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Original Article

Foxo4 gene polymorphism is associated with the risk of talipes equinovarus in Chinese Uyghur

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Abstract: Objective: To investigate the association between Forkhead Box O (FoxO) family polymorphism and talipes equinovarus (TEV) in Chinese Uyghur population. Methods: A total of 70 Uyghur patients with TEV and 30 control volunteers were included in this experiment. Blood and muscle samples were collected from each individual, from which RNA and DNA were extracted. Allele and genotype frequencies for single SNP were calculated and tested for departure from Hardly-Weinberg equilibrium (HWE). The associations between different SNPs and risk of TEV were evaluated by logistic regression analysis. Linkage disequilibrium and haplotype blocks were measured and visualized using Haploview 4.2 and SHEsis Softwere. Results: FoxO3 RNA levels increased significantly in tissues of TEV patients in comparison of control group, whereas FoxO1 and FoxO4 RNA levels decreased dramatically. Among all the 7 haplotypes of FoxO4, ACTTGCA, ATTTGCA and TTTTGCA showed significant differences in distributions between TEV and control groups. Conclusion: FoxO4 was strongly associated with TEV phenotype in the Uyghur population. Future studies concerning this subject would conduct with large sample size and focus on the underlying mechanism.

Keywords: Talipes equinovarus, FoxO, single nucleotide polymorphism, linkage disequilibrium, haplotype

Introduction

Talipes equinovarus (TEV) is a common birth defect with characteristic forefoot adduction, midfoot cavus, hindfoot varus and equinus, and hypoplastic lower limb musculature [1]. The birth prevalence of TEV is about 1/1000, varying among ethnic groups from 1/150-2500 [2]. TEV always occurs without any other anomaly or known causes. Many approaches including genome scans and candidate gene testing have been used to identify its underlying causes [2].

The Forkhead Box O (FoxO) family includes four members: FoxO1, FoxO3a, FoxO4, and FoxO6. FoxO1, FoxO3a, and FoxO4 are ubiquitously expressed where as FoxO6 is mostly expressed in the brain [3]. Regulation of FoxO activity by growth or stress stimuli was shown important in preventing diseases such as cancer, diabetes and neurodegenerative disorders [4]. FoxOs are crucial in maintaining bone homeostasis upon their effects on the interactions between osteoblasts and osteoclasts [5-8].

Given the role of the FoxO family in bone and skeleton muscle development [9-11], this study was undertaken to determine whether variation in FoxO1, FoxO3a or FoxO4 genes is associated with TEV in Chinese Uyghur.

Materials and methods

Study subject

The study population consisted of 70 TEV patients and 30 controls, whom were all from Chinese Uyghur nationality. All individuals were recruited from in the Center of Cardiology at First Affiliated Hospital of Xinjiang Medical University, between Jan. 2006 and Oct 2011. Seventy TEV patients were diagnosed using standardized clinical criteria and radiographic examination as previously described [12]. Cases were excluded if they were caused by chromosomal abnormality or were syndromic. Informed consent was obtained from all of the individuals. The study protocol was approved by the institutional Ethics Review Board of the First Affiliated Hospital of Xinjiang Medical University.

Table 1. Summary of primer design for FoxO genes

Gene		Sequence
FoxO1-2-1	Forward	5'-GCACCCAGCCACAGATATTG-3'
	Reverse	5'-TGGCGGGTACACCATAGAAT-3'
FoxO1-2-2	Forward	5'-GAGTACATTTCGCCCTCGAAC-3'
	Reverse	5'-AGATGCCTGGCTGCCATAG-3'
FoxO1-2-3	Forward	5'-TGCGCCTGGACTCTTGAA-3'
	Reverse	5'-TGTGCTAACCATGGCAAGTTAC-3'
FoxO3-2-1	Forward	5'-CATCTGGGTGCTCGGTTTTG-3'
	Reverse	5'-TCCAGCAGGTCGTCCATGAG-3'
FoxO3-2-2	Forward	5'-AGCCTGTCACCTTCAGTAAGC-3'
	Reverse	5'-GCCCATGTTGCTGACAGA-3'
FoxO3-2-3	Forward	5'-TGCTGCCCAGCCTAACCA-3'
	Reverse	5'-GAACTGGCTGGGAGCCATA-3'
FoxO4 CE	Forward	5'-TTCGCTCGGCAGAGGTTA-3'
	Reverse	5'-CCTGCCAGTCTTCAGAAAGT-3'
FoxO4-1-2	Forward	5'-AGGCTTCACTGAACGCTGAG-3'
	Reverse	5'-GGGCCCCAGAGTAGAAGTAA-3'
FoxO4-2-1	Forward	5'-AGGGTCCAGCATCCTCTT-3'
	Reverse	5'-GTGTGTAAGGGGCCGGTAA-3'
FoxO4-2-2	Forward	5'-TGGGCTCAATCTCACCTCTT-3'
	Reverse	5'-AGGAGCTCTGGCTACCACTTG-3'
FoxO4-3-1	Forward	5'-GGTTAGGTGCCACCATCTTC-3'
	Reverse	5'-TCTGCAGCCTTCTAAGGGTGAT-3'
FoxO4-3-2	Forward	5'-TTCTATGCAGTGGCCCCTTA-3'
	Reverse	5'-CCAGGCCTTCAATGTACTCTC-3'
FoxO4-3-3	Forward	5'-CAACTTGTGCCTGGGAGTGT-3'
	Reverse	5'-TTCTTCCTCCCGCCCTTT-3'

Sample collection and preparation

A total of 5 ml cubital vein blood was collected from each individual using vacuum tubes with or without EDTA, and plasma or serum was separated, respectively. Muscle samples were collected by biopsy gun and transferred into liquid nitrogen in micro tubes. All samples were stored at -80°C.

DNA isolation and genotyping

The DNA from whole blood was extracted by using Blood Genomic DNA Isolation Kit according to the manufacturer's protocol. Afterwards, 1 ul of DNA solution was transferred to NanoDrop Spectrophotometer (ND2000) to determine the purity and concentration. All purified genomic DNA samples were amplified by polymerase chain reaction (PCR). The design of detection primers were summarized in **Table 1**.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was extracted from muscle tissues using Trizol following the manufacturer's protocol. Reverse transcription was performed by using MLT RT Kit. And real-time quantitative polymerase chain reaction (qRT-PCR) was inducted using SYBR Green PCR Master Mix. The pairs of PCR primers for amplification of FoxO1, FoxO3, FoxO4, and β-actin were 5'-AATTCAATTCGTCATAATCTGTC-3'/5'-CGAAATGTACTCCAGTTATCAAAG-3'; 5'-AAAGGAAACCCTAACAGCTC-3'/5'-TCCGCTTCAAG-ACCTCATTTG-3'; 5'-TTCCCATTCCCTGCTATCTC-3'; and 5'-ACTGGAACGGTGAAGGTGAC-3'/5'-CAGTGTACAGGTAAGCCCTG-3', respectively. All the RNA expression levels were calculated using the $2^{(-\Delta CT)}$ values. And all qRT-PCR reactions were performed in triplicate.

Genotyping of SNPs

The tagging SNPs rs3751436, rs199728685 rs139436481 were selected for FoxO1, FOXO3-EXON2-461, rs199697163 were selected for FoxO3, and FOX04-EXON1-348, rs199927821, rs5980742, FOX04-ENON3-386, rs147716329, rs4503258, rs184012791 were selected for FoxO4.

Statistical ananlysis

Statistical analysis was performed with SPSS statistics package, version 18.0. Allele and genotype frequencies for single SNP were calculated and tested for departure from Hardly-Weinberg equilibrium (HWE) by χ^2 test. The associations between different SNPs and risk of TEV were evaluated by computing odds ratios (ORs) at 95% confidence intervals (CIs) using logistic regression analysis. Linkage disequilibrium and haplotype blocks were measured and visualized using Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview) and SHEsis Softwere (online version: http://analysis.bio-x.cn/SHEsisMain.htm). All statistical analyses were two-tailed, and a $P < 0.05$ was considered statically significant.

Results

Identification of PCR products

Of all the PCR products from whole blood samples, 13 SNPs were successfully identified, as

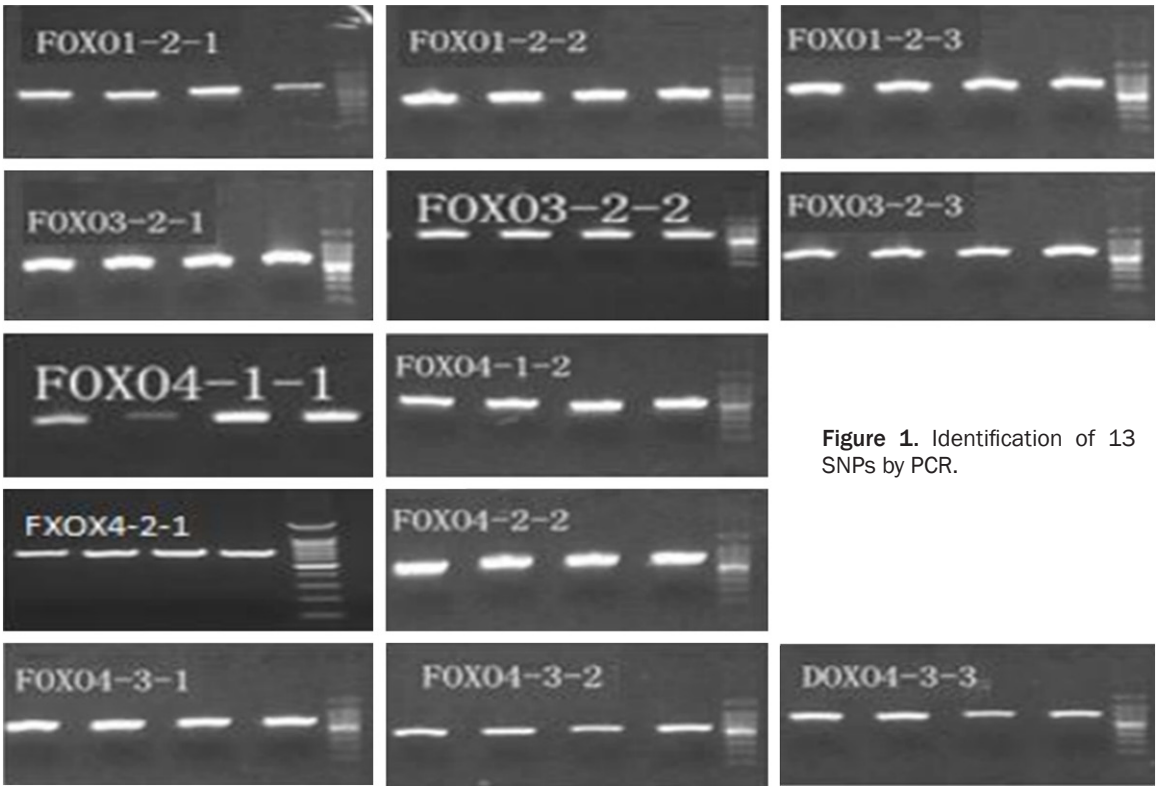

Figure 1. Identification of 13 SNPs by PCR.

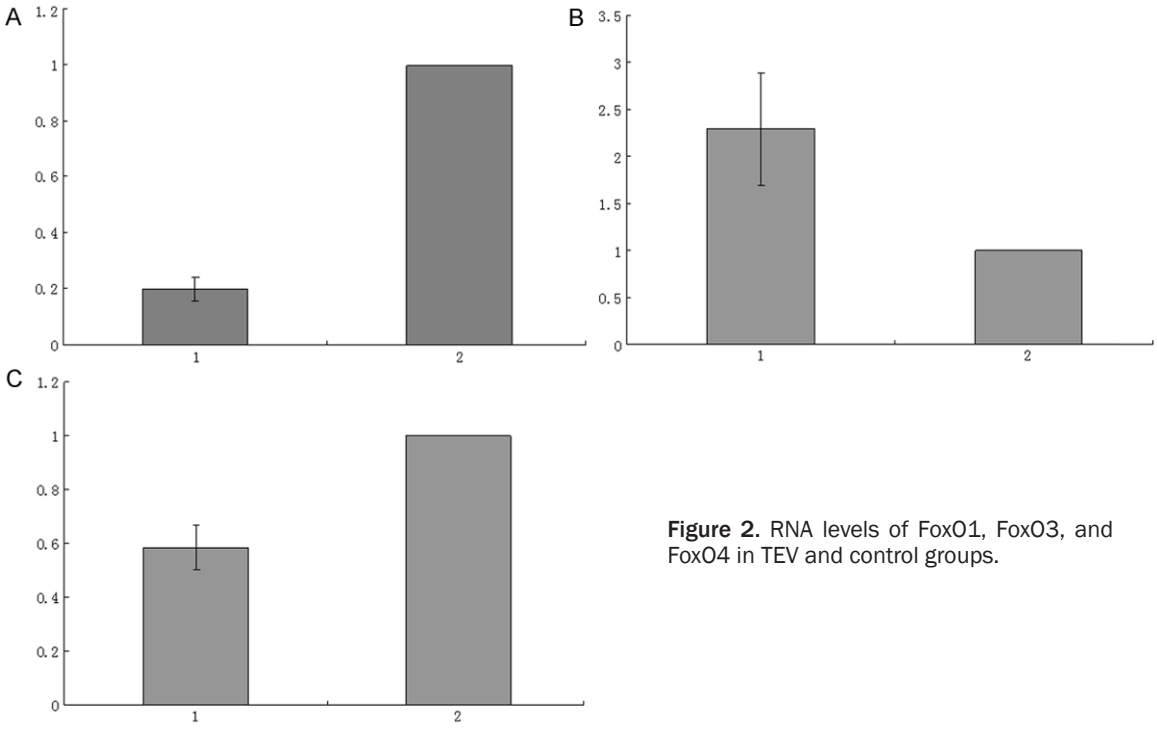

Figure 2. RNA levels of FoxO1, FoxO3, and FoxO4 in TEV and control groups.

shown in **Figure 1**. All the sequencing results were normal except that bimodal existed in FOXO3-2-2 which attributed to a base deletion or insertion.

RNA levels of FoxO1, FoxO3, and FoxO4

As shown in **Figure 2**, FoxO3 RNA levels increased significantly in tissues of TEV patients in

Table 2. Hardy-Weinberg equilibrium test for SNPs

SNP	Geno type	Case		P	Control		P
		Observed	Expected		Observed	Expected	
rs3751436	AA	40	40.89	0.555	11	12.45	0.234
	AG	27	25.22		16	13.10	
	GG	3	3.89		2	3.45	
rs199728685	CC	69	69.00	0.952	29	29.00	1.000
	CT	1	0.99		0	0.00	
	TT	0	0.00		0	0.00	
rs139436481	CC	70	70.00	1.000	28	28.01	0.952
	CT	0	0.00		1	0.98	
	TT	0	0.00		0	0.01	
rs199697163	CC	1	0.01	0.000	0	0.00	1.000
	CG	69	1.97		29	0.00	
	GG	0	68.01		0	29.00	
F0X04-EXON1-348	AA	14	0.70	0.353	21	3.68	0003
	AT	56	12.60		9	13.65	
	TT	0	56.70		0	12.68	
rs199927821	CC	1	0.00	0.952	2	0.03	0.850
	CT	69	0.99		28	1.93	
	TT	0	69.00		0	28.03	
rs5980742	GG	19	8.25	0.000	6	3.68	0.062
	GT	10	31.54		9	13.65	
	TT	41	30.23		15	12.68	
F0X04-EN0N3-386	CC	1	0.01	0.000	0	0.00	1.000
	CT	69	1.97		30	0.00	
	TT	0	68.01		0	30.00	
rs147716329	AA	1	0.00	0.952	0	0.00	1.000
	AG	69	0.99		30	0.00	
	GG	0	69.00		0	30.00	
rs4503258	CC	62	57.52	0.000	26	24.30	0.001
	CT	2	10.96		2	5.40	
	TT	5	0.52		2	0.30	
rs184012791	AA	69	69.00	1.000	29	29.03	0.000
	AC	0	0.00		1	1.93	
	CC	0	0.00		0	0.03	

comparison of control group, whereas Fox01 and Fox04 RNA levels decreased dramatically.

Hardy-Weinberg equilibrium for SNPs

Among 3 SNPs selected for Fox01 (rs3751436, rs199728685 rs139436481), no significant deviation from Hardy-Weinberg equilibrium test was found for all 3 SNPs in both groups. In 2 SNPs selected for Fox03, we found a significant deviation in TEV group for rs199697163 according to HWE (CC/CG/GG: 1/69/0 in observed counts, 0.01/0.97/68.1 in expected counts, P < 0.001). And among 7 SNPs for Fox04, we found significant deviations in control groups for F0X04-E XON1-348, rs4503258, rs184-012791, and in TEV groups for rs5980742, F0X04-EN0N3-386, rs4503258 (**Table 2**).

Univariate analysis of SNPs and talipes equinovarus

We listed frequency distributions of all the tag SNPs in both TEV group and control group, including 3 SNPs associated with Fox01 (rs-3751436, rs199728685, rs139436481), 2

Table 3. Frequency distributions of FoxO SNPs in TEV and control groups

Tag SNPs	Allele 1/2	Groups	Genotype (n, %)			P	MAF	P
			1/1	1/2	2/2			
rs3751436	A/G	Case	40 (0.571)	27 (0.386)	3 (0.043)	0.218	0.236	0.115
		Control	11 (0.379)	16 (0.552)	2 (0.069)		0.345	
rs199728685	C/T	Case	69 (0.986)	1 (0.014)	0 (0.000)	0.518	0.007	0.519
		Control	29 (1.000)	0 (0.000)	0 (0.000)		0.000	
rs139436481	C/T	Case	70 (1.000)	0 (0.000)	0 (0.000)	0.118	0.000	0.119
		Control	28 (0.966)	1 (0.034)	0 (0.000)		0.017	
F0X03-EXON2-461	G/C	Case	1 (0.014)	69 (0.986)	1 (0.014)	0.511	0.007	0.512
		Control	0 (0.000)	30 (1.000)	0 (0.000)		0.000	
rs199697163	G/C	Case	1 (0.014)	69 (0.986)	1 (0.014)	0.518	0.014	0.360
		Control	0 (0.000)	29 (1.000)	0 (0.000)		0.000	
F0X04-EXON1-348	T/A	Case	14 (0.200)	56 (0.800)	0 (0.000)	0.000	0.100	0.000
		Control	21 (0.700)	9 (0.300)	0 (0.000)		0.350	
rs199927821	T/C	Case	1 (0.014)	69 (0.986)	0 (0.000)	0.159	0.007	0.163
		Control	2 (0.067)	28 (0.933)	0 (0.000)		0.033	
rs5980742	T/G	Case	41 (0.586)	10 (0.143)	19 (0.271)	0.180	0.343	0.922
		Control	15 (0.500)	9 (0.300)	6 (0.200)		0.350	
F0X04-ENON3-386	T/C	Case	1 (0.014)	69 (0.986)	1 (0.014)	0.511	0.014	0.352
		Control	0 (0.000)	30 (1.000)	0 (0.000)		0.000	
rs147716329	G/A	Case	1 (0.014)	69 (0.986)	1 (0.014)	0.511	0.007	0.512
		Control	0 (0.000)	30 (1.000)	0 (0.000)		0.000	
rs4503258	C/T	Case	62 (0.899)	2 (0.029)	5 (0.072)	0.681	0.087	0.769
		Control	26 (0.867)	2 (0.067)	2 (0.067)		0.100	
rs184012791	A/C	Case	69 (1.000)	0 (0.000)	0 (0.000)	0.127	0.000	0.031
		Control	29 (0.967)	1 (0.033)	0 (0.000)		0.033	

1: major allele; 2: minor allele; MAF: minor allele frequency.

SNPs associated with FoxO3 (F0X03-EXON2-461, rs199697163), and 7 SNPs associated with FoxO4 (F0X04-EXON1-348, rs199927821, rs5980742, F0X04-ENON3-386, rs147716329, rs4503258, rs184012791). As shown in **Table 3**, only F0X04-EXON1-348 showed significant association with TEV ($P < 0.001$). However, according to HWE test, a significant deviation was found in control group for F0X04-EXON1-348 (CC/CG/GG: 1/69/0 in observed counts, 0.01/0.97/68.1 in expected counts, $P < 0.001$) (**Table 2**), which indicated that the result for F0X04-EXON1-348 was not reliable. Therefore, results of univariate analysis suggested that no significant difference was found among different SNPs.

Linkage disequilibrium and haplotype analysis

The linkage disequilibrium parameter (|D'|) of 3 SNPs selected for FoxO1 (rs3751436, rs199728685, rs139436481) were greater than 0.5 in both TEV and control groups, indicating that all the three SNPs located in the same haplotype block. Meanwhile, $r^2 < 0.5$ in both groups suggested haplotype could be established. Similarly, 1 haplotype could be established out of 2 SNPs associated with FoxO3, and 7 haplotypes could be established out of 7 SNPs associated with FoxO4 (**Figure 3**).

For FoxO1, the haplotypes ACC and GCC showed no significant difference in their distributions between TEV and control group. And the haplotype GG of FoxO3 present no significant difference in distributions either. The 7 haplotypes of FoxO4 were summarized in **Table 4**. Among all the 7 haplotypes, ACTTGCA, ATTTGCA and TTTTGCA showed significant differences in distributions between TEV and case groups (*P* values: 0.031, 0.002, and 0.014, respectively). And the 95% confidence intervals indicating that haplotypes ACTTGCA and ATTTGCA (95% CI, 0.082-0.604) were protective factors for

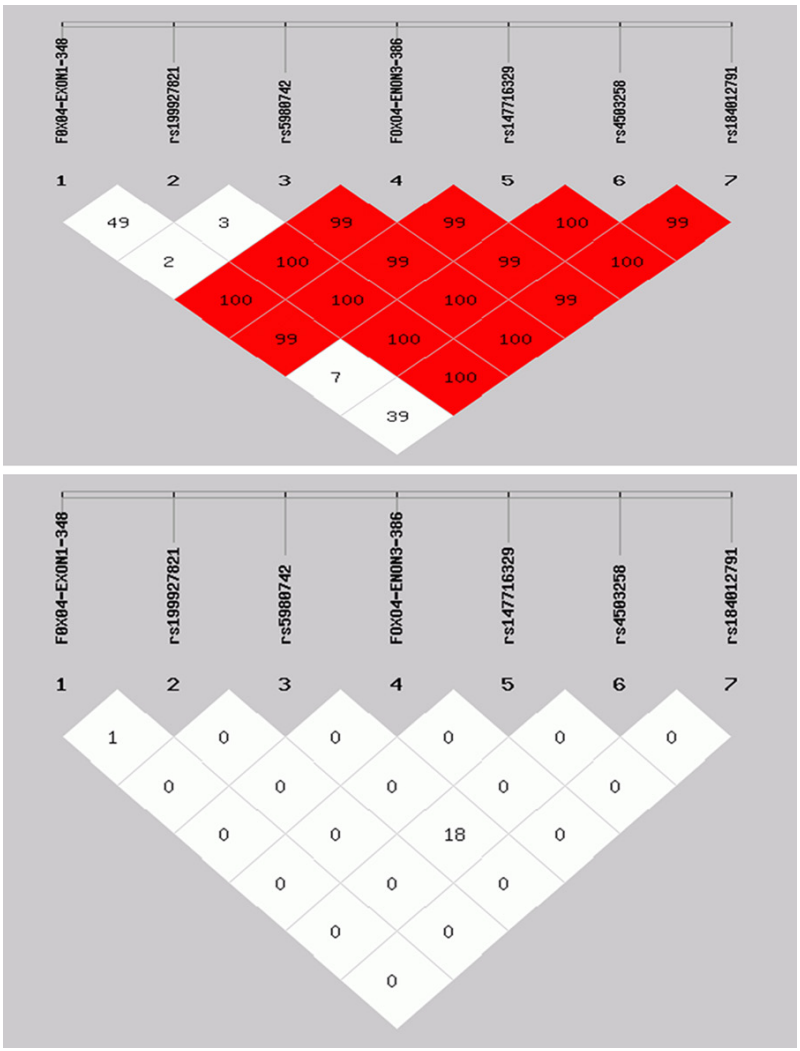

Figure 3. Linkage disequilibrium of 7 SNPs for FoxO4.

TEV and TTTTGCA (95% CI, 1.162-4.077) was risk factor.

Discussion

In this study, we demonstrated that FoxO4 is strongly associated with TEV phenotype in the Uyghur nationality population. Furthermore, this study identified protective and high-risk haplotypes for TEV. According to the haplotype analysis, 3 haplotypes established out of seven SNPs associated with FoxO4 were related with TEV phenotype, among which ACTTGCA and ATTTGCA were protective factors for TEV and TTTTGCA was risk factor.

There are two types of TEV, idiopathic and syndromic (those associated with other syndromes or conditions). In both the cause is not clear, although hypotheses include associated transient gene activity, neuromuscular disease [13], and lack of foetal movement [14]. TEV is a complex disorder, and emerging literature suggests a polygenic cause [15]. Apoptosis has been shown to play an important role in later limb development by shaping muscle and tendons recently. Studies have demonstrated that variations of many genes mediated apoptosis might affected muscle and limb development, which might contribute to TEV [16].

FoxO family are key regulators in major cellular processes, for example, proliferation, differentiation, metabolism as well as cell death [17]. Recent studies discovered the importance of FoxO signalling in bone, through FoxO deletion in early osteoblast [9]. FoxO loss in osteoblasts [5] and gain of function in osteoclasts [6] and dysregulation of FoxO activity in articular cartilage are found associated with osteoarthritis [18, 19]. Eelen *et al*. showed that complete loss of Fox expression in chondrocytes by stimulating Collagen2-Cre mediated inactivation of FoxO1, FoxO3a and FoxO4, would lead to severe growth plate abnormalities [11].

The FoxO family serve as the direct downstream signalling molecules of AKT1 in insulin/insulin-like growth factor signalling pathway. In vivo, FoxO family regulate the cell cycle and growth, apoptosis, DNA damage responses and angiogenesis. In skeletal muscle, FoxO activation may induce apoptosis in a DNA-biding-dependent manner in mature tubes [20]. Apoptosis can induct mitochondria associated pro-apoptotic genes and DNA fragmentation, leading to skeletal muscle atrophy [21]. The apoptotic

Table 4. Haplotype analysis for FoxO4

Haplotype	Case	Control	X^2	*P*	OR	95% CI
ACTTGCA	0.00 (0.000)	2.00 (0.033)	4.667	0.030801	0.030774	-
ATGTGCA	5.00 (0.036)	3.02 (0.050)	0.219	0.639530	0.639532	0.163-3.050
ATGTGTA	1.00 (0.007)	3.47 (0.058)	4.738	0.029547	0.029522	0.018-0.792
ATTTGCA	7.00 (0.051)	11.51 (0.192)	9.931	0.001635	0.001633	0.082-0.604
TTGTGCA	30.00 (0.217)	11.98 (0.200)	0.071	0.790083	0.790071	0.521-2.356
TTGTGTA	11.00 (0.080)	2.53 (0.042)	0.919	0.337913	0.337856	0.483-7.997
TTTTGCA	80.00 (0.580)	23.49 (0.392)	6.004	0.014308	0.014295	1.162-4.077

genes, Bcl-2-interactiong mediator and BNIP, can be induced by FoxO1 during muscle atrophy [22]. Taken together, previous findings suggested association between FoxO family and bone formation as well as skeleton muscle development.

In conclusion, the present study found that FoxO4 was strongly associated with TEV phenotype in the Uyghur nationality population. Future studies concerning this subject would conduct with large sample size and focus on the underlying mechanism.

Acknowledgements

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Disclosure of conflict of interest

None.

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