Original Article

The Relationship between Polymorphisms at 17 Gene Sites and Hypertension among the Aboriginal Tibetan People*

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Abstract

Objective The incidence of hypertension in Tibet ranks highest among all Chinese provinces. This may be due to genetic changes caused by Tibet's unique natural environment and agrarian lifestyle, prompting us to investigated the relationship between gene polymorphisms and hypertension.

Methods Blood samples were collected from 229 hypertensive participants and 372 healthy (control) participants from five Tibetan counties. Seventeen single nucleotide polymorphisms were investigated for their connection to hypertension.

Results The C allele at rs2070744 of the *NOS3* gene was shown to be significantly associated with hypertension (*P*=0.0443; OR=1.636). Additionally, the T allele of rs4961 of the *ADD* gene was correlated with hypertension in women (*P*=0.03124; OR=1.584).

Conclusion In this study we found that the *NOS3* and *ADD* genes were related to a high incidence of hypertension among Tibetans. *NOS3* gene plays a role in regulating vascular tone and blood vessel diameter, which may be altered by the low-oxygen environment of Tibet. *ADD* is involved in water and salt metabolism, which is consistent with the high-salt diet of Tibetans. The correlations elucidated by our study were different from those of other ethnic groups, indicating that these findings may be specific to the Tibetan people.

Key words: Genetic polymorphism; Hypertension; Tibetan

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INTRODUCTION

Tibet's landscape, climate, and high altitude are unlike those of any other region in China or the world. The combination of the atypical environmental factors specific to this region and the somewhat primitive and isolated lifestyle of the Tibetan people offers researchers a unique opportunity to study a variety of distinctive diseases commonly found in this agriculture-driven population. One such disease is hypertension, whose prevalence in Tibet ranks first

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amongst all of the provinces in China[1]. We expect that lifestyle might contribute to the high incidence of hypertension and coronary diseases, which are the major causes of death for Tibetan people.

For the reasons described above, Tibet is an ideal location for genetic studies. Such studies may facilitate the prevention, diagnosis, and treatment of diseases specific to the Tibetans. These studies may also allow for an in-depth investigation of genetic evolution of Tibetans. This paper describes a preliminary investigation to ascertain the connections between polymorphisms of genes such as *AGT*, *NOS3*, and *ADD1* and the high rate of hypertension in the aboriginal Tibetan people.

MATERIALS AND METHODS

Patient Samples

We selected participants from five representative Tibetan counties: ZhaRen of AnDuo County (Naqu Prefecture), ZhaXiZong of DingRi County (Shigatse Prefecture), XueBa of QiongJie County (Shannan Prefecture), Caigongtang of Chengguan Country (Lhasa City), and JiuBu of LinZhi County (LinZhi Prefecture). Two villages were randomly selected from each county, which represent typical pasturing areas, agricultural areas, and suburban areas. Hypertensive and healthy (control) participants were grouped using standard health examinations by experienced physicians from the People's Hospital of Tibet Autonomous Region. Laboratory examinations were performed in local hospitals.

Measurement Methods and Questionnaire

Conventional mercury sphygmomanometers were used to measure personal blood pressure twice on the left arm with a 5-minute break between the measurements. We took the average of these two measurements as the final blood pressure value.

A blood-pressure reading greater than or equal to 140/90 mmHg, as recommended by the World Health Organization, was taken as the criteria for hypertension. A questionnaire was administered to record each participant's medical history (including incidence of heart and lung disease) and hypertension treatment situation.

Genotyping Site Determination

We analyzed17hypertension-related gene sites that have been reported frequently in Chinese or international journals but have been investigated rarely in the Tibetan people (Table 1). The following six sites were selected for genotype analysis according to the criteria outlined by the Wellcome Trust Case Control Consortium's 2007 genome-wide association study (GWAS)[2]: HT-rs2820037, HT-rs3755351, HT-rs6997709, HT-rs7961152, HT-rs1937506, and HT-rs2398162.

Table 1. Seventeen Gene Sites that May Contribute to Hypertension

Gene	Sites	Common Name	Ref.
AGT	rs699	M235T	3, 4, 5
	rs5051	A-6G	
	rs3889728	G1035A	
AGTR1	rs5186	A1166C	
CYP11B2	rs1799998	C-344T	6, 7
ADRB2	rs1042714	Gln27Glu	8
	rs1042713	Arg16Gly	
ADD1	rs4961	Gly460Trp	9
NOS3	rs2070744	C-786/T	10, 11
	rs1799983	Glu298Asp	
GWAS	rs2820037		2
GWAS	rs6997709		
GWAS	rs7961152		
GWAS	rs11110912		
GWAS	rs1937506		
GWAS	rs3755351		
GWAS	rs2398162		

DNA Extraction and Genotyping

Genome DNA extraction from blood was conducted by salt fractionation and electrophoresis. Genotyping was performed using the MassARRAY platform (Sequenom, San Diego, CA, USA); however, as the SequenomMassARRAY system used in our study was selective to specific single-nucleotide polymorphisms (SNPs), some genes and SNP sites (e.g., Insertion/Deletion mutation in the ACE gene) could not be identified. The primer used in this study was designed using the SequenomASSAY-DESIGN software package and was subsequently synthesized by Invitrogen (Shanghai, China).

The standard protocol[12] for such genotyping is as follows. First, a locus-specific polymerase chain reaction (PCR) is carried out, followed by a locus-specific primer extension reaction in which an oligonucleotide primer anneals immediately to the upstream of the polymorphic site being genotyped.

In the iPLEX assay, the primer and the amplified target DNA are incubated using mass-modified dideoxynucleotide terminators. The primer extension is performed based on the sequence of the variant site and is a single complementary mass-modified base. Using MALDI-TOF mass spectrometry, the mass of the extended primer can be determined. The primer's mass indicates the sequence and, therefore, the alleles present at the polymorphic site SpectroTYPER, software from Sequenom, automatically translated the mass of the observed primers into a genotype for each reaction.

Statistical Analysis

SPSS (version 9.0) was used as a statistics tool in this study. Pearson's chi-squared test was used in the Hardy-Weinberg equilibrium test for all gene polymorphisms, where $P<0.01$ did not meet the Hardy-Weinberg equilibrium. The chi-squared test and Fisher's exact test were used to analyze the association of gene polymorphisms between hypertensive participants and healthy (control) participants. Logistic regression analysis was used to further justify the association between these two groups.

RESULTS

Sample Description

About 75%-86% of the population in each village participated in our study, and those who did not participate were mainly males that were working in other regions. The sample size varied by county and ranged from 302 participants (LinZhi) to 777 participants (DingRi). A total of 2 477 aboriginal Tibetan people, whose families had lived in the Tibetan region for at least three generations, participated in the study.

Determination of Case and Control

A total of 2455 participants from the initial sample population of 2477 individuals underwent blood pressure measurements as part of a standard in-hospital clinical examination. Two hundred eighty-nine participants (115 males and 174 females) were diagnosed (or determined by questionnaire) with hypertension. Among them, 30% were aware of their hypertensive status, 6.5% were undergoing hypertension treatment at the time of our study, and 1.5% kept their hypertension under control as revealed by our questionnaire. Participants with hypertension under control were excluded from the study because it was difficult to determine their blood pressure level.

Subjects were excluded from our study if they had secondary hypertension, chronic high-altitude sickness, diabetes, or other medical conditions that might skew our results; additionally, only subjects with an age between 18 and 65 years were included in our study. To test for possible secondary hypertension related to chronic-altitude sickness, diabetes, or other medical conditions, hypertensive participants underwent an array of examinations, including electrocardiogram and abdominal type-B ultrasound examinations, a chest x-ray, and a test of individual hemoglobin and blood sugar levels. After the screening, 78 males and 151 females remained in the hypertensive group.

The criteria for selecting the healthy (control) participants from those with normal blood pressure were that their age was between 18 and 65 years; they did not have any medical history of clinical problems related to the heart, brain, kidney, liver, or gallbladder; and their electrocardiogram, urinary, liver, and kidney exams and blood sugar and blood fat levels were all normal. After the screening, 372 subjects (128 males and 244 females) remained in the control group (Table 2).

Genotyping Results

The relevant genotyping statistics are shown in Table 3. As described previously, because of the characteristics of the MassARRAY system, some samples

Table 2. Characteristics of the Study Participants

	Control			Hypertension		
	N[a](Male/Female)	Mean	Std.	N[a]	Mean	Std.
Age (year)	372 (128/244)	42 (40/43)	10 (10/9)	229 (78/151)	49 (47/49)	10 (10/10)
BMI	367 (124/243)	22.0 (21.7/22.2)	2.8 (2.2/3.0)	225 (75/150)	23.5 (23.3/23.7)	3.6 (3.2/3.9)
SBP (mmHg)	372 (128/244)	109 (108/109)	12 (13/11)	229 (78/151)	143 (139/145)	22 (10/22)
DBP (mmHg)	372 (128/244)	74 (75/74)	8 (8/8)	229 (78/151)	98 (97/98)	11 (10/11)

Note. [a]The number of study participants. All of the data were shown as total (male/female).

Table 3. Results of Individual SNP Genotyping and Hardy-Weinberg equilibrium

SNP	Groups	Allele1(A1)	Allele2(A2)	Genotype (A1A1/A1A2/A2A2)	*P* Value[a]
AGT-rs699	All	T	C	53/230/303	0.3629
	Hypertensions	T	C	22/90/115	0.5229
	Control	T	C	31/140/188	0.5143
AGT-rs3889728	All	A	G	60/289/237	0.0453
	Hypertensions	A	G	20/118/88	0.0290
	Control	A	G	40/171/149	0.4182
AGT-rs5051	All	C	T	54/234/298	0.4241
	Hypertensions	C	T	23/91/112	0.5283
	Control	C	T	31/143/186	0.6074
HT-rs2820037	All	T	A	3/72/512	0.7352
	Hypertensions	T	A	0/34/193	0.6197
	Control	T	A	3/38/319	0.1358
HT-rs3755351	All	A	C	118/273/195	0.2081
	Hypertensions	A	C	49/97/80	0.0588
	Control	A	C	69/176/115	0.9148
AGTR1-rs5186	All	C	A	2/28/557	0.0632
	Hypertensions	C	A	0/13/214	1
	Control	C	A	2/15/343	0.0204
ADD1-rs4961	All	T	G	137/288/156	0.8682
	Hypertensions	T	G	61/110/55	0.6912
	Control	T	G	76/178/101	0.9154
ADRB2-rs1042713	All	G	A	138/294/143	0.6173
	Hypertensions	G	A	52/119/52	0.3504
	Control	G	A	86/175/91	0.9153
ADRB2-rs1042714	All	G	C	16/178/393	0.4851
	Hypertensions	G	C	5/70/152	0.4921
	Control	G	C	11/108/241	1
NOS3-rs2070744	All	C	T	3/75/507	0.7511
	Hypertensions	C	T	2/36/189	0.6849
	Control	C	T	1/39/318	1
NOS3-rs1799983	All	T	G	4/101/481	0.8068
	Hypertensions	T	G	2/40/185	1
	Control	T	G	2/61/296	0.7536
HT-rs6997709	All	T	G	5/77/505	0.2374
	Hypertensions	T	G	4/33/190	0.0914
	Control	T	G	1/44/315	1
CYP11B2-rs1799998	All	C	T	41/239/305	0.6036
	Hypertensions	C	T	14/97/115	0.3212
	Control	C	T	27/142/190	1
HT-rs7961152	All	A	C	10/120/455	0.5537
	Hypertensions	A	C	6/42/178	0.1033
	Control	A	C	4/78/277	0.8016
HT-rs11110912	All	G	C	5/79/503	0.3668
	Hypertensions	G	C	2/32/193	0.6365
	Control	G	C	3/47/310	0.4246
HT-rs1937506	All	A	G	6/125/455	0.5488
	Hypertensions	A	G	3/49/175	1
	Control	A	G	3/76/280	0.5991
HT-rs2398162	All	A	G	104/254/224	0.0375
	Hypertensions	A	G	42/104/80	0.4147
	Control	A	G	62/150/144	0.04358

Note. [a]The *P* value was established by the Hardy-Weinberg equilibrium test.

failed and were excluded from the genotyping analysis. Statistical testing supported the finding that all of the sites were in Hardy-Weinberg equilibrium, indicating that the proportions of these alleles remain relatively stable throughout generations.

Correlation between Gene Polymorphisms and Hypertension

After using the chi-squared test on the allele frequencies between hypertensive and control participants, we found that only rs2070744 of the *NOS3* gene was related to hypertension (details omitted). After adjusting by a permutation test, we still found that the frequencies of allele C and allele T were significantly different in hypertensive and control participants. These results were further verified by Fisher's exact test (Table 4), which indicated that the C/T polymorphism of rs2070744 of the *NOS3* gene was associated with hypertension. After taking age and body mass index (BMI) as covariants and then revising our calculations by logistic regression, the association was still significant, with *P*=0.0443 and OR value=1.636 (95% CI 1.012-2.643).

Table 4. The Association between Hypertension and Rs2070744

rs2070744	Allele	Allele	*P*[a]	OR (95% CI)
Hypertensions	40	414	0.04518	1.591(1.012-2.501)
Control	41	675		

Note. [a]The *P* value was established by Fisher's exact test.

We analyzed men and women separately (detailed results omitted) and found that the rs4961 locus of the ADD1 gene was associated with hypertension in females. The result of the Fisher's exact test related to this finding is shown in Table 5. Following revision by logistic regression, the correlation still held, with *P*=0.03124 and OR value =1.584 (95% CI 1.042-2.407).

Table 5. The Association between Hypertension and Rs4961 in Women

rs4961	Allele T	Allele C	*P*[a]	OR (95% CI)
Hypertensions	157	143	0.03252	1.378(1.031-1.84)
Control	212	266		

Note. [a]The *P* value was established by Fisher' s exact test.

DISCUSSION

As mentioned above, we found that particular alleles of *NOS3* and *ADD1* have statistically significant contributions to the high incidence of hypertension among the general Tibetan population and the female population, respectively. NOS3, also known as endothelial nitric oxide synthase (eNOS), produces nitric oxide (NO), which plays an important role in the regulation of vascular tone[13-15]. Furthermore, Ohashi et al. found that eNOS deficiency plus chronic hypoxia could induce hypertension in mice[16], which is similar to the hypoxic environment in Tibet. *ADD1* codes for alpha-adducin, which is a cytoskeleton protein. *ADD1* is known to be involved insodium metabolism. Torielli et al.[17] demonstrated that mutated forms of *ADD1* in rat and human increased Na/K pump activity and the number of pump units, which may further contribute to sodium-dependent hypertension. This is consistent with the high-salt diet of Tibetan people. Therefore, it is entirely rational for this study to identify *NOS3* and *ADD1* as candidate hypertension-related genes among Tibetans.

The rs2070744 mutation occurred in the promoter region of the *NOS3* gene. Previous research has shown that this mutation leads to down regulation of the *NOS3* gene, which might be associated with coronary spasm[18]. Hyndman et al.[19] found that people carrying genotype C endured higher systolic pressure and were at a higher risk of developing hypertension. However, Tsujita[20-21] reported that rs2070744 in the *NOS3* gene did not alter the rate of hypertension significantly in the Japanese population.

Hypertension-specific investigations into the role of rs2070744 among Chinese have been rare, with the exception of a report by Ma et al.[9] suggesting that single-site variance in rs2070744 does not contribute to hypertension on its own, but that it may be associated with hypertension when combined with other sites. However, our study showed that allele C of rs2070744 alone may be associated with hypertension among Tibetan people. Some studies have found that the nitric oxide (NO) level in the blood of Tibetans living in a plateau environment was significantly higher than that of the Han nationality[22]. Therefore, we hypothesized that a high NO level in Tibetans is an important factor in maintaining their normal blood pressure and that the mutation in rs2070744 reduces the synthesis of

the eNOS protein, causing the NO level to drop and the blood pressure to rise in parallel. We expect that this may be the mechanism of the relationship between rs2070744 and hypertension in Tibetan people.

NOS3-rs1799983 SNP that has been researched extensively by Chinese scholars was found not to be significantly associated with hypertension in this study. The mutation of rs1799983 would change glutamate at locus 298 to aspartate (Glu298Asp) in the resultant protein and the missense mutation may lead to dysfunction of eNOS[23]. This locus was also associated with NO synthesis, but it was not associated with hypertension among the Tibetan people. Thus, the role of gene polymorphisms of *NOS3* in hypertension needs to be further investigated.

With regard to the *ADD1* gene, its genetic product may influence the Na-K-ATP enzyme activity in such a way that the kidney tubules enhance the re-absorption of sodium, resulting in increased sodium retention and, subsequently, hypertension. Since 1995, researchers outside China have studied the relationship between the α-adducin gene and essential hypertension (EH); a case control study using hypertension and control subjects[24] found that the Gly460Trp mutation of α-adducin was related to EH in two separate Caucasian groups (Gly corresponding to base G, and Trp to base T in the study). In a case-control experiment performed by Glorioso et al.[25], positive results were obtained at Milano and negative ones at Sassar, indicating that the effect of Gly460Trp mutation on hypertension was ethnicity-dependent.

Chinese researchers also reached different opinions on the relationship between the mutation of the α-adducin gene and hypertension. Our study found that the rs4961 site of the *ADD1* gene was related to hypertension in the female Tibetan population, with *P*=0.03124 and OR value=1.584 (1.042-2.407), indicating that the T allele was a risk factor. Because we know that the *ADD1* gene is involved in water and salt metabolism, and that the traditional Tibetan diet involves considerable salt intake, we expect that mutations of the *ADD1* gene may be connected to the high incidence of EH among Tibetan people. Though other researchers found that the function of the *ADD1* gene was sex-dependent in people with hypertension[26], it is hard to conclude that the *ADD1* gene is associated with hypertension in females but not in males in the Tibetan population. This result may have been different if we could have increased the participate rate of males.

In this research, we focused solely on the relationship between gene alleles and hypertension without taking the effects of genotypes or haplotypes into consideration because of the limited number of subjects available for the study. It is also important to note that 13% of the participants were smokers, but the smoking rate between the hypertensive and healthy (control) groups was not significantly different. Smoking was, therefore, not analyzed in the context of our study.

No significant connections between other genes sites (including *AGT* and *AT1R*) and EH were found in this study. Additionally, the haplotype of the *AGT* gene (3 SNPs) was not associated with EH. Some previous reports hold opposing views to our findings, while others support our findings[27-28], indicating that the effects of these sites may be ethnicity-dependent. Interestingly, the seven sites identified in the GWAS that were found by other researchers to be related to hypertension were not significantly related to EH among Tibetans in our study. Therefore, the causes of hypertension appear to be influenced by environmental and genetic backgrounds, and research results obtained from non-Chinese populations cannot be applied blindly to the Chinese population and, in particular, to the Tibetans.

In conclusion, our research found that polymorphisms in the *NOS3* gene were significantly correlated with hypertension among Tibetan people, and polymorphisms in the *ADD1* gene were correlated with hypertension in the female population. *NOS3* plays a role in regulating blood vessel diameter, which may be altered by the low-oxygen environment of Tibet. *ADD1* is involved in water and salt metabolism, which is in line with the high-salt diet among Tibetans. Because hypoxia in the plateau and a high-sodium diet are two main environmental factors influencing the lives of Tibetans, we suggest that environmental factors may play an essential role in the high-incidence of hypertension among Tibetans.

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