi, oviduct and ovary tissues ($p < 0.05$). SMAD5 gene expression was the highest in ovary ($p < 0.05$), followed by lung and spleen ($p < 0.05$). SMAD7 gene expression was significantly higher in lung than in other tissues ($p < 0.05$).

![Figure 1. Cont.](image-url)
Figure 1. Expression of SMAD4 (A), SMAD5 (B) and SMAD7 (C) mRNAs in different tissues of Tibetan sheep. The expression of SMAD4, SMAD5 and SMAD7 genes was detected by qPCR, and the GAPDH gene was used as the internal control gene. Each sample was repeated to determine in triplicate, and the data were collected as mean ± standard deviation. Note: different superscripts indicate significant difference (p < 0.05).

3.2. cDNA Cloning and Bioinformatic Analysis of Genes

In this study, 1662 bp of Tibetan sheep SMAD4 gene was cloned. Sequencing analysis showed that the ORF of SMAD4 encodes a protein of 553 amino acids with a predicted molecular weight (MW) of 60.5 kD and isoelectric point (pI) of 7.63. A 1667 bp of SMAD5 sequence was cloned. Subsequent sequencing analysis showed that the ORF of SMAD5 contains 1398 bp and encodes a protein of 465 amino acids with a predicted molecular weight (MW) of 52.20 kD and isoelectric point (pI) of 7.63. After the Tibetan sheep, the SMAD7 gene was cloned and a 1408 bp sequence of SMAD7 was attained, containing 1284 bp ORF. The predicted Tibetan sheep SMAD7 protein consists of 427 amino acids with a molecular weight of 52.20 kD and an isoelectric point of 7.63. The homology analysis of SMAD4 proteins from different species showed that Tibetan sheep have 100%, 100%, 99.8%, 99.6%, 99.3%, 99.3%, 99.3%, 90.0%, 74.8% and 63.6% sequence similarity with the SMAD4 sequences of sheep, yak, cattle, dog, human, pig, chimpanzee, rhesus monkey, house mouse, chicken and zebra, respectively (Figure 2A). The predicted Tibetan sheep SMAD5 protein was aligned with its homologues in other species. The results showed that the sequence of Tibetan sheep SMAD5 has 100.0%, 99.6%, 99.6%, 99.6%, 99.6%, 99.6%, 99.6%, 98.5%, 97.2% and 90.7% identity with its counterparts in sheep, yak, cattle, dog, human, pig, chimpanzee, rhesus monkey, house mouse, chicken and zebra, respectively (Figure 2B). The sequence of SMAD7 proteins in Tibetan sheep has 100.0%, 99.1%, 98.8%, 98.4%, 98.1%, 98.1%, 97.9%, 97.4%, 97.4%, 82.4% and 81.1 identity with its homologues in sheep, yak, pig, human, cattle, rhesus monkey, chimpanzee, house mouse, dog, chicken and zebra, respectively (Figure 2C). The phylogenetic trees were constructed (Figure 3A–C), and they showed that Tibetan sheep SMAD4, SMAD5 and SMAD7 are positioned in one clade with sheep SMAD4, SMAD5 and SMAD7, respectively, indicating that these are the most closely related homologs, followed by yak, cattle and zebra, which has the farthest relationship with Tibetan sheep.
Figure 2. Cont.
Figure 2. Cont.
Figure 2. Amino acid sequence alignments of SMAD4 (A), SMAD5 (B) and SMAD7 (C) form Ovis aries. Bos Taurus, Bos mutus, Homo sapiens, Sus scrofa, Mus musculus, Maylandia zebra, Pan troglodytes, Macaca mulatta, Gallus gallus and Canis lupus familiaris.
Figure 2. Amino acid sequence alignment of SMAD4 (A), SMAD5 (B) and SMAD7 (C) form Ovis aries, Bos Taurus, Bos mutus, Homo sapiens, Sus scrofa, Mus musculus, Maylandia zebra, Pan troglodytes, Macaca mulatta, Gallus gallus and Canis lupus familiaris.

Figure 3. Phylogenetic tree of SMAD4 (A), SMAD5 (B) and SMAD7 (C) of Tibetan sheep, sheep, cattle, yak, house mouse, human, chimpanzee, rhesus monkey, pig, dog, chicken and zebra. Red word represents the sequence of Tibetan sheep.

Figure 4. Phylogenetic tree of SMAD4 (A), SMAD5 (B) and SMAD7 (C) of Tibetan sheep, sheep, cattle, yak, house mouse, human, chimpanzee, rhesus monkey, pig, dog, chicken and zebra. Red word represents the sequence of Tibetan sheep.
The secondary structure prediction of Tibetan sheep SMAD4, SMAD5 and SMAD7 proteins was performed (Figure 4). The results showed that the extension chain of sMAD4 composed of alpha-helix, extended strand, beta turn and random coil accounted for 53.35%, 24.41%, 15.55% and 6.69%, respectively, and the secondary structure components of the SMAD5 contained 57.63% alpha-helix, 21.51% extended strand, 16.99% beta turn and 3.87% random coil, and, for SMAD7 protein, alpha-helix, extended strand, beta turn and random coil were 33.76%, 15.92%, 3.82% and 46.50%, respectively.

![Figure 4. Secondary structure of SMAD4 (A), SMAD5 (B) and SMAD7 (C).](image)

The specific 3D structures (tertiary structure) of Tibetan sheep SMAD4, SMAD5 and SMAD7 domain areas were predicted (Figure 5). The results showed that all the SMAD4, SMAD5 and SMAD7 domain areas constituted curved spiral structures. The 3D struc-
tures of SMAD4, SMAD5 and SMAD7 proteins are consistent with the prediction of secondary structures.

Figure 4. Secondary structure of SMAD4 (A), SMAD5 (B) and SMAD7 (C).

Figure 5. Three-dimensional tertiary structures of SMAD4 (A), SMAD5 (B) and SMAD7 (C) were theoretically predicted using SWISS-MODEL.

3.3. Polymorphism of Genes in Sheep

Sequencing analysis identified two SNPs in SMAD5 and SMAD7 genes in Tibetan sheep. The g.51537A>G was detected in SMAD5, located in exon 3 and belonging to the synonymous type. We genotyped the identified SNPs of g.51537A>G in the SMAD5 gene. Three genotypes were found, namely AA, AG and GG. The genotype frequency of AA, AG and GG was 0.59, 0.35 and 0.06, respectively. The allele frequencies of A and G were 0.76 and 0.24. The observed homozygosity (Ho), heterozygosity (He) and effective allele numbers (Ne) were 0.64, 0.36 and 1.56, respectively. The polymorphism information content (PIC) analysis showed that the SNPs were in moderate diversity (0.25 < PIC < 0.5). The χ² test showed that identified SNP was in Hardy–Weinberg equilibrium. We screened out the g.319C>T in the SMAD7 gene of Tibetan sheep, which was located in exon 3 and belonged to synonymous types. Only two genotypes were identified: CC and CT, with frequencies of 0.94 and 0.06, respectively. The allele frequencies of C and T were 0.97 and 0.03. Ho, He and Ne were 0.95, 0.05 and 1.06, respectively. The PIC analysis indicated the SNPs in low diversity (PIC < 0.25). The identified SNP was in Hardy–Weinberg equilibrium. The distribution, genotype and allele frequencies of the two identified SNPs were presented in Table 2, and the population genetic analysis of the two SNPs was listed in Table 3.
Table 2. In Tibetan sheep, the genotype and allele frequencies of single nucleotide polymorphism loci of SMAD5 and SMAD7.

| Gene | Locus    | SNP Type    | Genotype | Genotype Frequency(no.) | Allele  | Allele Frequency | Exon |
|------|----------|-------------|----------|-------------------------|---------|-----------------|------|
| SMAD5 | g.51537A>G | Synonymous type | AA       | 0.59(256)               | A       | 0.76            | 3    |
|       |          |             | AG       | 0.35(150)               | G       | 0.24            |      |
|       |          |             | GG       | 0.06(27)                |         |                 |      |
| SMAD7 | g.319C>T  | Synonymous type | CC       | 0.94(409)               | C       | 0.97            | 3    |
|       |          |             | CT       | 0.06(24)                | T       | 0.03            |      |

Table 3. Population genetic analysis of single nucleotide polymorphism loci of SMAD5 and SMAD7 in Tibetan sheep.

| Gene | Locus    | Homozygosity (Ho) | Heterozygosity (He) | Effective Allele Numbers (Ne) | Polymorphic Information Content (PIC) | $\chi^2$ Test |
|------|----------|-------------------|---------------------|-------------------------------|---------------------------------------|--------------|
| SMAD5 | g.51537A>G | 0.64              | 0.36                | 1.56                          | 0.30                                  | 0.43         |
| SMAD7 | g.319C>T  | 0.95              | 0.05                | 1.06                          | 0.05                                  | 1.00         |

$df = 1, \chi^2_{0.05} = 3.841; df = 2, \chi^2_{0.05} = 5.991.$

3.4. Association between the SNP Loci of Genes and Litter Size of Tibetan Sheep

Possible associations between the SNPs and litter size were investigated in Tibetan sheep populations (Table 4). This analysis revealed that the different genotypes of SMAD5 and SMAD7 and litter size of Tibetan sheep had a lack of association. No significant difference was found between the litter size of individuals carrying different genotypes in the g.51537A>G site of SMAD5 ($p > 0.05$). Yet, no significant difference was found between the litter size of individuals carrying the CC and CT genotypes in g.319C>T of SMAD7 ($p > 0.05$).

Table 4. Association analysis of single nucleotide polymorphism of SMAD5 and SMAD7 and the litter size of Tibetan sheep.

| Gene | Locus    | Genotype | No. of Individuals | Litter Size       |
|------|----------|----------|-------------------|-------------------|
| SMAD5 | g.51537A>G | AA       | 256               | 1.07 ± 0.26       |
|       |          | AG       | 150               | 1.07 ± 0.27       |
|       |          | GG       | 27                | 1.00 ± 0.00       |
| SMAD7 | g.319C>T  | CC       | 409               | 1.07 ± 0.25       |
|       |          | CT       | 24                | 1.08 ± 0.28       |

4. Discussion

The SMAD family of proteins is the key component of TGF-β family signal transduction in cells [21]. In the present study, we mainly studied three genes: SMAD4, SMAD5 and SMAD7, and focused on the expression profiles, molecular characterization, polymorphism and association with the litter size of these genes in Tibetan sheep. The tissue expression profile revealed that SMAD4, SMAD5 and SMAD7 in Tibetan sheep exhibited broad expression patterns, including their expression in the hypothalamus, hypophysis, heart, liver, spleen, lung, kidney, ovary, oviduct, uterus, rumen, duodenum and longissimus dorsi. The SMAD4, SMAD5 and SMAD7 mRNA levels were unevenly distributed within all thirteen tissues. The SMAD4, SMAD5 and SMAD7 expressions were higher in the lung, spleen and uterus or ovary tissues than in other tissues, and their lowest expressions were in the
longissimus dorsi tissues. The members of the SMAD pathway are closely associated with reproduction and follicular development, regulating ovulation and the litter size of sheep [15]. Xu reported that SMAD4 and SMAD7 mRNAs were present in the ovary and other tissues, including the hypothalamus, pituitary, uterus, heart, liver, spleen, lung, kidney, muscle and oviduct of Hu sheep [22]. Rodriguez et al. uncovered that SMAD1/5/4-mediated signaling is indispensable for the structural and functional integrity of the oviduct and uterus. The loss of SMAD1/5/4 alters the development of the oviduct smooth muscle layer, impeding embryo transit [19]. SMAD4 expression was ubiquitous within the hypothalamus, pituitary, ovary, oviduct and uterus of small tail Han sheep and yaks [23,24]. SMAD5 was expressed in the heart, liver, spleen, lung, kidney, rumen, reticulum, omasum, abomasum, small intestine, large intestine, subcutaneous fat and longissimus dorsi tissues of Qinchuan cattle [6]. SMAD7 was expressed in domestic pigs' brains, cerebellum, hypothalamus, kidney, liver and lung tissues; however, its expression was low in the heart and small intestine tissues and was almost not expressed in muscle tissues [25]. Our experimental results were generally consistent with the above-reported results. SMAD4, SMAD5 and SMAD7 are ubiquitously expressed in nearly all cell types in the body. Therefore, the three genes may play a prominent role as ovary-specific SMAD-interacting proteins in TGF-β superfamily ligand-target-gene selection in the ovary [26].

In this study, the sequences of SMAD4, SMAD5 and SMAD7 in Tibetan sheep were cloned. The multiple sequence alignment analysis indicated that the protein sequences of SMAD4, SMAD5 and SMAD7 in the sheep were similar to those in other mammals (greater than 90% sequence homology for most analyzed species), with the greatest similarity with sheep for SMAD4, SMAD5 and SMAD7, which is consistent with the results of the phylogenetic analysis. These results showed that SMAD4, SMAD5 and SMAD7 are highly conserved among different species.

Understanding protein structures is essential for determining their functions [27]. DNA sequences are translated into amino acid sequences, and researchers determine their functions either by comparing protein sequences with each other or by comparing their specific 3D structures [28]. In the present study, the results showed all the SMAD4, SMAD5 and SMAD7 domain area structures. A clear understanding of their structures will facilitate further research on understanding SMAD functions in the ovary.

In this study, two SNPs from the candidate genes SMAD5 and SMAD7 were screened out to analyze the effects of SNPs on the litter size of Tibetan sheep. The g.51537A>G in SMAD5 and the g.319C>T in SMAD7 were synonymous changes, which could affect gene function by mRNA processing and protein translation and folding [29,30]. Thus, we hypothesized that the synonymous mutations might lead to the change in phenotype and affect litter size of sheep; nevertheless, association analysis revealed that the two SNPs of two genes were not associated with litter size in Tibetan sheep. Whether the two SNPs have no effect on the phenotype trait of litter size in other breeds of sheep remains to be further investigated.

5. Conclusions

In the present study, we showed that SMAD4, SMAD5 and SMAD7 genes were not only expressed in reproduction organs (ovary, uterus and oviduct) but also in other tissues: hypothalamus, hypophysis, heart, liver, spleen, lung, kidney, rumen, duodenum and longissimus dorsi, with the lowest expression in longissimus dorsi. SMAD4, SMAD5 and SMAD7 genes of Tibetan sheep were cloned and subjected to bioinformatics analysis. Based on the results of these experiments, SMAD4, SMAD5 and SMAD7 encoded 533, 465 and 427 amino acids, respectively. Additionally, the homology of three proteins between Tibetan sheep and other species is high. Two synonymous SNPs in the SMAD5 and SMAD7 genes were detected using DNA sequencing and then genotyped using iMLDR™ methods. The association analysis revealed that the two SNPs were not significantly associated with litter size in the Tibetan sheep in this study.
Supplementary Materials: The following is available online at https://www.mdpi.com/article/10.3390/ani12172232/s1, Table S1: Litter size in Tibetan sheep.

Author Contributions: The study was designed by R.S. and J.Z. and R.S., M.L. and N.H. performed all the experimental analyses and prepared all the figures and tables. R.S. and J.Z. analyzed the data and drafted the manuscript. R.S. and M.L. contributed to revisions of the manuscript. J.Z. and X.W. assisted in explaining the results and revised the final version of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by funds of the Science and Technology Planning Program of Qinghai (Science and Technology Department of Qinghai Province) (Grant No. 2020-ZJ-786); Outstanding person of Kunlong: rural revitalization Program (Grant No. (2020)9).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Qinghai University. (protocol code SL-2021027, 03/2021).

Data Availability Statement: All the data analyzed in this study are available from corresponding author.

Conflicts of Interest: All authors declare no conflict of interest.

References
1. Lu, Z.; Yuan, C.; Li, J.; Guo, T.; Yue, Y.; Niu, C.; Liu, J.; Yang, B. Comprehensive analysis of long non-coding RNA and mRNA transcriptomes related to hypoxia adaptation in Tibetan sheep. Front. Vet. Sci. 2020, 8, 801278. [CrossRef] [PubMed]
2. Zhao, P.; He, Z.; Xi, Q.; Sun, H.; Luo, Y.; Wang, J.; Li, S. Variations in HIF-1α contributed to high altitude Hypoxia adaptation via affected oxygen metabolism in Tibetan sheep. Animals 2022, 12, 58. [CrossRef] [PubMed]
3. He, Y.; Chen, W.; Ma, P.; Wei, Y.; Li, R.; Chen, Z.; Li, Y. Molecular detection of anaplasma spp.; babesia spp. and theileria spp. in yaks (Bos grunniens) and Tibetan sheep (Ovis aries) on the Qinghai-Tibetan Plateau, China. Parasites Vectors 2021, 14, 613. [CrossRef] [PubMed]
4. Lv, W.; Liu, X.; Sha, Y.; Shi, H.; Wei, H.; Luo, Y.; Pu, X. Rumen fermentation-microbiota-host gene expression interactions to reveal the adaptability of Tibetan sheep in different periods. Animals 2021, 11, 3529. [CrossRef]
5. Frick, C.L.; Yarka, C.; Nunns, H.; Goentoro, L. Sensing relative signal in the Tgf-β/Smad signaling pathway. Proc. Natl. Acad. Sci. USA 2017, 114, E2975–E2982. [CrossRef]
6. Kaivo-Oja, N.; Jeffery, L.A.; Ritvos, O.; Mottershead, D.G. Smad signalling in the ovary. Reprod. Biol. Endocrinol. 2006, 4, 21. [CrossRef]
7. Wang, R.N.; Green, J.; Wang, Z.; Deng, Y.; Qiao, M.; Peabody, M.; Shi, L.L. Bone morphogenetic protein (BMP) signaling in development and human diseases. Genes Dis. 2014, 1, 87–105. [CrossRef]
8. Yadin, D.; Knaus, P.; Mueller, T.D. Structural insights into BMP receptors: Specificity, activation and inhibition. Cytokine Growth Factor Rev. 2016, 27, 13–34. [CrossRef]
9. Gaarenstroom, T.; Hill, C.S. TGF-β signaling to chromatin: How Smads regulate transcription during self-renewal and differentiation. Semin. Cell Dev. Biol. 2014, 32, 107–118. [CrossRef] [PubMed]
10. Brorson, M.; Hougaard, D.; Nielsen, J.; Tornehave, D.; Larsson, L.-I. Expression of SMAD signal transduction molecules in the pancreas. Histochem. Cell Biol. 2001, 116, 263–267. [CrossRef]
11. Song, B.; Estrada, K.D.; Lyons, K.M. Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev. 2009, 20, 379–388. [CrossRef] [PubMed]
12. Wang, Q.Z.; Su, R.; Lv, X.Y.; Gao, W.; Chen, L.; Bao, J.J.; Sun, W. Analysis of the spatiotemporal expression of major genes in the TGF-β/Smad signaling pathway and correlation analysis using Hu sheep muscle tissue. Genet. Mol. Res. GMR 2016, 15, 15028133. [CrossRef] [PubMed]
13. Zhang, Y.; Feng, X.H.; Wu, R.Y.; Derynck, R. Receptor-associated mad homologues synergize as effectors of the TGF-β response. Nature 1996, 383, 168–172. [CrossRef]
14. Massagué, J.; Wotton, D. Transcriptional control by the TGF/Smad signalling system. EMBO J. 2000, 19, 4360–4369. [CrossRef]
15. Rodriguez, A.; Tripuran, S.K.; Burton, J.C.; Clementi, C.; Larina, I.; Pangas, S.A. Faculty Opinions recommendation of SMAD signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy in mice. Biol. Reprod. 2016, 95, 44. [CrossRef]
16. Sharum, I. Regulation of TGF/Smad Signalling during Early Follicle Development in the Mouse Ovary. Ph.D. Thesis, The University of Sheffield, Sheffield, UK, 2016.
17. Chu, M.X.; Liu, Z.H.; Jiao, C.L.; He, Y.Q.; Fang, L.; Ye, S.C.; Wang, J.Y. Mutations in BMPR-IB and BMP-15 genes are associated with litter size in Small Tailed Han sheep (Ovis aries). J. Anim. Sci. 2007, 85, 598–603. [CrossRef]
18. Bodin, L.; Di Pasquale, E.; Fabre, S.; Bontoux, M.; Monget, P.; Persani, L.; Mulsant, P. A Novel Mutation in the Bone Morphogenetic Protein 15 Gene Causing Defective Protein Secretion Is Associated with Both Increased Ovulation Rate and Sterility in Lacaune Sheep. *Endocrinology* 2007, 148, 393–400. [CrossRef]

19. Hanrahan, J.P.; Gregan, S.M.; Mulsant, P.; Mullen, M.; Davis, G.H.; Powell, R.; Galloway, S.M. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biol. Reprod.* 2004, 70, 900–909. [CrossRef]

20. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−ΔΔC_T) Method. *Methods* 2001, 25, 402–408. [CrossRef]

21. Xu, Y.F. Study on the Relationship between the Expression of Genes Related to Smad Signal Transduction Pathway and High Fertility of Hu Sheep. Ph.D. Thesis, Nanjing Agricultural University, Nanjing, China, 2010.

22. Li, Z.F.; Sun, W.; Chu, M.X. Expression analysis of TGF-β pathway-related genes in sheep gonad axis tissues. *J. Northeast. Agric. Univ.* 2021, 52, 43–49.

23. Wang, Y.H.; Xu, Y.F.; Niu, J.Q. Tissue expression of Smad4 gene mRNA in yak. *Chin. J. Anim. Sci. Vet. Med.* 2017, 48, 1882–1891.

24. Ning, Y. Regulation of SMAD5 Gene on Proliferation and Differentiation of Qinchuan Cattle Myoblasts and Correlation Analysis with Beef Quality Traits. Ph.D. Thesis, Northwest A&F University, Xianyang, China, 2019.

25. Chen, Q.R.; Li, J.; Wang, Y.J. Cloning, sequence analysis and tissue expression mapping of porcine Smad7 and Smad9 genes. *J. Sichuan Univ. Nat. Sci. Ed.* 2015, 52, 157–162.

26. Tanekhy, M.; Kono, T.; Sakai, M. Cloning, characterization, and expression analysis of Toll-like receptor-7 cDNA from common carp, *Cyprinus carpio* L. *Comp. Biochem. Physiol. Part D Genom. Proteom.* 2010, 5, 245–255. [CrossRef] [PubMed]

27. Ungerer, M.C.; Johnson, L.C.; Herman, M.A. Ecological genomics: Understanding gene and genome function in the natural environment. *Heredity* 2007, 100, 178–183. [CrossRef] [PubMed]

28. Diederichs, S.; Bartsch, L.; Berkmann, J.C.; Fröse, K.; Heitmann, J.; Hoppe, C.; Iggien, D.; Jazmati, D.; Karschnia, P.; Linsenmeier, M.; et al. The dark matter of the cancer genome: Aberrations in regulatory elements, untranslated regions, splice sites, non-coding RNA and synonymous mutations. *EMBO Mol. Med.* 2016, 8, 442–457. [CrossRef]

29. Sharma, Y.; Miladi, M.; Dukare, S.; Boulay, K.; Caudron-Herger, M.; Groß, M.; Backofen, R.; Diederichs, S. A pan-cancer analysis of synonymous mutations. *Nat. Commun.* 2019, 10, 2569. [CrossRef]

30. Lin, C.; Li, F.; Zhang, X.; Zhang, D.; Li, X.; Zhang, Y.; Wang, W. Expression and polymorphisms of CD8B gene and its associations with body weight and size traits in sheep. *Anim. Biotechnol.* 2021, 1–9, Online ahead of print. [CrossRef]