RESEARCH ARTICLE

Identification of *MEF2B* and *TRHDE* Gene Polymorphisms Related to Growth Traits in a New Ujumqin Sheep Population

Li Zhang‡*, Xiaomeng Ma‡*, Junli Xuan, Huihua Wang, Zehu Yuan, Mingming Wu, Ruizao Liu, Caizhu Zhu, Caihong Wei, Fuping Zhao, Lixin Du*

Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

☯ These authors contributed equally to this work.
‡ These authors are co-first authors on this work.
* zhangli07@caas.cn (LZ); lxdu@263.net (LD)

Abstract

2 SNPs were discovered in our previous genome-wide association study (GWAS): s58995.1 (rs420767326 A>G) in *MEF2B* gene and OAR3_115712045.1 (rs401775061 A>C) in *TRHDE* gene, which were significantly associated with post-weaning gain in sheep. Herein, we performed a replication experiment to investigate single nucleotide polymorphisms (SNPs) within the *MEF2B* and *TRHDE* gene exons, the 5′ untranslated regions (within 1000bp), the 3′ untranslated regions (within 1000bp) and their associations with Ujumqin sheep growth traits in 4-month age and 6-month age, respectively. Finally, 3 SNPs were selected to be investigated including 1 SNP in 3′ untranslated regions in *MEF2B* gene (rs417014745 A>G) and 2 SNPs in *TRHDE* gene (rs426980328 T>C and rs430810656 G>A). The χ² test showed all the 3 variations were in Hardy–Weinberg equilibrium (P>0.05) status. Association analysis suggested that rs426980328 T>C was significantly associated with body weight and chest girth in 4-month age (P<0.05). rs430810656 G>A exhibited extremely significant association with body weight and chest girth in 4-month age (P<0.01). rs417014745 A>G was extremely significantly associated with body weight and chest girth in 4-month age and chest girth in 6-month age (P<0.01), and it was also significantly associated with body weight in 6-month age (P<0.05). Combined effect analysis indicated significant associations between the combinations of rs426980328-rs417014745, rs430810656-rs417014745 and several growth traits (P<0.05). These results suggested *MEF2B* and *TRHDE* genes affected growth traits in Ujumqin sheep and the combination effect of the two genes also played a significant effective role. These SNPs might have potential value as genetic markers for growth traits and it could be used in Ujumqin sheep breeding in future. Further studies are necessary to confirm our findings.
Introduction

Growth traits are significant economic traits in sheep, advances of molecular genetics have opened appealing perspectives for the identification of functional genes through genomics technology, SNPs have been widely used for QTL detection and localization for complex traits in many species[1]. Abundant SNPs have been discovered by using modern sequencing technologies and bioinformatics tools, which allowed us to better understand the association between genomic variations and different traits. Our previous GWAS results showed that 2 SNPs: s58995.1(rs420767326 A>G) in MEF2B gene (Genbank XM_015095748) and OAR3_115712045.1 (rs401775061 A>C) in TRHDE gene (Genbank XM_004007369) were significantly associated with post-weaning gain in sheep [2]. MEF2B gene is one of the myocyte enhancer factor-2 gene family members (MEF2A, MEF2B, MEF2C and MEF2D) in vertebrates, which play a significant role in the regulation of muscular growth and development. MEF2B gene had a lower homology in different species, and it was also distinct from the rest gene family members. It was located on chromosome 5 and widely expressed in various organizations [3, 4], and it is crucial to cell development, embryonic development, the differentiation and regeneration of muscle tissue, the differentiation of nervous system and liver fibrosis. Expression of MEF2B mRNA and myocyte fibroblast proteins were higher in spindle cells than that in cobblestone cells. Knockdown of MEF2B in a cobblestone cell line abolished EGF-induced upregulation of MEF2, including vimentin and non-muscle caldesmon proteins, yet enhanced basal expression of mesenchymal vimentin and fibronectin[5]. MEF2B mutations could be linked to CREBBP and EP300 mutations, and to recurrent Y641 mutations in EZH2 [6]. Study revealed MEF2B-Nox1 signaling was critical for stretch-induced phenotypic modulation of vascular smooth muscle cells [7]. In mouse, MEF2B was co-expressed with MEF2C throughout the early stages of cardiogenesis, it was up-regulated in MEF2C mutant mouse, consistent with the possibility that it may partially substitute for MEF2C[8]. MEF2B also had an impact on the expression of MEF2 target genes owing to the reduced acetylation of nucleosomes near these genes. As a novel candidate gene for growth traits detected by GWAS, however, no study was found on associations between TRHDE gene and sheep growth traits. TRHDE is thyrotropin-releasing hormone degrading enzyme which encodes a member of the peptidase M1 family. It is reported TRHDE gene was associated with neuroglioma in human[9], OAR3_115712045.1 identified by our GWAS analysis was located within a QTL which was found to affect internal fat amount in Merino sheep[10]. TRHDE protein was an extracellular peptidase that specifically cleaved and inactivated the neuropeptide thyrotropin-releasing hormone, which regulated appetite and metabolism [11, 12]. Knockdown of TRHDE gene in drosophila sensory neurons resulted in altered impaired nociception and cellular morphology[13]. TRHDE gene in goat was identified by the multiple genomic signatures of selection study, the results showed it could directly or indirectly influence traits for adaptation to hot arid environments[14]. Based on these previous studies, further study on MEF2B and TRHDE genes were taken to investigate the relationship between SNPs within them and Ujumqin sheep growth traits, respectively, as well as their combined effect on Ujumqin sheep growth traits, in order to validate the GWAS results and screen casual genetic variants as genetic markers that benefit for the sheep growth performance in an independent sheep population.

Experimental Section

Ethic statement

The methods of this study were conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China). All
experimental protocols were approved by the Science Research Department (in charge of animal welfare) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (CAAS) (Beijing, China).

**Phenotypic data collection and DNA extraction**

Totally of 343 Ujumqin sheep were selected as experimental population, including 218 samples (111 rams and 107 ewes) collected at Dong Ujumqin Banner (Inner Mongolia Autonomous Region, China, N45° 30', E116° 57') and the remaining 125 samples (54 rams and 71 ewes) were from Zhonglan Banner (Inner Mongolia Autonomous Region, China, N42°14', E115°30'). The feeding was in line with the Instructive Notions with Respect to Caring for Laboratory Animals that was published in 2006 by the Science and Technology Department of China (Approval No. S20072911). We collected 343 blood samples and each was 25 mL in 4-month age. We also measured the growth traits including body weight, body height, body length, chest girth, shin circumference in 4-month age and body weight, body height, body length, chest girth, shin circumference, chest width and chest depth in 6-month age. 343 genomic DNA were isolated from blood samples using a TIANamp Genomic DNA kit (TianGen, Beijing, China) according to the manufacturer’s instructions finally. In order to identify potential SNPs, a DNA pool (50 ng/ul/sheep) was constructed using 30 blood samples and primers were designed with Primer3 web based Program (v.0.4.0.) (http://bioinfo.ut.ee/primer3-0.4.0/), to amplify all the exons and 1000 bp of 5’-UTR and 3’-UTR based on the reference sequence. The Primer pairs used for scanning SNPs were in Table 1.

**SNP detection and genotyping**

The pooled DNA were amplified with a programmable thermal cycler, (Bio-Rad, Thermal Cycler, Mexico). The PCR reaction mixture consisted of 25 μL, containing 2 μL pooled DNA, 1 μL of 5’ primers, 1 μL of 3’ primers, 12.5 μL 2× Taq PCR MasterMix, and 8.5 μL double-distilled water. The PCR reaction protocol was 5 min at 94°C for initial denaturing followed by 35 cycles at 94°C for 30 s; Tm for 30 s; 72°C for 30 s; a final extension at 72°C for 10 min. The PCR products were stored at 4°C. Then PCR products were detected via 2% agarose gel and visualizing under UV rays to confirm the amplification. The desired product were sequenced by Tianyi-Huiyuan Gene Sequencing Co.Ltd (Beijing, China). Then the results of the sequencing were aligned with standard sequence and the SNPs were detected using DNAMAM 5.2.10 software (Lynnon BioSoft, Quebec, ONT, Canada) and Chromas 2 software. Considering the compatibility of the primers used to extend the SNPs genotyped in the next MALDI-TOF assay process, 3 SNPs were then genotyped in 343 Ujumqin sheep using MALDI-TOF assay (Mass ARRAY; Sequenom Inc, San Diego, CA, US).

**Statistical analysis**

Microsoft Excel 2013 (Microsoft Inc, Redmond, WA, USA) was used to calculate the allele frequencies, polymorphic information content (PIC), heterozygosity (He), and homozygosity (Ho). SPSS 22.0 software (SPSS Inc, Chicago, IL, USA) was used to analyze the associations between the SNPs and growth traits of the sheep. The Hardy–Weinberg equilibrium were tested for each site through the chi-square test. The following model was used to check the single gene effect on the sheep growth traits:

\[ Y_i = \mu + G_i + p_i + m_i + \epsilon_i \]

where \( Y_i \) was the trait measured in individual, \( \mu \) was the overall mean, \( G_i \) was a fixed effect corresponding to the genotype of polymorphisms, \( p_i \) was the gender effect, \( m_i \) was the farm effect,
Table 1. Primer pairs used for scanning SNPs within sheep MEF2B and TRHDE gene.

| PRIMER | Primer sequence(5'-3') | Annealing Temperature(°C) | Product (bp) | Extend region |
|--------|------------------------|---------------------------|--------------|--------------|
| TRH-2F | TGGCGAAATATGCATGAAGT    | 59.3                      | 595          | Exon 2       |
| TRH-2R | CCTCTCCGACCTGGAACCA    |                          |              |              |
| TRH-3F | CAGCTGCTTTGATATATGAACA | 59                        | 300          | Exon 3       |
| TRH-3R | TCCTTACACAAACATTAGGGA   |                          |              |              |
| TRH-4F | ACTGATGTTTGAAGAGCTTTG   | 60.3                      | 226          | Exon 4       |
| TRH-4R | ACTTACACCTGTACCTTTTCTCA |                          |              |              |
| TRH-5F | GAGTGAGTTTATTCTGATGCCA | 59.5                      | 232          | Exon 5       |
| TRH-5R | GAGTGATTTTATCTGATGCCA   |                          |              |              |
| TRH-6F | ACCCTGATGATTTTACCCACC  | 59.6                      | 229          | Exon 6       |
| TRH-6R | AGAATTTGCGCAGGAGAAGTA   |                          |              |              |
| TRH-7F | TGCAGAACCAGCTTTTGAA     | 60.6                      | 683          | Exon 7       |
| TRH-7R | TCGATTTCCAACAGCTGTGAA   |                          |              |              |
| TRH-8F | ACTGAAATATGCCTGATTTTG   | 58.8                      | 249          | Exon 8       |
| TRH-8R | GCATACAGAAGAGACGAAAGCT  |                          |              |              |
| TRH-9F | TGTGATGCTGTCTCCATTTT    | 58.3                      | 840          | Exon 9       |
| TRH-9R | ACACTTCTGATGATGCTGCAC   |                          |              |              |
| TRH-10F| CTCTTCACATTAGTACTGGGG   | 59.2                      | 266          | Exon 10      |
| TRH-10R| TGAAGACCAGAAGTGTGACAG   |                          |              |              |
| TRH-11F| CCTCTTACCTCTGAAAAATGCTGT| 59.8                      | 533          | Exon 11-12   |
| TRH-11R| TGATGCAGCTCTCCTCGC      |                          |              |              |
| TRH-13F| GCCCTCATCATGATATTTTTGCC  | 60.3                     | 350          | Exon 13      |
| TRH-13R| TGCATTTAGTCTAGATGCA     |                          |              |              |
| TRH-14F| TAATTTGCTGCAAGCAAGTGA   | 59                        | 824          | Exon 14-15   |
| TRH-14R| TCTGCTCTTGTTCCTCCAGT    |                          |              |              |
| TRH-16F| ACTGACTAAGCCGACATTGTC   | 59.8                      | 999          | Exon 16-17   |
| TRH-16R| TCGGATCTGTCTCTCCACACA   |                          |              |              |
| TRH-18F| CACAGCCTGAGACACGGTTA    | 59                        | 295          | Exon 18      |
| TRH-18R| ACTGGAAGAATATGTTGAAAGTC |                          |              |              |
| TRH-19F| TGTCGTGTCTGTTCATTCT    | 60                        | 247          | Exon 19      |
| TRH-19R| TTCCTCTACCATCACAAGCAAGT |                          |              |              |
| ME-5'-1F| TGGTGTTGTTGGTGTTGTTGTT  | 57.9                      | 545          | 5'-UTR 500bp |
| ME-5'-1R| CAGCAGGGCGACAGTATCT    |                          |              |              |
| ME-5'-2F| AGTCTGCTGTCTGAGCTGATGA | 58.2                      | 600          | 5'-UTR500-1000bp |
| ME-5'-2R| TGGCTCCAGAATGCTCTGAGAT |                          |              |              |
| MEF2B-1F| TCTGACTCCACTGCTGTTT    | 60.1                      | 234          | Exon 1       |
| MEF2B-1R| CAGGCTGCTTGTCTCTCTG    |                          |              |              |
| MEF2B-2F| CCTCTCAACACCCTGAAACT   | 58.8                      | 335          | Exon 2       |
| MEF2B-2R| AACGGAACGCTCTCATTTTTT  |                          |              |              |
| MEF2B-3F| CTGCTCCTGCTCTCTCCACAG  | 59.9                      | 242          | Exon 3       |
| MEF2B-3R| AGTCTCTGCTCTCTGACTCAG  |                          |              |              |
| MEF2B-5F| CAGCCTCTCCTCTCTCTCTTT  | 61                        | 360          | Exon 5       |
| MEF2B-5R| GTGAGAACCAGGACCATTT    |                          |              |              |
| ME-3'-1F| TCCCTTACCTTGTGCTCTC   | 58.6                      | 568          | 3'-UTR500bp  |
| ME-3'-1R| CAGCATTACACTCAGGATCC   |                          |              |              |
| ME-3'-2F| GCCTGGCTATTACCCCTCCA  | 60.6                      | 664          | 3'-UTR500-1000bp |
| ME-3'-2R| TACAGAAAGGCTGCTTGAGAG  |                          |              |              |

doi:10.1371/journal.pone.0159504.t001
and $e_i$ was a random residual effect. The model of association analysis between combined genotypes and growth traits was as follow:

$$Y_i = \mu + G_i + p_i + m_i + e_i$$

$Y_i$ was the trait measured in individual, $\mu$ was the overall mean, $G_i$ was the combined genotype effect of the 2 genes, $p_i$ was the gender effect, $m_i$ was the farm effect, and $e_i$ was a random residual effect.

Significant differences among genotypes and their corresponding mean growth trait values were calculated using Duncan’s multiple-range test. $P<0.05$ was considered significant. $P<0.01$ was considered extremely significant. Numbers less than 6 of the combined genotypes were not included in the association analysis.

**Results**

**SNP detecting and genotyping**

DNA pool sequencing showed totally 2 SNPs were identified in $MEF2B$ gene and 5 SNPs in the $TRHDE$ gene, considering the compatibility of the primers used to extend the single site in the next MALDI-TOF assay process, rs417014745 A>G in the 3'-UTR of $MEF2B$ gene and 2 SNPs in the exon2 (rs426980328 T>C), exon14 (rs430810656 G>A) respectively were selected to be genotyped in a new Ujumqin sheep validation group including 343 samples. All the 3 SNPs were classed into 3 genotypes in the experimental population.

**Genetic parameters calculation**

Genetic parameters were analyzed in the experimental population (Table 2), the results showed all the sites were in Hardy-Weinberg equilibrium status ($P>0.05$). rs426980328 and rs417014745 were in low polymorphic information content status (PIC<0.25), while rs430810656 was in moderate polymorphic information content status (0.25<PIC<0.5). The homozygosity of the 3 sites were higher than the heterozygosity.

**Association of single nucleotide polymorphism with growth traits in sheep**

The results of single nucleotide polymorphism association analysis revealed the rs417014745 that located in 3'-UTR of $MEF2B$ gene was extremely significantly associated with body weight, chest girth in 4-month age ($P<0.01$) and chest girth in 6-month age ($P<0.01$), it was also significantly associated with body weight in 6-month age ($P<0.05$). On rs417014745, sheep carrying AA genotype were significantly better than the GG or GA carriers on body weight and

| Locus       | Genotype | Genotypic Frequency | Allele | Allelic frequency | He  | Ho  | PIC  | H-W test (P value) |
|-------------|----------|---------------------|--------|-------------------|-----|-----|------|-------------------|
| rs426980328 | CC(291)  | 0.86                | C      | 0.93              | 0.14| 0.86| 0.13 | 0.7845            |
|             | CT(44)   | 0.13                | T      | 0.07              |     |     |      |                   |
|             | TT(2)    | 0.01                |        |                   |     |     |      |                   |
| rs430810656 | AA(172)  | 0.51                | G      | 0.27              | 0.39| 0.61| 0.31 | 0.0826            |
|             | GA(144)  | 0.43                | A      | 0.73              |     |     |      |                   |
|             | GG(18)   | 0.06                |        |                   |     |     |      |                   |
| rs41701474  | GG(242)  | 0.72                | G      | 0.84              | 0.27| 0.73| 0.24 | 0.5152            |
|             | GA(85)   | 0.25                | A      | 0.16              |     |     |      |                   |
|             | AA(9)    | 0.03                |        |                   |     |     |      |                   |

doi:10.1371/journal.pone.0159504.t002
chest girth in 4-month age. As to the growth traits in 6-month age, sheep with AA genotype performed extremely significantly better than the GG or GA genotype carriers on body weight, the allele A might be the superior allele on rs417014745.

rs426980328 that located on exon2 of TRHDE gene was significantly associated with body weight, chest girth in 4-month age ($P<0.05$). On rs426980328, sheep with TT genotype were significantly greater than those CC or CT genotype carriers on body weight and chest girth in 4-month age, which could be observed with overall 3.91kg heavier and 3.21cm bigger from phenotype, respectively. Individuals with homozygous genotype TT on rs426980328 showed better growth performance on body weight, body length, chest girth and chest width in 6-month age as well. The association analysis results were in Tables 3 and 4.

**Association of combined markers with growth traits in sheep**

When combined the SNPs in MEF2B and TRHDE genes respectively, highly significant interactions were observed, the combination of the rs426980328 in TRHDE gene and rs417014745 in MEF2B gene showed significant association with body weight and chest girth in 4-month age ($P<0.05$), and chest girth in 6-month age($P<0.05$). Sheep with the combined genotype CCAA had higher body weight, body length, chest girth in 4-month age and higher body weight, chest girth in 6-month age than the individuals with the other 4 combined genotypes. CCAA was the preponderant genotype. The combination of rs430810656 and rs417014745 exhibited significantly association with body weight and chest girth in 4-month age ($P<0.05$), the rs430810656-rs417014745 combined genotypes were also significantly associated with chest girth in 6-month age ($P<0.05$), GGAA was the preponderant combined genotype. The association analysis results were in Tables 5 and 6.
Table 4. Associations of single marker of the genes with 6-month age growth traits. Data marked with the lower letters within the same rank mean significant difference (P<0.05), different uppercase letters indicate extremely significant difference (P<0.01).

| Locus     | Genotype | Body weight (kg) | Body height (cm) | Body length (cm) | Chest girth (cm) | Shin circumference (cm) | Chest width (cm) | Chest depth (cm) |
|-----------|----------|------------------|------------------|------------------|-----------------|------------------------|-----------------|-----------------|
| rs426980328 |          |                  |                  |                  |                 |                        |                 |                 |
| CC        | 32.43±5.67 | 60.14±3.22       | 64.16±4.01       | 81.65±3.33       | 7.56±0.43       | 14.89±2.54            | 31.20±4.15      |                 |
| CT        | 33.06±5.42 | 60.28±3.25       | 64.82±3.63       | 81.55±6.80       | 7.61±0.44       | 15.21±3.29            | 31.47±2.69      |                 |
| TT        | 36.00±4.94 | 60.00±1.41       | 66.00±1.24       | 83.50±3.53       | 7.80±0.28       | 15.00±2.22            | 30.50±2.07      |                 |
| P         | 0.171     | 0.950            | 0.179            | 0.460            | 0.539           | 0.805                  | 0.972           |                 |
| rs430810656 |          |                  |                  |                  |                 |                        |                 |                 |
| AA        | 33.22±5.39 | 60.36±3.2        | 64.63±4.01       | 82.68±5.81       | 7.59±0.41       | 15.20±2.18            | 31.42±2.72      |                 |
| GG        | 29.84±4.86 | 58.80±3.62       | 61.93±3.61       | 77.80±6.60       | 7.48±0.41       | 13.60±2.09            | 29.80±2.83      |                 |
| GA        | 31.95±5.82 | 60.05±3.16       | 64.04±3.70       | 80.83±6.73       | 7.53±0.45       | 14.79±3.12            | 31.14±5.16      |                 |
| P         | 0.907     | 0.911            | 0.551            | 0.810            | 0.753           | 0.794                  | 0.933           |                 |
| rs417014745 |          |                  |                  |                  |                 |                        |                 |                 |
| AA        | 36.26±4.52 | 61.25±2.76       | 66.00±4.00       | 85.25±7.59A      | 7.75±0.23       | 15.5±2.56             | 30.37±2.44      |                 |
| GG        | 32.41±5.73 | 60.09±3.28       | 64.21±4.11       | 81.27±6.45B      | 7.54±0.45       | 15.03±2.67            | 31.07±2.82      |                 |
| GA        | 32.50±5.35 | 60.15±3.02       | 64.42±3.36       | 82.35±5.96B      | 7.62±0.38       | 14.57±1.83            | 31.78±2.43      |                 |
| P         | 0.037     | 0.698            | 0.568            | 0.001            | 0.058           | 0.311                  | 0.369           |                 |

doi:10.1371/journal.pone.0159504.i004

Table 5. Combined genotype effect analysis of rs426980328- rs417014745 and rs430810656- rs417014745 with 4-month age growth traits. Data marked with the lower letters within the same rank mean significant difference (P<0.05), different uppercase letters indicate extremely significant difference (P<0.01).

| Loci       | Genotype | Body weight(kg) | Body height(cm) | Body length(cm) | Chest girth(cm) | Shin circumference(cm) |
|------------|----------|-----------------|-----------------|-----------------|-----------------|------------------------|
| rs426980328- rs417014745 |          |                  |                  |                  |                 |                        |                 |
| CCAA       | 32.18±5.02 | 60.75±2.81       | 62.62±2.61       | 79.87±4.43A     | 8.00±0.26       |                        |                 |
| CCGA       | 28.75±5.60 | 59.17±3.01       | 61.94±2.20       | 76.74±5.93Bc    | 7.75±0.50       |                        |                 |
| CTGA       | 31.55±2.11 | 59.05±1.77       | 61.72±1.56       | 79.05±3.04Ab    | 8.00±0.25       |                        |                 |
| CCGG       | 28.16±5.31 | 58.96±2.31       | 61.50±2.46       | 75.83±6.13C     | 7.70±0.44       |                        |                 |
| CTGG       | 29.93±5.81 | 59.98±2.33       | 62.26±3.37       | 77.04±5.96Bc    | 8.73±0.61       |                        |                 |
| P          | 0.009     | 0.195            | 0.364            | 0.034           | 0.483           |                        |                 |
| rs430810656- rs417014745 |          |                  |                  |                  |                 |                        |                 |
| GAAA       | 29.85±5.31 | 59.32±3.21       | 62.10±2.10       | 78.04±5.66Ab    | 7.77±0.54       |                        |                 |
| GAGA       | 28.83±5.16 | 59.12±2.20       | 61.68±2.06       | 76.37±5.61Ab    | 7.80±0.40       |                        |                 |
| GAGG       | 26.68±5.71 | 58.85±2.79       | 61.35±1.37       | 74.35±4.24Ab    | 7.78±0.48       |                        |                 |
| GGAA       | 29.51±5.11 | 59.22±2.24       | 61.86±2.36       | 77.04±5.81Ab    | 7.75±0.47       |                        |                 |
| GGGA       | 27.60±5.48 | 58.99±2.35       | 61.30±2.63       | 75.21±6.19Abc   | 7.68±0.47       |                        |                 |
| GGGG       | 25.14±5.80 | 59.13±2.84       | 60.72±2.53       | 73.16±7.29C     | 7.72±0.14       |                        |                 |
| P          | 0.025     | 0.613            | 0.502            | 0.027           | 0.496           |                        |                 |

doi:10.1371/journal.pone.0159504.i005

Discussion

To validate the effect of promising SNPs identified by our previous genome-wide association study, we successfully genotyped 3 SNPs in a new validation group of 343 Urumqi sheep with growth traits measured at 4-month age and 6-month age respectively. It showed all the 3 sites were in Hardy-Weinberg equilibrium (P>0.05) status, this might be owe to the closed environment where the Urumqi sheep lived, which indicated that the sheep were not be extensively artificially selected. According to the standard of polymorphic information content, rs426980328 and rs417014745 were in low polymorphic information content status (PIC<0.25) and the heterozygosity of the 2 sites were also very low, indicating the genetic variation of these sites were not high, this might give rise to the experimental population were not
rs426980328- rs417014745 and rs430810656- rs417014745 with 6-month age growth traits. Data marked with the lower letters within the same rank mean significant difference (P<0.05), different uppercase letters indicate extremely significant difference (P<0.01).

| Loci               | Genotype | Body weight (kg) | Body height (cm) | Body length (cm) | Chest girth (cm) | Shin circumference (cm) | Chest width (cm) | Chest depth (cm) |
|--------------------|----------|------------------|-------------------|------------------|------------------|-------------------------|------------------|-----------------|
|                    |          |                  |                   |                  |                  |                         |                  |                 |
| rs426980328-        |          |                  |                   |                  |                  |                         |                  |                 |
| rs417014745        | CCAA     | 36.98±4.36       | 61.85±2.34        | 64.71±4.23       | 86.14±7.73       | 7.78±0.22               | 15.85±2.54       | 30.42±2.63      |
|                    | CCGA     | 31.94±5.32       | 60.17±2.98        | 64.20±3.29       | 82.20±6.13       | 7.62±0.38               | 14.43±1.92       | 31.78±6.63      |
|                    | CCGG     | 32.37±5.73       | 60.06±3.32        | 64.08±4.21       | 81.23±6.29       | 7.52±0.45               | 15.02±2.73       | 30.99±2.79      |
|                    | CTGA     | 34.62±3.53       | 60.00±3.54        | 64.75±3.10       | 82.50±5.34       | 7.48±0.31               | 15.50±0.92       | 31.62±1.50      |
|                    | CTGG     | 32.70±5.89       | 60.48±3.22        | 64.77±3.37       | 81.37±7.32       | 7.65±0.47               | 15.20±3.73       | 31.48±2.99      |
|                    | GAAA     | 32.81±5.05       | 60.19±2.80        | 64.50±2.99       | 83.63±6.11       | 7.63±0.36               | 14.69±1.93       | 31.58±2.51      |
|                    | GGAA     | 33.29±5.55       | 60.40±3.37        | 64.58±4.34       | 82.44±5.72       | 7.57±0.43               | 15.42±2.23       | 31.46±3.04      |
|                    | GAGA     | 32.23±5.35       | 60.37±3.14        | 64.46±3.42       | 81.09±5.72       | 7.60±0.37               | 14.46±1.60       | 32.28±3.09      |
|                    | GAGG     | 31.62±5.94       | 59.87±3.15        | 63.95±3.77       | 80.35±6.82       | 7.50±0.48               | 14.81±3.51       | 30.71±2.77      |
|                    | GGGG     | 30.42±5.65       | 58.28±2.92        | 62.57±3.50       | 80.57±5.31       | 7.50±0.41               | 14.28±2.49       | 30.00±2.00      |
|                    | GGGG     | 29.33±4.38       | 59.25±4.30        | 61.37±3.85       | 75.37±6.96       | 7.46±0.45               | 13.00±1.60       | 29.62±3.54      |
|                    | P        | 0.127            | 0.558             | 0.886            | 0.010            | 0.055                   | 0.365            | 0.622           |

doi:10.1371/journal.pone.0159504.t006

Identification of MEF2B and TRHDE Gene Polymorphisms Related to Growth Traits in a Sheep Population

highly selected, prompting that we could strengthen the artificial selection. While, the rs430810656 site was in moderate polymorphic information content status (0.25<PIC<0.5) and the genetic variation of rs430810656 was high, indicating that we could obtain more selection effect.

No research was found before we carried a relationship analysis between variations in MEF2B, TRHDE genes and growth traits in Urumqin sheep. The 3 SNPs were proved to be significantly associated with several growth traits in a new population, implying these SNPs might be linked to a QTL which had an effect on different stages of sheep growth and development. MEF2B was involved in the transcription process of muscle specific growth factors and the cell differentiation of skeletal muscle and myocardium development. However, its genetic structure has not been widely investigated and the relationship with sheep growth traits remains unknown. Previous study revealed that the MEF2B gene was significantly associated with the meat trait and reproduction traits in pigs [15]. Genes preferentially regulated by MEF2B were associated with hepatic fibrotic pathways, ovarian cancer signaling and human stem cell pluripotency [16]. Study revealed expression level of MEF2B was significantly positively correlated with myocyte fiber diameter (P<0.05) and significantly negatively correlated with myocyte fiber density (P<0.01) [17]. MEF2B was considered to be a good candidate gene for growth traits as it was a transcription factors which possessed DNA binding activities, it could be directly bonded to the specific promoters and enhancers of genes related to the muscle development. Previous study revealed in the absence of any MEF2 family members, genes related to the development of muscle showed partial transcriptional disruption [18]. In the study of muscle development mechanism, MRFs (myogenic regulatory factors) was considered as a core factor in the signal transduction net, in the process of muscle cell development, MRF must interact with other factors to play its effect, including the interaction with the MEF2 gene family. MRF4 promoter contains abundant information of muscle specific gene expression, but its full expression required MEF2 binding sites, mutations in MEF2B gene might alter binding site thus influenced the muscle development [19].

The expression pattern of Mef2b mRNA indicates that MEF2B, like its paralogs, may contribute to development and maintenance of a variety of tissue types [20]. MEF2B and its gene
family members play an important role in cell proliferation and differentiation, embryonic development, muscle production, neural system differentiation and liver fibrosis. Therefore, it is reasonable to suggest that \textit{MEF2B} mutations had a critical role in affecting the development and phenotype.

In single-locus analysis, the results showed the substitution of A with G on rs417014745 site lead to a consequence that sheep with mutation genotype AA had higher body weight, chest girth in 4-month age and higher body weight, chest girth in 6-month age than that in the wild genotype GG carriers. The detected SNP in the \textit{MEF2B} gene in our study was located in 3′–UTR, studies proved that mutations in the 3′–UTR may affect the appropriate expression of genes\cite{21, 22}, the 3′–UTR of mRNA could affect the rates of localization, translation and mRNA specific degradation\cite{21}, more importantly, miRNA could interact with the 3′–UTR of mRNA to negatively regulate the gene transcripts, which might cause a gene to be silent\cite{23}. The importance of the variations in the 3′–UTR could not be ignored, it might either directly have an effect on the expression of the gene or be linked to other variation in critically important regions of the genes that regulate gene expression. The substitution of rs417014745 might take effect through this way, further research of the miRNA and rs417014745 are necessary. Herein the allele A might be useful to improve the growth performance of sheep.

While the GWAS identified s58995.1 (rs420767326 A > G) in \textit{MEF2B} gene showed no polymorphism in this experiment, Mass array genotyping results indicated that all the Ujumqin sheep were with GG genotype on the GWAS identified SNP locus. This might be caused by the following reasons: some mutations are specific to certain groups, and they can not be found when they are validated by other groups \cite{24} or the numbers of the Ujumqin sheep were not that rich. In order to detect the SNP locus, we could expand the numbers of experiment population next.

As a novel candidate gene for sheep growth traits, seldom research on \textit{TRHDE} gene were performed in human and sheep until now. TRHDE enzyme was an extracellular peptidase, it was considered to have a critical role in inhibiting the release of the neuropeptide effectively. A Genome-wide scan in porcine revealed a SNP near the \textit{TRHDE} gene was associated with ear size at genome significance level\cite{25}. Our study revealed rs426980328 and rs430810656 were significantly associated with Ujumqin sheep growth traits. Both rs426980328 and rs430810656 sites were synonymous mutations, it is widely considered that synonymous mutations had no effect on gene function and animal phenotype because these mutations could not lead to the change of amino acid in the process of protein synthesis\cite{26}. However, recent reports proved synonymous mutations were usually under the evolutionary pressure, which affected the gene function by changing the shear mode and stability of mRNA as well as changing the characteristics of protein subunits \cite{27, 28}. Because of purifying selection, synonymous mutations might be fixed with a higher probability than neutral ones during evolution. The effect of synonymous mutations on gene function and phenotype were controlled by various factors. Zuo reported a synonymous 93G > A mutation in the \textit{MC4R} gene which was significantly associated with back fat thickness, sheep with mutation genotype AA was significantly higher than those with wild genotype GG \cite{22}. In our study, the rs426980328 synonymous mutation improved the body weight, chest girth in 4-month age and body weight, chest girth in 6-month age compared with the wild genotype carriers. Generally speaking, although one amino acid could be encoded by several codons, the organism had a certain preference for the choice of the codon or tRNA in the translation process. On the rs426980328 site, the usage of the codon(UAC) to encode tyrosine was 25.2%, while the mutation type(UAU) was 14.3%. On rs430810656 site, the usage of the codon (GCA) to encode the alanine was 13%, while the mutation type (GCG) was 5.3%. (http://www.kazusa.or.jp/codon/). The usage rate change might lead to a deceleration in the incorporation rate of the amino acid in protein peptide synthesis process, and finally
affect the phenotype of the organisms. The results suggested that rs426980328 and rs430810656 may be functional polymorphisms which could affect Ujumqin sheep growth traits. To the TRHDE gene, according to the compatibility of the primers used for Mass array genotyping, we could not obtain the genotypes of the sheep on the GWAS identified SNP locus.

Growth traits were quantitative traits controlled by multiple genes, they were not only influenced by the effect of single polymorphic loci but also the combined effect of multiple mutation sites [29]. Previous study was reported in small tailed Han ewes carried BB/G+ genotype (BMPR-IB and BMP-15) showed more litter size than those with either mutation alone, which proved the effect of multi-marker combination on the economic traits [30]. Based on our data, we found that individuals with CCAA combined genotype of rs426980328 and rs417014745 were overall 4.12kg heavier and 4.04cm bigger than the CCGG combined genotype carriers on body weight and chest girth in 4-month age, and overall 4.74kg heavier and 4.94cm bigger than the CCGG carriers on body weight and chest girth in 6-month age, respectively. Individuals with CCAA combined genotype performed better on body weight, chest girth in 4-month age and body weight, chest girth in 6-month age than those with either mutation CC or AA genotype alone, which indicated that combined genotype might play a more effective role in body weight and chest girth in sheep, the combined genotype CCAA was the superior genotype in the experimental population. When we analyzed the combined effect of rs430810656 and rs417014745 on growth traits in sheep, the results showed that the individuals with GAAA genotype were overall 4.71 kg heavier and 4.88cm bigger on chest girth in 4-month age than those with GGGG carriers. Sheep with GGAA genotype were overall 3.96kg heavier in 4-month age and 7.07cm bigger on chest girth in 6-month age than those with the GGGG carriers. Both GAAA and GGAA were advantageous genotypes affecting the sheep growth performance and homozygous genotypes, and they were easily to be selected and fixed in sheep production, which suggested that we can look the sites as genetic markers and strengthen the artificial selection in sheep breeding.

We particularly noted that most of the SNPs were significantly associated with growth traits in 4-month age, but were not remarkably associated with growth traits in 6-month age, this might give rise to the weaning time, the weaning time was always in 4-month age, during 3-month age to 4-month age was the growth peak of sheep the candidate genes might play a part together with the growth environment and feed nutrition to jointly influence the growth traits effectively in this crucial time.

**Conclusions**

Our results provide evidence that the MEF2B and TRHDE genes might be better candidate genes for Ujumqin sheep growth traits. These SNPs within them have potential value for growth traits of Ujumqin sheep. rs426980328-rs417014745 and rs430810656-rs417014745 pyramiding provided a way to accelerate the sheep growth traits improvement.

**Acknowledgments**

This study was supported by the National Natural Science Foundation of China “Studies on the Characteristics and Molecular Mechanisms of Formation of Sheep Skeletal Muscle” (U1503285) and the 863 project of China “Genome-wide association study on meat traits in sheep” (2011AA100307-02).

Authors thank the two sheep studs that mentioned above, thank the workers in the studs for help in collecting the blood samples.
Author Contributions
Conceived and designed the experiments: LZ LD. Performed the experiments: XM. Analyzed the data: XM. Contributed reagents/materials/analysis tools: JX HW ZY MW RL CZ. Wrote the paper: LZ XM. Contributed to the amendment of the manuscript: CW FZ.

References

1. Daw EW, Heath SC, Lu Y. Single-nucleotide polymorphism versus microsatellite markers in a combined linkage and segregation analysis of a quantitative trait. BMC Genet. 2005; 6 Suppl 1:S32. Epub 2006/02/03. doi:10.1186/1471-2156-6-s1-s32 PMID: 16451642; PubMed Central PMCID: PMCPmc1866753.

2. Zhang L, Liu J, Zhao F, Ren H, Xu L, Lu J, et al. Genome-wide association studies for growth and meat production traits in sheep. PLoS One. 2013; 8(6):e66569. Epub 2013/07/05. doi:10.1371/journal.pone.0066569 PMID: 23825544; PubMed Central PMCID: PMCPmc3692449.

3. Firulli AB, Miano JM, Bi W, Johnson AD, Casscells W, Olson EN, et al. Myocyte enhancer binding factor-2 expression and activity in vascular smooth muscle cells. Association with the activated phenotype. Circ Res. 1996; 78(2):196–204. Epub 1996/02/01. PMID:8575062.

4. Iida K, Hidaka K, Takeuchi M, Nakayama M, Yutani C, Mukai T, et al. Expression of MEF2 genes during human cardiac development. Tohoku J Exp Med. 1999; 187(1):15–23. Epub 1999/08/24. PMID:10458848.

5. Sun Q, Sattayakhom A, Backs J, Stremmel W, Chamulitrat W. Role of myocyte enhancing factor 2B in epithelial myofibroblast transition of human gingival keratinocytes. Exp Biol Med (Maywood). 2012; 237(2):178–85. Epub 2012/02/04. doi: 10.1258/ebm.2011.011261 PMID: 22302709.

6. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin. Nat Genet. 2010; 42(2):181–5. Epub 2010/01/19. doi: 10.1038/ng.518 PMID: 20081860; PubMed Central PMCID: PMCPmc2850970.

7. Rodríguez Al, Csatnyi G, Ranayhossaini DJ, Feck DM, Blose KJ, Assatourian L, et al. MEF2B-Nox1 signaling is critical for stretch-induced phenotypic modulation of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2015; 35(2):430–8. Epub 2015/01/01. doi: 10.1161/atvbaha.114.304936 PMID: 25550204; PubMed Central PMCID: PMCPmc4409426.

8. Lin Q, Schwarz J, Bucana C, Olson EN. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science. 1997; 276(5317):1404–7. Epub 1997/05/30. PMID: 9162005; PubMed Central PMCID: PMCPmc4437729.

9. Bowers PM, Cokus SJ, Eisenberg D, Yeates TO. Use of logic relationships to decipher protein network organization. Science. 2004; 306(5705):2246–9. Epub 2004/12/25. doi: 10.1126/science.1103330 PMID: 15618515.

10. Cavanagh CR, Jonas E, Hobbs M, Thomson PC, Tammen I, Raadsma HW. Mapping Quantitative Trait Loci (QTL) in sheep. Ill. QTL for carcass composition traits derived from CT scans and aligned with a meta-assembly for sheep and cattle carcass QTL. Genet Sel Evol. 2010; 42:36. Epub 2010/09/18. doi: 10.1186/1297-9686-42-36 PMID: 20846385; PubMed Central PMCID: PMCPmc2949606.

11. Schomburg L, Turwitt S, Prescher G, Lohmann D, Horsthemke B, Bauer K. Human TRH-degrading ectoenzyme cDNA cloning, functional expression, genomic structure and chromosomal assignment. Eur J Biochem. 1999; 265(1):415–22. Epub 1999/09/22. PMID: 10491199.

12. Freudenberg J, Lee HS, Han BG, Shin HD, Kang YM, Sung YK, et al. Genome-wide association study of rheumatoid arthritis in Koreans: population-specific loci as well as overlap with European susceptibility loci. Arthritis Rheum. 2011; 63(4):884–93. Epub 2011/04/01. doi: 10.1002/art.30235 PMID: 21452313.

13. Nagy V, Cole T, Van Campenhout C, Khoun TM, Leung C, Vermeiren S, et al. The evolutionarily conserved transcription factor PRDM12 controls sensory neuron development and pain perception. Cell Cycle. 2015; 14(12):1799–808. Epub 2015/04/22. doi: 10.1080/15384101.2015.1036209 PMID: 25891934; PubMed Central PMCID: PMCPmc4615559.

14. Kim ES, Elbeltagy AR, Aboul-Naga AM, Rischkowsky B, Sayre B, Mwacharo JM, et al. Multiple genomic signatures of selection in goats and sheep indigenous to a hot and environment. Heredity (Edinb). 2015. Epub 2015/11/12. doi: 10.1038/hdy.2015.94 PMID: 26555032.

15. HE Bo, ZHENG Rong,XIONG Yuanzhu,Chunyan HU.Culture, Identification and biological characteristics of skeletal muscle satellite cells of the Neonatal pig.Acta veterinaria et Zootecnica sinica,2006, (06):555–559.
Identification of MEF2B and TRHDE Gene Polymorphisms Related to Growth Traits in a Sheep Population

16. Estrella NL, Desjardins CA, Nocco SE, Clark AL, Maksimenko Y, Naya FJ. MEF2 transcription factors regulate distinct gene programs in mammalian skeletal muscle differentiation. J Biol Chem. 2015; 290(2):1256–68. Epub 2014/11/25. doi:10.1074/jbc.M114.589838 PMID: 25416778; PubMed Central PMCID: PMCPmc4294490.

17. Chen L, Cheng B, Li L, Zhan S, Wang L, Zhong T, et al. The molecular characterization and temporal-spatial expression of myocyte enhancer factor 2 genes in the goat and their association with myofiber traits. Gene. 2015; 555(2):223–30. Epub 2014/12/03. doi: 10.1016/j.gene.2014.11.007 PMID: 25447896.

18. Molkentin JD, Olson EN. Combinatorial control of muscle development by basic helix-loop-helix and MADS-box transcription factors. Proc Natl Acad Sci U S A. 1996; 93(18):9366–73. Epub 1996/09/03. PMID: 8790335; PubMed Central PMCID: PMCPmc38433.

19. Naidu PS, Ludolph DC, To RQ, Konieczny SF. Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. Mol Cell Biol. 1995; 15(5):2707–18. Epub 1995/05/01. PMID: 7799551; PubMed Central PMCID: PMCPmc230501.

20. Pon JR, Marra MA. MEF2 transcription factors: developmental regulators and emerging cancer genes. Oncotarget. 2016; 7(3):2297–312. Epub 2015/10/28. doi: 10.18632/oncotarget.6223 PMID: 26506234.

21. Reamon-Buettner SM, Cho SH, Borlak J. Mutations in the 3′-untranslated region of GATA4 as molecular hotspots for congenital heart disease (CHD). BMC Med Genet. 2007; 8:38. Epub 2007/06/27. doi: 10.1186/1471-2350-8-38 PMID: 17592645; PubMed Central PMCID: PMCPmc1933416.

22. Zuo B, Liu G, Peng Y, Qian H, Liu J, Jiang X, et al. Melanocortin-4 receptor (MC4R) polymorphisms are associated with growth and meat quality traits in sheep. Mol Biol Rep. 2014; 41(10):6967–74. Epub 2014/07/23. doi: 10.1007/s11033-014-3583-x PMID: 25048291.

23. Dumortier O, Van Obberghen E. MicroRNAs in pancreas development. Diabetes Obes Metab. 2012; 14 Suppl 3:22–8. Epub 2012/09/07. doi: 10.1111/j.1463-1326.2012.01656.x PMID: 22928561.

24. Chanock SJ, Manolio T, Boehnke M, Boerwinkle E, Hunter DJ, Thomas G, et al. Replicating genotype-phenotype associations. Nature. 2007; 447(7145):655–60. Epub 2007/06/08. doi: 10.1038/447655a PMID: 17554299.

25. Zhang L, Liang J, Luo W, Liu X, Yan H, Zhao K, et al. Genome-wide scan reveals LEMD3 and WIFI1 on SSC5 as the candidates for porcine ear size. PLoS One. 2014; 9(7):e102085. Epub 2014/07/07. doi: 10.1371/journal.pone.0102085 PMID: 25006967; PubMed Central PMCID: PMCPmc4090188.

26. Komar AA. Silent SNPs: impact on gene function and phenotype. Pharmacogenomics. 2007; 8(8):1075–80. Epub 2007/08/25. doi: 10.2217/14622416.8.8.1075 PMID: 17716239.

27. Liu Y, Chu A, Chakroun I, Islam U, Blais A. Cooperation between myogenin regulatory factors and SIX family transcription factors is important for myoblast differentiation. Nucleic Acids Res. 2010; 38(20):6857–71. Epub 2010/07/06. doi: 10.1093/nar/gkq585 PMID: 2061407; PubMed Central PMCID: PMCPmc2978361.

28. Orozco G, Hinks A, Eyre S, Ke X, Gibbons LJ, Bowes J, et al. Combined effects of three independent SNPs greatly increase the risk estimate for RA at 6q23. Hum Mol Genet. 2009; 18(14):2693–9. Epub 2009/05/07. doi: 10.1093/hmg/ddp193 PMID: 19417005; PubMed Central PMCID: PMCPmc2701332.

29. Chu MX, Liu ZH, Jiao CL, He YQ, Fang L, Ye SC, et al. 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(3):598–603. Epub 2006/10/17. doi: 10.2527/jas.2006-324 PMID: 17040942.