Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (1): 11-18, 2025
DOI: 10.9775/kvfd.2024.32412

RESEARCH ARTICLE

Identification and Expression of the Target Gene SLC24A2 of oar-miR-377 and Its Novel SNPs Effects on Wool Traits in Sheep

Huaqian ZHOU [1,2] Hua YANG [1,2 (*)] Wenhua FAN [1,2] Hanyulu YANG [2]
Zongsheng ZHAO [1 (*)] Qian YU [2] Yonglin YANG [2] Wenzhe ZHANG [2]

[1] College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, 832000, China
[2] State Key Laboratory of Sheep Genetic Improvement and Healthy Production, Xinjiang Academy of Agricultural and Reclamation Science, Shihezi, Xinjiang, 832000, China

[(*)] **Corresponding authors:**
Hua YANG & Zongsheng ZHAO
Phone: +86 135 65735767 (Z. Zhao);
+86 136 77568622 (H. Yang)
E-mail: 2944138946@qq.com (Z. Zhao);
yhxjcn@sina.com (H. Yang)

How to cite this article?
Zhou H, Yang H, Fan W, Yang H, Zhao Z, Yu Q, Yang Y, Zhang W: Identification and expression of the target gene SLC24A2 of oar-miR-377 and its novel SNPs effects on wool traits in sheep. *Kafkas Univ Vet Fak Derg*, 31 (1): 11-18, 2025.
DOI: 10.9775/kvfd.2024.32412

Article ID: KVFD-2024-32412
Received: 30.05.2024
Accepted: 19.12.2024
Published Online: 23.12.2024

Abstract

Hair follicle development is closely associated with wool traits. Current studies reveal the crucial role of microRNAs in regulating the specific gene expression by binding to target mRNA involution in hair follicle growth and development, thereby regulating the wool traits. Our previous miRNA sequencing showed that oar-miR-377 has special expression in secondary hair follicle development and SLC24A2 may be a new candidate target using bioinformatics analysis. In this study, the regulatory relationship of oar-miR-377 and its specific target gene SLC24A2 was determined in sheep fibroblasts by dual-luciferase reporter assay, RT-qPCR, and western blot. The variation of oar-miR-377 precursors was detected using PCR and Sanger sequencing, and the association between polymorphisms of oar-miR-377 and wool quality traits was analyzed in Chinese Merino. The result showed that SLC24A2 was a target gene of oar-miR-377. A SNP (276T>C) of oar-miR-377 upstream sequences was identified and extremely significant associated with the coefficient of variation of wool fiber diameter in Chinese Merino sheep (P<0.01). These results suggest that oar-miR-377 promotes secondary hair follicle development by downregulation of SLC24A2 gene expression, and its SNP might be a useful marker for wool quality.

Keywords: oar-miR-377, SLC24A2, SNP, Wool traits, Sheep

INTRODUCTION

Wool is a healthy natural product from sheep, the economic traits of wool include fiber length, fiber diameter, fiber diameter standard deviation, coefficient of variation of wool fiber diameter, fiber crimp, fiber density, and so on. Wool quality is determined by the development of hair follicles. Hair follicles (HFs) are complex and composed of 8 unique cell populations that are derived from the ectoderm and mesoderm, comprised of primary hair follicles and secondary hair follicles [1,2], the secondary hair follicles are a key factor in determining wool quality [3]. In mammals, hair follicles are crucial for temperature regulation, physical protection, sweat and sebum dispersion, sensory and tactile functions, and social interactions [4]. The number of dermal papilla cells and the size of the hair placode are associated with the diameter, crimp, and density of wool fibers, which are of high economic value in the sheep industry [5-7]. Many reports have indicated that several genes may be involved in hair follicle development, such as Wnt10a [8], Sox9 [9], and BMP4 [10]. Additionally, some signaling pathways, such as BMP [11], Eda [12], Shh [13], and TGF-β [14] signaling were revealed to promote or suppress the process of hair follicle development. In animal husbandry, elucidating the genetic mechanisms of the development of hair follicles and wool-related traits is important to improve sheep breeding.

MicroRNAs are non-coding RNAs that include 22nt nucleotide and are widely present in animals and plants, which can negatively regulate gene expression by base pairing of 5-end with the 3 untranslated regions (3'UTRs) of target mRNAs [15,16]. Research has found that microRNAs are expressed in a variety of different hair follicle cells, such as hair follicle stem cells, matrix cells,

outer root sheath cells, and inner root sheath cells, and lots of miRNAs are verified to be specifically expressed in hair follicle cells [17]. Both MiR-203 [18], MiR-206 [19], miR-125b [20], and miR-205 [21] are expressed in hair follicle stem cells. Several SNPs in miRNA genes have been proven to be associated with human diseases by affecting the miRNA-mediated regulatory function. The SNP of mature miR-125a sequences can reduce miR-125a expression in cancer [22]. The SLC24A2 gene is a cation exchanger and serves as the second member of the solute carrier 24 family [23,24]. Although there is no direct evidence that SLC24A2 is related to skin hair follicles, SLC24A5 has been confirmed to be related to animal hair color [25]. RT-PCR results showed that the SLC24A5 gene was highly expressed in skin and eyes and low expressed in other tissues, while the expression level in mouse melanoma was more than 100 times higher than that in normal skin and eyes [26,27].

Our previous research showed that SLC24A2 is a key molecule in hair follicle development signal transduction which also is the target gene of the oar-miR-377 by microRNA-sequencing analysis. However, the potential relationship between SLC24A2 and oar-miR-377, and its SNP effects on wool traits has not been illustrated. In this study, the target binding relationship of SLC24A2 and oar-miR-377 was investigated at cellular levels, and functional mechanisms of SNP of oar-miR-377 were elucidated in sheep populations levels of Chinese Merino (Xinjiang Junken type). The study may provide a basis for the genetic mechanism of wool traits and fine wool sheep breeding.

Materials and Methods

Ethical Statement

This study was approved by the Experimental Animal Care and Use Committee of Xinjiang Academy of Agricultural and Reclamation Sciences (Approval no: XJNKKXY-2020-34).

Experimental Animals and Sample Collection

A total of 265 female Chinese Merino sheep (Xinjiang Junken type) were selected from the sheep breeding farm of the Xinjiang Academy of Agricultural and Reclamation Science. The flock included 55 one-year-old sheep, 110 two-year-old sheep and 100 three-year-old sheep. All the ewes were sourced from a single flock and fed under the same conditions. The peripheral blood was collected from the jugular vein and placed in anticoagulant tubes containing EDTA (1 mg/mL) for genomic DNA extraction. The wool samples were collected to detect the wool quality from 265 Chinese Merino. The ear tissue from three-month-old lamb was collected for cell culture.

Cell Culture of Sheep Skin Fibroblasts

Ear tissue (10x10 mm) was sterilized with 75% alcohol and collected from 3-month-old healthy Merino lambs. After sterilized with 75% alcohol and washed by 1xPBS (pH 7.2) containing penicillin and streptomycin double antibody (1:100), the skin was minced pieces and put into 90 mm dishes containing 10 mL of Dulbecco's Modified Eagle's Medium (DMEM, The Gibco Company) supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). The cells were placed in Galaxy® 48R CO_2 Incubator from Eppendorf at 37°C and 5% CO_2, the culture medium was changed every 3 days and the growth status of cells was observed. When cell confluency reached 80%, the cells were detached using trypsin-EDTA (0.10% trypsin and 0.02% EDTA, The Gibco Company) for 5 min at 37°C, followed by trypsin digestion method for culture in the next passage.

Prediction of Target Genes and Construction of Recombinant Plasmids

Using Bibiserv *(https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/)* online website to predict oar-miR-377 and sheep SLC24A2 gene 3'-UTR region binding sites. The primers of wild carriers were designed according to the binding site sequence of miRNA and target SLC24A2 gene searched on the NCBI website by Primer5.0 software *(Table 1)*. The upstream primer was introduced to the Not I restriction site, and the downstream primer was introduced to the Xho I restriction site, the primers were synthesized by Sangon Biotech (Shanghai, China). Total RNA was extracted from sheep skin samples using Trizol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Beijing Transgene Biotech Co. Ltd., Beijing, China). SLC24A2 gene 3'-UTR sequence containing microRNA binding sites was amplificated by RT-PCR, and the reactions were incubated in a 96-well plate at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, a final extension of 72°C, PCR products were purified using gel recovery kit, and ligated to pEASY - T1

Table 1. *Sequences of primers*

Primer Names	Primer Sequence	Length (bp)
SLC24A2-F	ATAAGAATGCGGCCGCgccaccatggTGGAAGCGCCTCACAA	589
SLC24A2-R	CCGCTCGAGcgccaccatggCTCTGACCAGCAAGGAGTA	

The lowercase letters indicate NotI and XhoI restriction sites

Table 2. Experimental groups	
Groups	**Experiment Project**
A	pCHECK-W and mimic
B	pCHECK-W and mimic negative control
C	pCHECK-M and mimic
D	pCHECK-M and mimic negative control
E	psiCHECK-2 and mimic negative control

Simple vector, then cloned into a psiCHECK-2 vector and constructed wild-type plasmid (pCHECK-W, *Table 2)*. The mutation sequence of SLC24A2 gene 3'-UTR region binding sites was synthesized in Jikai Gene Company (Shanghai, China), and ligated to psiCHECK-2 vector constructed mutation-type plasmid (pCHECK-M, *Table 2)*. The reconstructed plasmids were sequenced in Sangon Biotech (Shanghai, China). Mimics of oar-miR-377 were designed and synthesized by Jikai Gene Company (Shanghai, China).

Cell Transfection and Dual Luciferase Activity Assay

The skin fibroblasts were digested after the cell density reached approximately 85% confluence and transfected with oar-miR-377 mimic, oar-miR-377 NC (negative control), psiCHECK-2, pCHECK-W and pCHECK-M plasmid using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Experimental groups were divided into 5 groups *(Table 2)*. Each group had three replicates. At 48 h of transfection, the luciferase activity was detected by the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. The luciferase activity of the firefly and renal luciferase was detected using a microplate reader (Thermo Scientific varioskan flash, MA, USA). The firefly and *Renilla* luciferase enzyme activities were measured for each biological sample. The firefly luciferase enzyme activity was normalized to the *Renilla* luciferase enzyme activity. Mean±SD of the relative luciferase activity data were calculated by repeated three times independently.

Western Blotting

The cell proteins were extracted using a whole protein extraction kit (Applygen Technologies, Beijing, China). Protein concentrations in cell lysates were determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE), and adjusted to the same concentration. Heat-denatured protein samples (25 μg per lane) were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on duplicate gels and transferred to nitrocellulose membrane (Boster, Wuhan, China). The membranes were incubated for 60 min in 10% nonfat dry milk to block nonspecific binding, followed by incubation for 12 h at 4°C with a primary rabbit monoclonal antibody against oar-miR-377 (Boster, Wuhan, China), which was diluted 1:1000 in tris-buffered saline Tween-20 (TBST). The membrane was then washed 2 times for 10 min in TBST, 1 time for 10 min in TBS, and incubated for 1 h at room temperature with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) (Boster, Wuhan, China) diluted 1:2500 in TBST. The membrane was washed 2 times for 10 min in TBST and 1 time for 10 min in TBS, and the bound antibody was detected colorimetrically using a DAB detection kit (Boster, Wuhan, China) according to the manufacturer's instructions. The intensity of signals for SLC24A2 was quantified using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The grey values of bands were measured using ImageJ software (v1.48, NIH, Bethesda, MD). The relative intensity ratio of SLC24A2 and GAPDH was calculated based on grey values. Mean±SD of the grey value was calculated by repeated three times independently.

RT-qPCR

Sheep skin fibroblasts were cultured after transfection, and their mRNA levels were detected by qPCR after 48 h culture. Total RNA was extracted according to the manufacturer's instructions using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quality was detected using electrophoresis on 1% agarose gel in 1x TAE buffer. The purity and concentration of RNA were detected using Nanodrop 2000. Gene sequences were obtained from the NCBI gene bank, primers of SLC24A2 were designed by Primer 5, and miRNA primers of oar-miR-377 were designed by miR primer 2 *(Table 1)*. The 20 μL PCR reaction mixture contained 10 μL Platinum SYBR GreenImaster, 1 μL forward primer (10 μM), 1 μL reverse qPCR primer (10 μM), 6 μL ddH_2O, 2 μL DNA template. The reactions were incubated in a 96-well plate at 95°C for 5 min, followed by 45 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 20 sec. All RT-qPCR experiments were performed at least in triplicate, and relative mRNA quantification was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$) method. The ΔCt values for the SLC24A2 gene were calculated using the Ct values [Ct (test) – Ct (reference)]. Mean±SD of the $2^{-\Delta\Delta Ct}$ data were calculated.

Measurement of Wool Traits

The wool fiber diameter, fiber diameter standard deviation and coefficient of variation of wool fiber diameter were automatically measured from 265 Chinese Merino (Xinjiang Junken type) according to the guidelines of the China Fiber Inspection Bureau, International Wool Trade Organization standards IWTO-TM47 and 57 using OFDA 2000 instrument (Cottle DJ, 2010).

Table 3. *Primers sequences for detection SNPs of oar-miR-377*

Primer Name	Sequences	Annealing Temperature (°C)	Product Length	Chromosome Position/bp
miR-377-1	CCTTGGGAGGACCTTGCT	60	485	chr18:64558969-64559453
	AGAAGCCATCCCAAGCAG			
miR-377-2	CTCTCTGTTCAATCGCAGCTC	60	460	chr18:64558923-64559382
	AATTCACCAAAGGCAACCTC			

Preparation of the Genomic DNA

The genomic DNA was extracted using a Tiangen blood genomic DNA extract kit following the manufacturer's protocol, the concentration and purity of genome DNA were detected by using 1.5% gel electrophoresis.

PCR Amplification and SNP Detection of oar-miR-377

The mature sequence of oar-miR-377 was obtained according to miRBase *(http://www.mirbase.org/)*. 700bp of oar-miR-377 upstream and downstream flanking sequences were searched by the UCSC Genome Browser *(http://genome.ucsc.edu/)*. The primers were designed using Primer 5.0 software, and synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China) *(Table 3)*.

The mature sequence of oar-miR-377 was individually amplified using miR-377-1 primers and miR-377-2 primers. The PCR reactions were performed by mixing 2.5 µL of genome DNA, 25 µL of 2 x EasyTaq PCR Supermix, 1 µL (10 µM) each of the upstream primers and downstream primers, 20.5 µL of DDW, in a final volume of 50 µL. The cycling was performed on a thermocycler (A200, Longgene Scientific Instrument Company, Hangzhou, China), by the following program: 94°C for 5 min, 35 cycles of 94°C for 30 sec, annealing at 60°C for 30 sec, and 72°C for 30 sec, a final extension of 72°C for 5 min. The PCR products were detected using 1.5% agarose gel electrophoresis and sequenced by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China), The SNP was identified, and the location was determined by Blast against miRBase database (v19, *http://microrna.sanger.ac.uk)*.

Statistical Analysis

Genotype frequencies, allele frequencies, and Hardy-Weinberg equilibriums tests were performed using the Popgene 32 software. For the tested population, the statistical models associated between different genotypes with average wool fiber diameter, fiber diameter standard deviation, and coefficient of variation of wool fiber diameter were: $Y_{ij}=\mu+G_i+A_j+G_ixA_j+e_{ij}$, which Y_{ij} was the phenotypic value of the wool traits, µ was overall population mean, Gi was the fixed effect of genotype, A_j was the fixed effect of age, G_ixA_j was genotype x age interaction, e_{ij} was the random residual.

Data were subjected to the GLM procedures of John's Macintosh Program (JMP version 16.0.0, SAS Institute Inc.), which was used to examine the correlations between genotypes and continuous traits, and to evaluate the least squares means. For all the data, $P<0.05$ was significant, and $P<0.01$ was highly significant.

Results

Culture of Sheep Skin Fibroblasts

Primary sheep fibroblast cells migrated from tissue pieces 5-12 days after explanting. Then, cells continued to proliferate and were passaged when reached 90% confluences. The cells were morphologically consistent with the fibroblast phenotype.

Prediction of miRNA Binding Sites with Target Genes

The secondary structure of oar-miR-377 was obtained by sequencing results, which had two typical stem-loop structures. The 3'-UTR sequence of the SLC24A2 gene was obtained on the NCBI website, and the binding site sequence of the mature sequence of oar-miR-377 was matched with the 3'-UTR of the SLC24A2 gene by Bibiserv software. The mfe was -26.1 keal/mol.

Construction of psiCHECK-2 Wild-type Vector and Mutant Vector

The binding site sequence of the SLC24A2 gene was obtained by PCR amplification. The 589bp PCR production appeared by 1.5% agarose gel electrophoresis. The target gene products were incorporated into the psiCHECK-2 vector using a T4 DNA ligase at 4°C overnight. The sequences of constructed pCHECK-W and pCHECK-M were confirmed by standard BLAST alignment analysis and showed 100% and 97% Identities with Ovis aries solute carrier family 24 member 2 (SLC24A2) mRNA (Genbank accession number XM_015093243.4). The results indicated that pCHECK-W and pCHECK-M vectors were successfully constructed.

Dual Luciferase Activity Assay of SLC24A2 Targeted by oar-miR-377

The results showed that the relative luciferase activity in pCHECK-W and mimic was significantly lower than the pCHECK-W and mimic NC (negative control), psiCHECK-2 and mimic NC (negative control) by using dual luciferase activity assay ($P<0.05$) *(Fig. 1)*. The

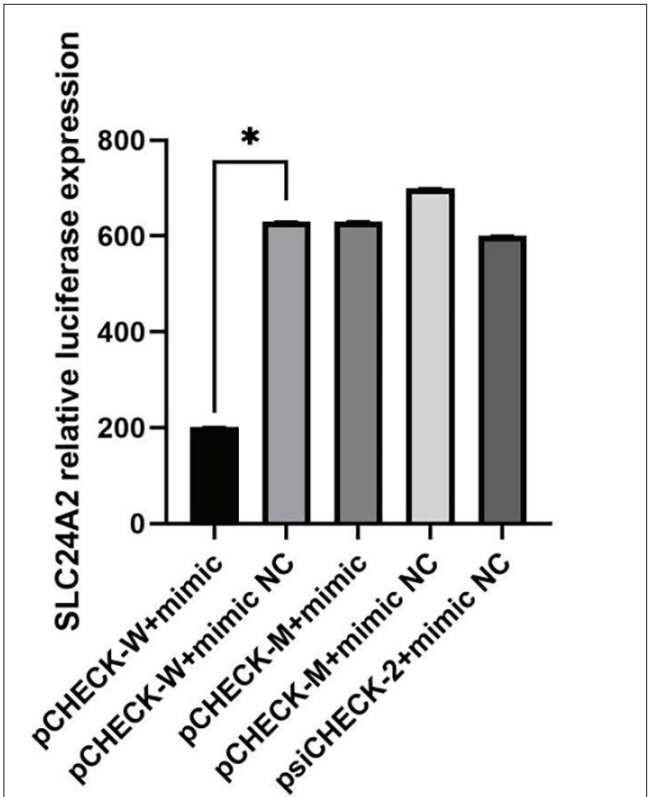

Fig 1. Analysis of dual-luciferase reporter assay

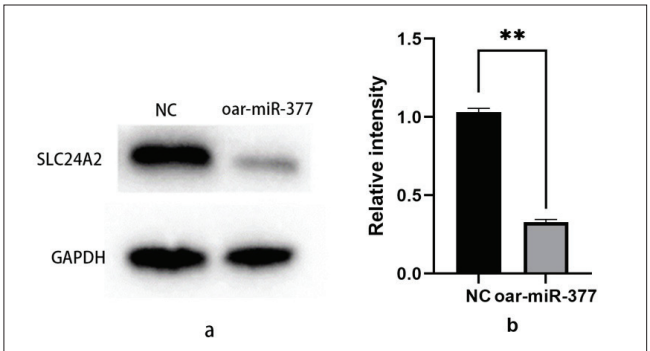

Fig 2. Protein levels of SLC24A2 regulated by oar-miR-377. **A:** Western blot images, **B:** Relative intensity of Western blot

relative luciferase activities were not significantly different among pCHECK-M and mimic, pCHECK-M and mimic NC, psiCHECK-2 and mimic NC (P>0.05). These results indicated that oar-miR-377 was binding to the 3'-UTR in the regulation of SLC24A2 mRNA.

The Effect of SLC24A2 Protein Level Regulated by oar-miR-377

The regulation of SLC24A2 protein level by oar-miR-377 in sheep skin fibroblasts was detected by Western blot *(Fig. 2-a)*. The results show that the protein relative expression of SLC24A2 regulated by oar-miR-377 was extremely significantly lower than NC (P<0.01), the expression level

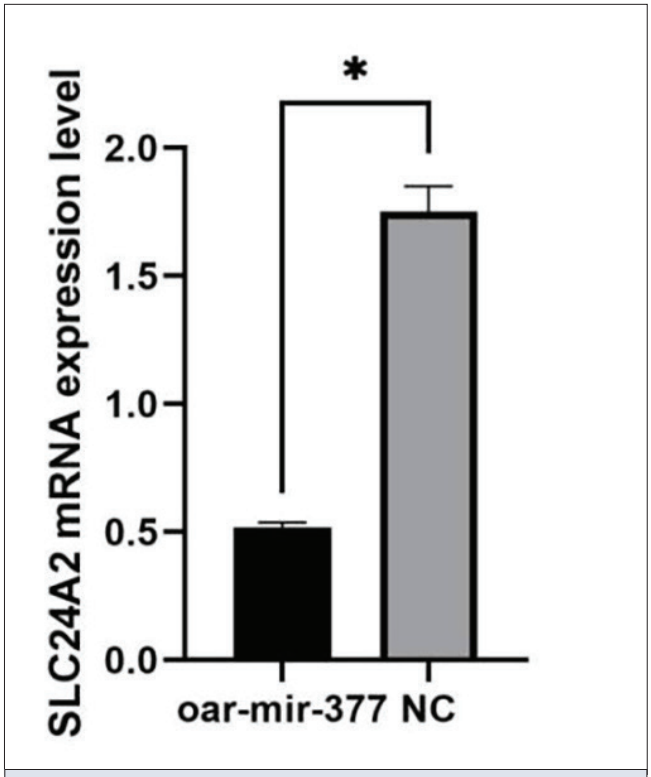

Fig 3. mRNA expression levels of SLC24A2 gene regulated by oar-miR-377

of oar-miR-377 was 3.28 times lower than the negative control *(Fig. 2-b)*.

The Effect of SLC24A2 Gene Expression Level Regulated by oar-miR-377

The mRNA expression level of SLC24A2 was regulated by oar-miR-377 and negative control was detected using RT-qPCR. The results showed that SLC24A2 relative expression level was significantly lower than NC (P<0.05), the expression level of SLC24A2 was 3.37 times lower than the NC *(Fig. 3)*.

PCR Amplification and SNP Identification of oar-miR-377 Precursors

PCR productions of oar-miR-377 were detected by 1.5% agarose gel electrophoresis, and the 460bp bands were observed. The sequence of PCR productions was aligned to the known oar-miR-377 precursors sequence after DNA sequencing. T > C mutation site was detected at 276bp upstream flanking region of oar-miR-377 by miR-377-1 primer in Chinese Merino sheep, and located in chr18: 64559086 against the miRBase miRNA database *(Fig. 4)*.

Association of oar-miR-377 Polymorphisms with Wool Traits

Three genotypes of TT, TC, and CC were identified in Chinese Merino sheep (Xinjiang Junken type), by using genetic polymorphism analysis of -276T>C locus of oar-

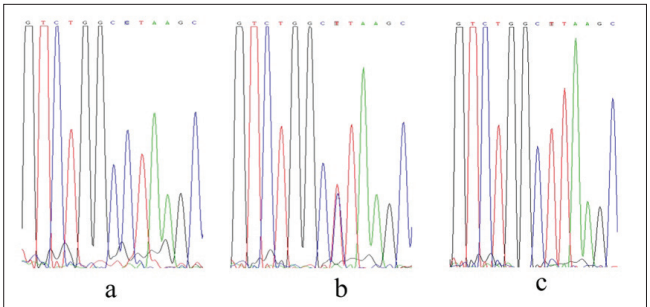

Fig 4. Three genotypes of oar-miR-377 gene by sequencing. **A:** CC genotype, **B:** CT genotype, **C:** TT genotype

Table 4. *The distribution of genotypic frequency and gene frequency of -276T>C in oar-miR-377 in Chinese Merino sheep*

Genotype Frequency			Allele Frequency		χ^2	P-value
CC	TC	TT	C	T		
0.39 (103)	0.55 (146)	0.06 (16)	0.67	0.33	1.7	0.19

Table 5. *Effects of oar-miR-377 SNP on wool traits (LSM)*

Genotype	Numbers	Wool Fiber Diameter (μm)	Wool Fiber Diameter Standard Deviation	Coefficient of Variation of Wool Fiber Diameter
CC	103	19.64±0.19	3.94±0.06	19.96±0.21 ^{A}
TC	146	19.48±0.16	3.86±0.05	18.02±0.18 ^{B}
TT	16	19.72±0.56	3.89±0.19	17.81±0.62 ^{B}

Different uppercase letters indicate the difference is extremely significant (P<0.01).

miR-377. The genotype frequencies of CC, TC, and TT in the Chinese Merino sheep were 0.39, 0.55, and 0.06 *(Table 4)*. The SNP site was under the Hardy-Weinberg equilibrium in Chinese Merino sheep.

The results of the LSM analysis showed that the coefficient of variation of wool fiber diameter of the CC genotype was extremely significantly larger than that of the TC and TT genotypes (P<0.01). The wool fiber diameter was no significant difference among the CC, TT, and TC genotypes. The wool fiber diameter standard deviation was no significant difference among the CC, TT, and TC genotypes *(Table 5)*.

Discussion

MicroRNA is a small non-coding RNA [28], several research had proved that miRNAs were related to the development of hair follicles, 22 new miRNAs and 316 conserved miRNAs were identified in the growth of skin and hair follicles in adult inner Mongolia cashmere goats [29]. MiR-203 was abundantly expressed in the epidermis and hair follicles, and closely related to the development of skin and hair follicles [18]. MiR-206 regulated the periodic changes of hair follicles by affecting the expression of genes related to hair follicle initiation and development in Shanbei white cashmere goats [19]. MiR-125b may act as a repressor to suppress hair follicle stem cell differentiation [20]. MiR-205 has a positive effect on hair follicle stem cells and the proliferation of their progenies [21].

oar-miR-377 has been reported to enhance fibronectin protein production, regulate angiogenesis, suppress cell proliferation, predict clinical outcomes in patients with gastric cancer, induce tumorigenesis, and promote oxidative stress [30]. Owing to the pleiotropic functions and DNMT1 targeting potential of oar-miR-377 may regulate human skin fibroblast (HSF) senescence by targeting DNMT1 [31]. Studies have shown that oar-miR-377 controls the occurrence and development of esophageal cancer by inhibiting the expression of CD133 and VEGF [32], and can regulate the NF-κB signaling pathway in melanoma cells by targeting the E2F3 gene [33]. The SLC24A2 gene is a cation exchanger and serves as the second member of the solute carrier 24 family. Although there is no direct evidence that SLC24A2 is related to skin hair follicles, SLC24A5 is related to animal hair color. Due to the mutation of the SLC24A5 gene, the golden mutation is accompanied by the decreased pigmentation of the skin melanophore and the retinal epithelium, and the number and density of melanosomes are reduced, resulting in the delay and reduction of melanin deposition. In this study, the Dual-Luciferase reporter assay was used to study the SLC24A2 targeting relationship with oar-miR-377,

and results showed that oar-miR-377 could significantly reduce the gene expression and protein expression of the SLC24A2 gene compared with NC, it was indicated that SLC24A2 is also regulated by oar-miR-377 to affect the quality of wool in sheep. Previous studies found that oar-miR-377 can regulate CD133, VEGF, and E2F3 genes in humans, and this study found that oar-miR-377 can regulate the SLC24A2 gene in sheep. This result indicates that oar-miR-377 is species-specific gene regulation.

The SNPs affect gene expression and complex diseases. However, because the thermodynamics of RNA-RNA binding plays a crucial role in the interaction of miRNA with target mRNA, it can be expected that sequence variants such as SNP in miRNA binding sites may affect the expression of miRNA targets. There was also evidence that SNPs in miRNA binding sites in oncogenes are associated with increased gene expression in papillary thyroid cancer [34]. Due to the mutation of the SLC24A5 gene, the golden mutation is accompanied by the decreased pigmentation of the skin melanophore and the retinal epithelium, and the number and density of melanosomes are reduced, resulting in the delay and reduction of melanin deposition [35]. In this study, T > C mutation was found at 276bp upstream flanking region of the oar-miR-377 by sequencing and sequence alignment. TT, TC, and CC genotypes were identified in Chinese Merino sheep (Xinjiang Junken type), and coefficient of variation of wool fiber diameter of the TC and TT genotypes was extremely significantly less than that of the CC genotype, it was indicated that the T allele is closely related to wool quality. These results suggest that SNP at 276bp upstream flanking region of the oar-miR-377 affected the binding with SLC24A2 and regulation of SLC24A2 expression. Currently, there were lack of clear research on the mutation of miRNA relationship to phenotypic traits, we speculated that this SNP of oar-miR-377 flanking region affected the expression level of mature miR-377 and SLC24A2 target gene, and indirectly regulated secondary hair follicle development and wool quality. As a result, the mutation of oar-miR-377 influences the coefficient of variation of wool fiber diameter. It was indicated that oar-miR-377 regulated hair follicle development, the mutation of oar-miR-377 affected wool quality. The T allele of 276T>C in oar-miR-377 could promote the secondary hair follicle development, the individuals with T allele could be selected in fine wool sheep breeding.

oar-miR-377 could significantly reduce the gene expression of the SLC24A2 at the level of transcription and translation, SLC24A2 was regulated by oar-miR-377 to affect the wool quality. T > C mutation found at 276bp upstream flanking region of the oar-miR-377 extremely affected coefficient of variation of wool fiber diameter in Chinese Merino sheep (Xinjiang Junken type), TC and TT genotypes could be favorable genotypes for improving wool quality in fine wool sheep breeding.

Declarations

Availability of Data and Materials: The data presented in this study are available on request from the corresponding author (H. Yang).

Acknowledgments: None.

Funding Support: This work was supported by the National Natural Science Foundation of China (No. 32360818), Youth Science and Technology Innovation Talent of Tianshan Talent Training Program in Xinjiang (No. 2022TSYCCX0122); Agricultural Science and Technology Innovation Project of Xinjiang Production and Construction Corps (No. NCG202211), Sheep Breeding and Upgrading Plan of duel-purpose breed both for mutton and wool, cashmere and wool of Xinjiang Uygur Autonomous Region (No. 2024XJRMY-03), China.

Ethical Approval: This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Experimental Animal Care and Use Committee of Xinjiang Academy of Agricultural and Reclamation Sciences (Shihezi, China, ethic committee approval number: XJNKKXY-2020-34, 30 December 2020).

Competing Interests: The authors declared that there is no competing interest.

Declaration of Generative Artificial Intelligence: The article, tables and figures were not written by AI and AI-assisted technologies.

Authors Contributions: Conceptualization and writing-original draft preparation: H. Zhou and H. Yang; Methodology and data curation: H. Yang; Validation: W. Fan; Investigation and samples: Qian Yu and Wenzhe Zhang; Writing-review and editing: Z. Zhao and Y. Yang; Funding acquisition: H. Yang. All authors have read and agreed to the published version of the manuscript.

References

1. Li C, He X, Wu Y, Li J, Zhang R, An X, Yue Y: Single-cell transcriptome sequence profiling on the morphogenesis of secondary hair follicles in Ordos fine-wool sheep. *Int J Mol Sci,* 25 (1):584, 2024. DOI: 10.3390/ijms25010584

2. Tian D, Pei Q, Jiang H, Guo J, Ma X, Han B, Li X, Zhao K: Comprehensive analysis of the expression profiles of mRNA, lncRNA, circRNA, and miRNA in primary hair follicles of coarse sheep fetal skin. *BMC Genomics,* 25 (1):574, 2024. DOI: 10.1186/s12864-024-10427-7

3. Watabe R, Yamaguchi T, Kabashima-Kubo R, Yoshioka M, Nishio D, Nakamura M: Leptin controls hair follicle cycling. *Exp Dermatol,* 23 (4): 228-229, 2014. DOI: 10.1111/exd.12335

4. Fuchs E: Epithelial skin biology: Three decades of developmental biology, a hundred questions answered and a thousand new ones to address. *Curr Top Dev Biol,* 116, 357-374, 2016. DOI: 10.1016/bs.ctdb.2015.11.033

5. Azzawi S, Penzi LR, Senna MM: Immune privilege collapse and alopecia development: is stress a factor. *Skin Appendage Disord,* 4 (4): 236-244, 2018. DOI: 10.1159/000485080

6. Bertolini M, McElwee K, Gilhar A, Bulfone-Paus S, Paus R: Hair follicle immune privilege and its collapse in alopecia areata. *Exp Dermatol,* 29 (8): 703-725, 2020. DOI: 10.1111/exd.14155

7. Sun H, Meng K, Wang Y, Wang Y, Yuan X, Li X: LncRNAs regulate the cyclic growth and development of hair follicles in Dorper sheep. *Front Vet Sci,* 10:1186294, 2023. DOI: 10.3389/fvets.2023.1186294

8. Mo R, Chen Z, Yang Y, Jiang Y: Novel variant in WNT10A caused short anagen hair syndrome in a Chinese pedigree. *J Eur Acad Dermatol Venereol,* 38 (11): e976-e978, 2024. DOI: 10.1111/jdv.20035

9. Wang J, Wu X, Zhang L, Wang Q, Sun X, Ji D, Li Y: miR-133a-3p and miR-145-5p co-promote goat hair follicle stem cell differentiation by regulating NANOG and SOX9 expression. *Anim Biosci,* 37 (4): 609-621, 2024. DOI: 10.5713/ab.23.0348

10. Iwabuchi T, Ogura K, Hagiwara K, Ueno S, Kitamura H, Yamanishi H, Tsunekawa Y, Kiso A: Ginsenosides in *Panax ginseng* extract promote anagen transition by suppressing BMP4 expression and promote human hair growth by stimulating follicle-cell proliferation. *Biol Pharm Bull,* 47 (1): 240-244, 2024. DOI: 10.1248/bpb.b23-00276

11. Xiong L, Zhevlakova I, West XZ, Gao D, Murtazina R, Horak A, Brown JM, Molokotina I, Podrez EA, Byzova TV: TLR2 Regulates hair follicle cycle and regeneration via BMP signaling. *bioRxiv,* 20, 2023.08.14.553236, 2023. DOI: 10.1101/2023.08.14.553236

12. Wu Z, Wang Y, Han W, Yang K, Hai E, Ma R, Di Z, Shang F, Su R, Wang R, Wang Z, Zhang Y, Li J: EDA and EDAR expression at different stages of hair follicle development in cashmere goats and effects on expression of related genes. *Arch Anim Breed,* 63 (2): 461-470, 2020. DOI: 10.5194/aab-63-461-2020

13. Zhang J, Liu Y, Chang J, Zhang R, Liu Z, Liang J, Wang D, Feng J, Zhao W, Xiao H: Shh gene regulates the proliferation and apoptosis of dermal papilla cells to affect its differential expression in secondary hair follicle growth cycle of Cashmere goats. *Animals (Basel),* 14 (14):2049, 2024. DOI: 10.3390/ani14142049

14. Kang JI, Choi YK, Han SC, Nam H, Lee G, Kang JH, Koh YS, Hyun JW, Yoo ES, Kang HK: 5-bromo-3,4-dihydroxybenzaldehyde promotes hair growth through activation of Wnt/β-catenin and autophagy pathways and inhibition of TGF-β pathways in dermal papilla cells. *Molecules,* 27 (7):2176, 2022. DOI: 10.3390/molecules27072176

15. Vattathil SM, Tan SSM, Kim PJ, Bennett DA, Schneider JA, Wingo AP, Wingo TS: Effects of brain microRNAs in cognitive trajectory and Alzheimer's disease. *Acta Neuropathol,* 148 (1):59, 2024. DOI: 10.1007/s00401-024-02818-7

16. Zhang J, Chen L, Yu J, Tian W, Guo S: Advances in the roles and mechanisms of mesenchymal stem cell derived microRNAs on periodontal tissue regeneration. *Stem Cell Res Ther,* 15 (1):393, 2024. DOI: 10.1186/s13287-024-03998-5

17. Pickup ME, Hu A, Patel HJ, Ahmed MI: MicroRNA-148a controls epidermal and hair follicle stem/progenitor cells by modulating the activities of ROCK1 and ELF5. *J Invest Dermatol,* 143 (3): 480-491.e5, 2023. DOI: 10.1016/j.jid.2022.06.028

18. Warshauer E, Samuelov L, Sarig O, Vodo D, Bindereif A, Kanaan M, Gat U, Fuchs-Telem D, Shomron N, Farberov L, Pasmanik-Chor M, Nardini G, Winkler E, Meilik B, Petit I, Aberdam D, Paus R, Sprecher E, Nousbeck J: RBM28, a protein deficient in ANE syndrome, regulates hair follicle growth via miR-203 and p63. *Exp Dermatol,* 24 (8): 618-622, 2015. DOI: 10.1111/exd.12737

19. Zou X, Lu T, Zhao Z, Liu G, Lian Z, Guo Y, Sun B, Liu D, Li Y: Comprehensive analysis of mRNAs and miRNAs in the ovarian follicles of uniparous and multiple goats at estrus phase. *BMC Genomics,* 21 (1):267, 2020. DOI: 10.1186/s12864-020-6671-4

20. Qu H, Wu S, Li J, Ma T, Li J, Xiang B, Jiang H, Zhang Q: MiR-125b regulates the differentiation of hair follicles in fine-wool sheep and Cashmere goats by targeting MXD4 and FGFR2. *Anim Biotechnol,* 34 (2): 357-364, 2023. DOI: 10.1080/10495398.2021.1968884

21. Liu G, Li S, Liu H, Zhu Y, Bai L, Sun H, Gao S, Jiang W, Li F: The functions of ocu-miR-205 in regulating hair follicle development in Rex rabbits. *BMC Dev Biol,* 20:8, 2020. DOI: 10.1186/s12861-020-00213-5

22. Huang X, Zhang T, Li G, Guo X, Liu X: Regulation of miR-125a expression by rs12976445 single-nucleotide polymorphism is associated with radiotherapy-induced pneumonitis in lung carcinoma patients. *J Cell Biochem,* 120 (3): 4485-4493, 2019. DOI: 10.1002/jcb.27736

23. Wang F, Luo Q, Chen Y, Liu Y, Xu K, Adhikari K, Cai X, Liu J, Li Y, Liu X, Ramirez-Aristeguieta LM, Yuan Z, Zhou Y, Li FF, Jiang B, Jin L, Ruiz-Linares A, Yang Z, Liu F, Wang S: A genome-wide scan on individual typology angle found variants at SLC24A2 associated with skin color variation in Chinese populations. *J Invest Dermatol,* 142 (4): 1223-1227.e14, 2022. DOI: 10.1016/j.jid.2021.07.186

24. Wang L, Shao Z, Chen S, Shi L, Li Z: A SLC24A2 gene variant uncovered in pancreatic ductal adenocarcinoma by whole exome sequencing. *Tohoku J Exp Med,* 241 (4): 287-295, 2017. DOI: 10.1620/tjem.241.287

25. Jorgenson E, Choquet H, Yin J, Hoffmann TJ, Banda Y, Kvale MN, Risch N, Schaefer C, Asgari MM: Genetic ancestry, skin pigmentation, and the risk of cutaneous squamous cell carcinoma in Hispanic/Latino and non-Hispanic white populations. *Commun Biol,* 3 (1):765, 2020. DOI: 10.1038/s42003-020-01461-8

26. Childebayeva A, Rohrlach AB, Barquera R, Rivollat M, Aron F, Szolek A, Kohlbacher O, Nicklisch N, Alt KW, Gronenborn D, Meller H, Friederich S, Prufer K, Deguilloux MF, Krause J, Haak W: Population genetics and signatures of selection in early neolithic European farmers. *Mol Biol Evol,* 39 (6):msac108, 2022. DOI: 10.1093/molbev/msac108

27. Saito T, Okamura K, Kosaki R, Wakamatsu K, Ito S, Nakajima O, Yamashita H, Hozumi Y, Suzuki T: Impact of a SLC24A5 variant on the retinal pigment epithelium of a Japanese patient with oculocutaneous albinism type 6. *Pigment Cell Melanoma Res,* 35 (2): 212-219, 2022. DOI: 10.1111/pcmr.13024

28. Cai Y, Yu X, Hu S, Yu J: A brief review on the mechanisms of miRNA regulation. *Genomics Proteomics Bioinformatics,* 7 (4): 147-154, 2009. DOI: 10.1016/S1672-0229(08)60044-3

29. Liu Z, Xiao H, Li H, Zhao Y, Lai S, Yu X, Cai T, Du C, Zhang W, Li J: Identification of conserved and novel microRNAs in cashmere goat skin by deep sequencing. *PLoS One,* 7 (12):e50001, 2012. DOI: 10.1371/journal.pone.0050001

30. Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X, Quigg RJ: MicroRNA-377 is up-regulated and can lead to increased fibronectin production in diabetic nephropathy. *FASEB J,* 22 (12): 4126-4135, 2008. DOI: 10.1096/fj.08-112326

31. Azizi M, Fard-Esfahani P, Mahmoodzadeh H, Fazeli MS, Azadmanesh K, Zeinali S, Teimoori-Toolabi L: MiR-377 reverses cancerous phenotypes of pancreatic cells via suppressing DNMT1 and demethylating tumor suppressor genes. *Epigenomics,* 9 (8): 1059-1075, 2017. DOI: 10.2217/epi-2016-0175

32. Li B, Xu WW, Han L, Chan KT, Tsao SW, Lee NPY, Law S, Xu LY, Li EM, Chan KW, Qin YR, Guan XY, He QY, Cheung ALM: MicroRNA-377 suppresses initiation and progression of esophageal cancer by inhibiting CD133 and VEGF. *Oncogene,* 36 (28): 3986-4000, 2017. DOI: 10.1038/onc.2017.29

33. Zehavi L, Schayek H, Jacob-Hirsch J, Sidi Y, Leibowitz-Amit R, Avni D: MiR-377 targets E2F3 and alters the NF-kB signaling pathway through MAP3K7 in malignant melanoma. *Mol Cancer,* 14:68, 2015. DOI: 10.1186/s12943-015-0338-9

34. Hou Y, Zhou M, Li Y, Tian T, Sun X, Chen M, Xu W, Lu M: Risk SNP-mediated LINC01614 upregulation drives head and neck squamous cell carcinoma progression via PI3K/AKT signaling pathway. *Mol Carcinog,* 61 (8): 797-811, 2022. DOI: 10.1002/mc.23422

35. Reis LB, Bakos RM, Vianna FSL, Macedo GS, Jacovas VC, Ribeiro-Dos-Santos AM, Santos S, Bakos L, Ashton-Prolla P: Skin pigmentation polymorphisms associated with increased risk of melanoma in a case-control sample from southern Brazil. *BMC Cancer,* 20 (1):1069, 2020. DOI: 10.1186/s12885-020-07485-x