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Gender flip-flop association between genetic variations of *NEDD4L* and metabolic syndrome in the Kazakh general population

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ABSTRACT. Genetic variation is thought to contribute to etiology of metabolic syndrome (MS). Neural precursor cell expressed developmentally downregulated 4-like gene (*NEDD4L*) is a candidate gene for MS. This study investigated the relationship between variations of *NEDD4L* and MS in the Kazakh, which is an ideal population to study the genetic mechanisms of complex diseases such as MS. We screened the promoter and exons of *NEDD4L* in 48 Kazakh individuals with MS to identify representative variations. By genotyping the representative variations [271420T>C (rs2288774), 271454A>G (rs2288775), and 296921-296923delTTG] in the Kazakh general population, we conducted a case-control study. In female subjects, the distribution of genotypes and alleles of rs2288775 and 296921-296923delTTG differed significantly between the MS pacients and controls. In male subjects, the genotype distributions of 296921-296923delTTG were significantly different between the MS pacients and controls in the dominant model (P = 0.047). After adjustment for age, smoking, and drinking, multivariate logistic regression analysis showed that rs2288775 was significantly associated

with MS [for the A/A genotype, odds ratio (OR) = 3.296, P = 0.011] in female subjects. For 296921-296923delTTG, the I/D+D/D genotype was the high-risk genotype for MS in female subjects (OR = 2.791, P = 0.035) and was a protective factor for MS in male subjects (OR = 0.580, P = 0.045). The 296921-296923delTTG variation of *NEDD4L* is a gender flip-flop associated with MS in Kazakh individuals. The A allele of rs2288775 may be an independent risk factor for MS in Kazakh women. The results suggest that the genetic variations of *NEDD4L* might be involved in the pathogenesis of MS.

Key words: Metabolic syndrome; Kazakh; Flip-flop; *NEDD4L* gene; Neural precursor cell expressed developmentally downregulated 4-like

INTRODUCTION

Metabolic syndrome (MS), which includes elevated blood pressure, dyslipidemia, hyperglycemia, or insulin resistance, and obesity, has become the major health problem worldwide (Gami et al., 2007), but the pathophysiology and etiology of MS remain poorly understood. Evidence indicates that genetic factors modulate individual predisposition for the development of these components of MS (Bray and Bouchard, 2002; Elbein et al., 2002; Fava et al., 2004). Therefore, identifying susceptible genes in populations is important for the prevention and treatment of the disease.

Systemic inflammation plays an important role in obesity, and growing evidence suggests that cellular and molecular mechanisms involving inflammatory mediators are upregulated in patients with MS (Das, 2002). The ubiquitin-proteasome system, the major pathway for nonlysosomal intracellular protein (up to 80-90% of all intracellular proteins) degradation in eukaryotic cells, is required for the activation of nuclear factor κB (NF-κB) (Palombella et al., 1994). NF-κB is the key transcription factor in the inflammatory response, and activated NF-κB controls the expression of many genes, including those encoding inflammatory cytokines such as tumor necrosis factor alpha and interleukin-6, chemokines such as interleukin-8, adhesion molecules such as intercellular adhesion molecule-1, and cell receptors (Willerson and Ridker, 2004). Therefore, the ubiquitin-proteasome system may play an important role in the pathogenesis of MS. Neural precursor cell expressed developmentally downregulated 4-like (NEDD4L), a ubiquitin ligase enzyme, is a critical component of the ubiquitin-proteasome system. Therefore, *NEDD4L* is a candidate gene for MS. The variation of *NEDD4L* has a crucial role in the pathogenesis of hypertension (Russo et al., 2005; Araki et al., 2008; Manunta et al., 2008; Wen et al., 2008). However, no reports have been published about the relationship between genetic variations in human *NEDD4L* and MS.

The Kazakh, a nomadic population predominantly made up of herders who dwell primarily north of Xinjiang in northwest China, is characterized by a higher prevalence of hypertension, obesity, and dyslipidemia than that in other ethnic populations living in the same area (Li et al., 2003). Moreover, Kazakhs rarely marry individuals of other ethnicities as a matter of custom, and their cultural background and dietary habits differ from those of other ethnic groups. Therefore, the Kazakh is a relative isolated population with a pure genetic background, making it an ideal population to study the genetic mechanisms of complex diseases such as MS.

The National Center for Biotechnology Information and HapMap project provide no genetic information for the Xinjiang Kazakh, and we were unable to use the tag single nucleotide polymorphisms (SNPs) specific to the Kazakh in this study. Hence, we sequenced the promoter and all exons of *NEDD4L* in 48 Xinjiang Kazakh MS individuals to identify representative SNPs and then genotyped the representative variations in the general population to study systemically whether genetic variations in *NEDD4L* are implicated in MS in the Xinjiang Kazakh general population.

MATERIAL AND METHODS

Study population

A total of 1000 Kazakh subjects aged 30 to 60 years without mixed marriages within the past three generations were randomly recruited for this study using multistage cluster sampling from the Fukang area in Xinjiang Uygur Autonomous Region. Written consent was obtained from all subjects before any data collection and measurements. A total of 956 individuals completed the survey during the 1-month period from January to February 2008, with an overall response rate of 95.6%. Subjects with cancer, pregnancy, or other acute and chronic inflammation were excluded from this study (N = 61). Participants with missing body mass index, high-density lipoprotein cholesterol, triglycerides, fasting glucose, systolic blood pressure, or diastolic blood pressure measurements were also excluded from the analyses (N = 54), leaving a final study population of 841 subjects, including 359 men and 482 women. A case-control study was then conducted. This study was approved by the Ethics Committee of the People's Hospital of Xinjiang.

Diagnostic criteria and measurements

A set of completed questionnaires containing demographic data, anthropometric measurement (e.g., blood pressure, height, weight), and fasting blood were obtained. Serum was separated immediately and stored at -80ºC. Serum lipids and fasting blood glucose were determined using enzymatic methods with an auto analyzer (7600-010 Automatic Analyzer; HITACHI Medical System, Suzhou, China). Quality controls were conducted with a special docimaster. All blood samples were examined within a month in the Clinical Center of People's Hospital of Xinjiang Uygur Autonomous Region. MS was diagnosed according to the criteria of the Chinese Diabetes Society (Expert Panel on Metabolic Syndrome of Chinese Diabetes Society, 2004).

Screening of genetic variations in *NEDD4L* in MS patients

We sequenced all exons and the promoter region of *NEDD4L*. Blood samples were obtained from 48 MS patients (24 men and 24 women) randomly chosen from the MS group of the study population, and genomic DNA was isolated from peripheral blood leukocytes using a PAXgene Blood DNA kit (QIAGEN GmbH, Hiden, Germany). All exons with their flanking sequences and approximately 500 bp of the upstream region of the promoter were directly sequenced with an ABI 3130Xl genetic analyzer (Applied Biosystems, Foster City, CA, USA) using 35 sets of primers described elsewhere (Okuda et al., 2002). The obtained sequences

were examined for the presence of variations using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) followed by visual inspection. The A of the ATG of the initiator Met codon was denoted nucleotide +1. The nucleotide sequence (National Center for Biotechnology Information accession ID: NM-015277) was used as a reference sequence.

Genotyping novel polymorphisms in the general population

Three representative variations with a minor allele frequency greater than 10% were selected for genotyping (Table 1). TaqMan SNP Genotyping Assays were performed for genotyping in a 7900HT Fast Real-Time PCR system (Applied Biosystems). Finally, three representative variations were successfully genotyped in all participants.

Statistical analysis

Data analyses were performed using the SPSS software for Windows (version 16; SPSS Inc., Chicago, IL, USA). The distribution of patient characteristics between the metabolic and control groups in the Kazakh general population was analyzed using the Student *t*-test or a chi-square test. The differences in distributions of genotypes and alleles between the MS patients and controls were analyzed using a chi-square test. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors (including smoking, drinking, and age). A case-control-based haplotype study (permutation test), linkage disequilibrium, and Hardy-Weinberg equilibrium (HWE) were analyzed using SNPAlyze, version 7.0 Pro (DYNACOM Co. Ltd., Mobara, Japan). Statistical significance was established at P values of <0.05.

RESULTS

Eight genetic variations in *NEDD4L* were identified by sequencing 48 MS individuals. Three common variations were found with a minor allele frequency of >10% (271420T>C, 271454A>G, and 296921-296923delTTG). No missense mutations in *NEDD4L* were identified. None of the variations were in tight linkage disequilibrium ($r^2 < 0.5$; see Table 1).

Table 1. Sequence variations in the promoter region and exons in *NEDD4L* identified in Kazakh metabolic syndrome patients.

SNP name	LD	Region	Amino acid substitution	Allele 1 frequency	Allele 2 frequency	Flanking sequence	Typing	db SNP ID
-608C>G	-	promoter		0.9896	0.0104	cgccgcgccg[c/g]gctggaggcc		
271420T>C	-	intron 6		0.6146	0.3854	ttgtagtcag[t/c]gtattttcag	Taqman	rs2288774
271454A>G	-	intron 6		0.7708	0.2292	caccacggtg[a/g]agaaggctga	Taqman	rs2288775
296921-296923delTTG	-	intron 13		0.6979	0.3021	ttgcgttg[ttg/---]tttgggtt	Taqman	
312644T>G	-	intron 18		0.9792	0.0208	tcttgtgtat[t/g]actgataacg		
312671C>T	-	intron 18		0.9687	0.0313	caagggacaa[c/t]ggtgattgag		
325828A>G	-	intron 22		0.9792	0.0208	atcaccgggc[a/g]ctcgtgtctt		
351555C>T		exon 30	A944A	0.9896	0.0104	TTCTCATGGC[C/T]GTGGAAAATG		

Sequence variations were screened in 48 metabolic syndrome patients. The apparent linkage disequilibrium (LD) was defined by r-square more than 0.5 and there is no LD between all sequenced variations in the LD column. Taqman, the single nucleotide polymorphism (SNP), was successfully genotyped by the Taqman method. The A from the ATG of the initiator Met codon is denoted nucleotide +1, as recommended by the Nomenclature Working Group (Antonarakis, 1998).The nucleotide sequence (NCBI accession ID NM-015277) was used as a reference sequence.

Next, we looked for an association in the Kazakh general population among the three polymorphisms of *NEDD4L* (271420T>C, 271454A>G, and 296921-296923delTTG) and MS. Table 2 shows the clinical characteristics of the study participants. For men, women, and total participants, the following values were significantly higher in MS patients than in controls: body mass index, systolic blood pressure, diastolic blood pressure, triglycerides, high-density lipoprotein cholesterol, and fasting blood glucose (all $P < 0.001$).

Table 2. Basic characteristics of metabolic syndrome patients and controls.

	Total			Male			Female		
	Control (N = 760)	Case (N = 81)	P	Control (N = 301)	Case (N = 58)	P	Control (N = 459)	Case (N = 23)	P
Age (years)	43.71 ± 7.745	45.42 ± 7.389	0.058	43.93 ± 7.944	44.81 ± 7.466	0.436	43.56 ± 7.616	46.96 ± 7.119	0.037
BMI (kg/m^2)	26.05 ± 3.915	31.02 ± 4.747	<0.001	26.13 ± 3.375	30.68 ± 5.000	<0.001	26.00 ± 4.234	31.88 ± 4.009	<0.001
SBP (mmHg)	129.29 ± 21.082	152.15 ± 20.529	<0.001	129.76 ± 19.193	148.47 ± 19.346	<0.001	128.97 ± 22.249	161.43 ± 20.905	<0.001
DBP (mmHg)	84.88 ± 12.238	100.27 ± 10.119	<0.001	85.97 ± 11.180	99.51 ± 9.258	<0.001	84.15 ± 12.850	102.20 ± 12.034	<0.001
TG (mM)	0.98 ± 0.521	2.17 ± 0.843	<0.001	1.16 ± 0.643	2.39 ± 0.821	<0.001	0.86 ± 0.378	1.61 ± 0.618	<0.001
HDL-C (mM)	1.49 ± 0.380	1.17 ± 0.245	<0.001	1.32 ± 0.332	1.15 ± 0.226	<0.001	1.59 ± 0.370	1.20 ± 0.291	<0.001
FBG (mM)	4.84 ± 0.736	5.85 ± 2.271	0.001	4.91 ± 0.602	5.85 ± 2.492	<0.001	4.80 ± 0.810	5.84 ± 1.632	<0.001
Life style									
Smoking (%)	21.6	40.7	<0.001	53.2	56.9	0.601	0.9	0.0	-
Drinking (%)	20.1	35.5	0.001	48.5	50.0	0.853	1.5	0.0	-

BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; TG = triglyceride; HDL-C = high-density lipoprotein cholesterol; FBG = fasting blood glucose. Values are reported as mean ± standard deviation or percentage. $P < 0.05$ was considered to be significant. The differences between case and control groups were performed by the *t*-test or χ^2 test.

Significant differences in age for women and in lifestyle (smoking and drinking) for the total population were found ($P = 0.038$, $P < 0.001$, and $P = 0.001$, respectively).

Table 3 shows the distribution of the genotypes and alleles of the three common variations. The genotype distribution of each variation was in HWE (data not shown). Power analyses revealed that our study had powers of 0.90 (for the D-carrying genotype of 296921-296923delTTG) and 0.80 (for the G-carrying genotype of rs2288775) to detect differences across cases and controls ($\alpha = 0.05$). For total participants, the genotype distribution of the three SNPs did not differ significantly between the MS patients and controls. For female subjects, the distribution of the additive, dominant, and recessive models and alleles of rs2288775 ($P = 0.004$, $P = 0.011$, $P = 0.004$, and $P = 0.001$, respectively) and 296921-296923delTTG ($P = 0.013$, $P = 0.034$, $P = 0.009$, and $P = 0.004$, respectively) differed significantly between MS patients and controls. In male subjects, the genotype distributions of 296921-296923delTTG were significantly different between the MS patients and controls in the dominant model ($P = 0.047$). Moreover, for 296921-296923delTTG, the frequency of I/I was higher than that of I/D+D/D in male MS patients and lower than that of I/D+D/D in female MS patients.

After adjustment for confounding factors (age, smoking, and drinking), multivariate logistic regression analysis also showed that rs2288775 was significantly associated with MS [A/A genotype: odds ratio (OR) = 3.296, 95% confidence interval (95%CI) = 1.322-8.221, $P = 0.011$] in female subjects (Table 4). Moreover, for 296921-296923delTTG, the I/D+D/D genotype was a high-risk genotype for MS in female subjects (OR = 2.791, 95%CI = 1.078-7.246, $P = 0.035$) and a protective factor for MS in male subjects (OR = 0.580, 95%CI = 0.304-0.988, $P = 0.045$).

Table 3. Genotype and allele distributions in metabolic syndrome patients and controls.

	Total			Male			Female		
	Case (N = 760)	Control (N = 81)	P	Case (N = 301)	Control (N = 58)	P	Case (N = 459)	Control (N = 23)	P
rs2288774 N (%)									
Additive model									
T/T	248	29	0.768	97	25	0.220	151	4	0.261
C/T	351	37		147	25		204	12	
C/C	153	14		54	7		99	7	
Dominant model									
T/T	248	29	0.555	97	25	0.100	151	4	0.113
T/C+C/C	504	52		201	32		303	19	
Recessive model									
T/T+C/T	599	66	0.546	244	50	0.284	355	16	0.332
C/C	153	14		54	7		99	7	
Allele									
T	657	65	0.458	341	75	0.089	402	26	0.103
C	847	95		255	39		506	20	
rs2288775 N (%)									
Additive model									
A/A	435	47	0.589	172	40	0.275	263	7	0.004
A/G	276	27		110	16		166	11	
G/G	45	7		16	2		29	5	
Dominant model									
A/A	435	47	0.931	172	40	0.110	263	7	0.011
A/G+G/G	321	34		126	18		195	16	
Recessive model									
A/A+AG	711	74	0.341	282	56	0.541	429	18	0.004
G/G	45	7		16	2		29	5	
Allele									
A	1146	121	0.756	454	96	0.122	692	25	0.001
G	366	41		142	20		224	21	
296921-296923delTTG N (%)									
Additive model									
I/I	374	44	0.611	154	38	0.113	220	6	0.013
I/D	312	29		122	18		190	11	
D/D	66	8		24	2		42	6	
Dominant model									
I/I	374	44	0.433	154	38	0.047	220	6	0.034
D/D+I/D	378	37		146	20		232	17	
Recessive model									
I/I+I/D	686	73	0.741	276	56	0.221	410	17	0.009
D/D	66	8		24	2		42	6	
Allele									
I	1060	117	0.643	430	94	0.037	630	23	0.004
D	444	45		170	22		274	23	

Table 4. Odds ratios and 95% confidence intervals for two variations of the *NEDD4L* gene associated with metabolic syndrome after adjusting gender, smoking, drinking, and age.

Polymorphism	Genotype	Odds ratio	95% confidence interval	P
Female				
rs2288775	A/G+G/G	1		
	A/A	3.296	1.322-8.221	0.011
296921-296923delTTG	I/I	1		
	I/D+D/D	2.791	1.078-7.246	0.035
Male				
296921-296923delTTG	I/I	1		
	ID+D/D	0.580	0.304-0.988	0.045

In the haplotype-based case-control analysis, haplotypes were established in three representative common SNPs (Table 5). For the total population, the distribution of the haplotypes was not significantly different between the MS patients and controls. In male subjects, the frequency of the I-T-A haplotype (established by 296921-296923delTTG, rs2288774, and rs2288775) was significantly higher in MS patients than in controls (P = 0.022). However, for females subjects, the frequency of the D-C-G haplotype (established by another allele of 296921-296923delTTG, rs2288774, and rs2288775) was significantly higher in MS patients than in controls (P < 0.001).

Table 5. Haplotype analysis in metabolic syndrome patients and controls.

	Haplotype			Total			Male			Female		
	1	2	3	Case	Control	Permutation	Case	Control	Permutation	Case	Control	Permutation
				(N = 81)	(N = 760)	P	(N = 58)	(N = 301)	P	(N = 23)	(N = 459)	P
H1	I	T	A	0.586	0.532	0.180	0.658	0.538	0.022	0.408	0.528	0.137
H2	D	C	G	0.230	0.211	0.557	0.147	0.196	0.290	0.433	0.220	<0.001
H3	I	C	A	0.106	0.144	0.202	0.121	0.141	0.667	0.068	0.146	0.193
H4	D	C	A	0.044	0.053	0.731	0.046	0.053	0.821	0.041	0.053	1.000

1 = 296921-296923delTTG; 2 = rs2288774; 3 = rs2288775.

DISCUSSION

By systemically screening variations of *NEDD4L* in this study, we identified no functional mutations in the gene. By studying the associations of three representative common SNPs with MS, we found that rs2288775 was significantly associated with MS in female Kazakh. Moreover, we found a gender flip-flop association between genetic variation 296921-296923delTTG of *NEDD4L* and MS in the Kazakh general population.

Considering the *a priori* biological function of the ubiquitin-proteasome system and NEDD4L, we presumed that *NEDD4L* is an important candidate gene for MS. Importantly, by studying some functional SNPs of *NEDD4L* chosen from public genetic databases, several studies have recently demonstrated that genetic variations of *NEDD4L* are associated with hypertension (Russo et al., 2005; Araki et al., 2008; Manunta et al., 2008; Wen et al., 2008). Therefore, study of the association of *NEED4L* with MS comprehensively from a genetic point of view is necessary. In this study, we systemically examined the association of genetic variations of *NEDD4L* with MS in Xinjiang Kazakh through a research strategy in which we first screened genetic variations of *NEDD4L* and then studied the relationship of the representative polymorphisms to MS. This research strategy was selected based on the following: 1) the HapMap project does not provide genetic information for Xinjiang Kazakh, so we could not use tag SNPs specific for Kazakh in this study, and 2) by sequencing the functional regions with their flanking sequences of *NEDD4L* in 48 Kazakh MS patients, we found not only common and rare SNPs or mutations, both of which are considered to contribute to the pathogenesis of MS, but also race-specific genetic variations.

The most important finding of the present study is the gender flip-flop association in genetic markers (296921-296923delTTG) and MS in the general Kazakh population. This flip-flop association means that the observed high-risk allele in one studied population is a protective factor in another population (Lin et al., 2007). Such interesting flip-flop findings

have extremely important implications (Lin et al., 2007) and require cautious explanation: 1) flip-flop risk alleles might come from population differences. Such flip-flops of risk alleles have been reported across various ethnic groups in other studies (Singleton et al., 2003; Tan et al., 2003). Flip-flop associations may indicate heterogeneous effects of the same variant that are due to differences in genetic background or environment. 2) The flip-flop result might be a spurious finding owing to population stratification, misclassification of outcome, variation in power between studies, and failure to exclude chance as an explanation in some studies (Colhoun et al., 2003). 3) Multilocus effects and variation in interlocus correlations might contribute to the flip-flop phenomenon. When the investigated allele at the noncausal variant is positively associated with the disease-risk allele in a population, the target allele at this variant appears to be a risk allele. When the target allele at the variant is negatively associated with the disease-risk allele in another population, the target allele at this variant appears to be a protective allele.

Interestingly, Russo et al. (2005) have discovered an additional flip-flop phenomenon at rs3865418 polymorphisms of *NEDD4L*: the T allele of rs3865418 was associated with essential hypertension in Greek whites, but the C allele of rs3865418 was associated with essential hypertension in United States whites. Careful review of the characteristics of the participants from the Russo et al. (2005) study has shown that the gender composition of the two groups was different: the male proportion was 48.3% in white hypertensive subjects in the United States (case group), whereas it was 58.0% in Greek white hypertensive subjects (case group). Therefore, a plausible prediction is that the difference in male proportions may be a causative factor for the flip-flop phenomenon in the two populations.

In the present study, male and female subjects were selected from the same population: Xinjiang Kazakh with pure genetic backgrounds and the same environmental exposure. Moreover, TaqMan-PCR experimental results are reliable (the consistent rate of repeat testing was 100% in the present study). Furthermore, statistical power analyses revealed that our study had a power of 0.90 (for the I/D+D/D genotype of 296921-296923delTTG) to detect differences across cases and controls ($\alpha = 0.05$). Therefore, we exclude population stratification, misclassification of outcome, and variation in statistical power as contributors to the flip-flop effect in this study. The gender flip-flop associations in 296921-296923delTTG and MS in the Kazakh population were relatively reliable.

The mechanisms of the gender flip-flop association in 296921-296923delTTG and MS are unknown. This inconsistency might be derived from sex hormone differences. *NEDD4L* expression is reportedly downregulated in prostate cancer and upregulated after androgen treatment (Qi et al., 2003; Hu et al., 2009), suggesting that *NEDD4L* is associated with androgen-mediated gender differences in MS. We were unable to obtain data on plasma sex hormone, as we could not obtain informed consent to collect blood samples for this measurement. Moreover, locus 296921-296923delTTG is not the causative factor, and causal variant(s) are in tight linkage disequilibrium with this marker, suggesting that additional variants within or near this gene might exist. Therefore, the differences in its correlation with other causal variants between male and female subjects might contribute to the observed gender flip-flop.

In the present study, we found no missense mutations in *NEDD4L*, which may be indicative of the relatively high conservation of NEDD4L and the importance of this molecule. One polymorphism, rs2288775 in *NEDD4L*, was found to be significantly associated with MS in female Kazakh. Because significant association of rs2288775 with MS was uncovered in a

multivariable analysis with adjustment for confounding risk factors including age and lifestyle (current smoking and drinking), the G allele of rs2288775 may be an independent protective factor for MS. Moreover, statistical power analyses revealed that our study had a power of 0.80 (for the AA genotype of rs2288775) to detect the difference across cases and controls (α = 0.05). Furthermore, the distribution of rs2288775 conformed to HWE (data not shown), which suggests that the results of this study are unlikely to be biased by population stratification or admixture for MS. These results strongly suggest that the association between rs2288775 and MS might be relatively reliable. However, as association studies are not consistently reproducible as a result of false positives, false negatives, and problems with true variability between populations (Lohmueller et al., 2003), the association of rs2288775 with MS must be re-examined in another population.

In summary, 296921-296923delTTG of *NEDD4L* was found to be gender flip-flop-associated with MS in the general Kazakh population. Moreover, the G allele of rs2288775 may be an independent protective factor for MS in female Kazakh. The genetic variations of *NEDD4L* might be involved in the pathogenesis of MS.

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