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ORIGINAL ARTICLE

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The *ADD1* G460W polymorphism is not associated with variation in blood pressure in Canadian Oji-Cree

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Abstract Since adducin modulates cellular sodium retention, its follows that *ADD1*, which encodes the α-subunit of adducin, is an attractive candidate gene for blood pressure variation. Association studies examining the relationship between polymorphism at *ADD1* codon 460 (G460W) and both hypertension and blood pressure, which were performed in a variety of human population samples derived from different genetic backgrounds, have given inconsistent results. We examined the association between the *ADD1* G460W polymorphism and variation in blood pressure in a sample of non-diabetic, largely normotensive Canadian Oji-Cree from an isolated community in Northern Ontario. Among 481 Oji-Cree subjects, we measured blood pressure and related clinical phenotypes and determined genotypes of *ADD1* G460W. We observed an allele frequency of 0.08 for the *ADD1* W460 variant, which is among the lowest so far observed in human populations. We found significant associations between variation in both systolic and diastolic blood pressure and gender, age, body mass index (BMI), and treatment for hypertension. However, we found no association between the *ADD1* W460 allele and increased blood pressure, nor did we observe a higher frequency of the W460 allele in a hypertensive subgroup compared with normotensive subjects. While the low sample frequency of *ADD1* W460 is consistent with the low sample prevalence of hypertension, the absence of a specific association with both blood pressure and hypertension suggests that the *ADD1* W460 variant is not an important determinant of blood pressure among individuals of this genetic background.

Key words Complex disease · Cardiovascular · Sodium · Aboriginal populations

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Introduction

Blood pressure (BP), an archetypal complex trait, is influenced by many genetic and non-genetic factors (Williams et al. 1994; Harrap 1996). A common method to further the understanding of genetic determinants of BP variation is association analysis using candidate genes. In such an approach, a population sample is genotyped for a common polymorphism of a gene that has a potentially important physiological role. The associations found by the candidate gene strategy can be inconsistent between populations due to artefacts such as population admixture (Harrap 1996; O'Byrne and Caulfield 1998). One approach to reduce such artefacts may be to select genetic variants for which functional studies have already established a likely biological impact for the genetic variation. Another approach is to test for consistency of the association in several population samples that come from different genetic backgrounds.

Alpha (α)-adducin was first identified as a cytoskeletal protein in erythrocytes (Bianchi et al. 1994). α-Adducin may play a role in the formation of spectrin-actin complexes, which anchor some transmembrane proteins, such as the Na^+-K^+ ATPase (Tripodi et al. 1996). Genetic variation of α-adducin has been shown to affect renal sodium transport at the cellular level in the Milano Hypertensive (MHS) rat strain (Tripodi et al. 1996). This variation appears to account for the BP differences between MHS rats and normotensive control rat strains (Bianchi et al. 1994). In humans, the α-adducin gene (*ADD1*) locus on chromosome 4p16.3 was found to be associated with essential

hypertension (Casari et al. 1995). In addition, a common polymorphism at codon 460 of *ADD1*, namely G460W, has been associated with hypertension in samples of French (Cusi et al. 1997); Italian (Manunta et al. 1998; Castellano et al. 1997; Cusi et al. 1997), and some Japanese subjects (Iwai et al. 1997), but not in another Japanese sample (Ishikawa et al. 1998), nor in a Scottish sample (Kamitani et al. 1998). Furthermore, subjects with the *ADD1* W460 allele had lower plasma renin activity (Manunta et al. 1998), and also a greater reduction in BP in response to salt-loading followed by salt-depletion or diuretic treatment (Cusi et al. 1997).

Ferrandi et al. (1996) observed significant increases in BP in young pre-hypertensive MHS rats, which suggests that the *ADD1* W460 allele may also be a determinant of BP variation in a normotensive population. Some authors have suggested that genetic determinants of variation in BP may also be determinants of hypertension, since hypertension is defined by a threshold value imposed on BP (Harrap 1996; Hegele et al. 1996; 1997; 1998). Harrap (1996) has suggested that in order to understand the genetic determination of BP, analytic strategies should examine both BP as a continuous trait and hypertension as a discrete trait in variety of populations. We have investigated the association of the *ADD1* G460W polymorphism and BP variation in a generally normotensive sample of Canadian Oji-Cree.

Methods

Study subjects

The Sandy Lake Health and Diabetes Project studied a total of 728 participants aged 10 years and older, which accounts for 72% of the population of the community (Harris et al. 1997). Sandy Lake is an isolated native reserve within the Sioux Lookout Zone, located about 2000 km northwest of Toronto in the sub-arctic boreal forest region of central Canada, and is accessible only by air during most of the year. Most members of the community speak both English and the traditional language of Ojibway-Cree (Oji-Cree), one of the Algonkian languages (Harris et al. 1997). The ancestors of the current residents of this reserve lived an active, nomadic, hunting-gathering lifestyle typical of other Algonkian-speaking groups of the sub-arctic. As a result of imposition of the reservation and residential school systems, the traditional nomadic lifestyle has recently changed to a more sedentary existence, and the diet has become richer in saturated fat and processed foods, and poorer in dietary fiber (Gittelsohn et al. 1988).

The Sandy Lake Health and Diabetes Project was undertaken to determine the prevalence of non-insulin-dependent diabetes mellitus (type 2 DM) in Sandy Lake. Medical history was determined by a questionnaire, which included inquiry into use of antihypertensive medications. A physical examination determined body mass index (BMI), defined as weight divided by height squared (kg/m^2), and blood pressure (BP). A single highly trained observer who used a mercury manometer took two separate BP measurements in the right arm with each subject seated. Systolic BP (SBP) was recorded to the nearest 2 mmHg at the appearance of the first Korotkoff sound (phase I), and diastolic BP (DBP) was recorded to the nearest 2 mmHg at the disappearance of the fifth Korotkoff sound (phase V). Blood samples were obtained with informed consent after the subjects had fasted for 10–12 h. The only exclusion criterion for the current study was a diagnosis of type 2 DM. The project was approved by The University of Toronto Ethics Review Committee.

Genetic analysis

Sufficient DNA and phenotypic data were obtained for analysis from 481 subjects between the ages of 10 and 79. Blood collected for DNA isolation was centrifuged at 1300 *g* for 15 min; the buffy coat was retained and the leukocyte DNA was extracted using the phenol:chloroform method (Hegele 1996). DNA amplification to determine the *ADD1* G460W genotype was performed using the polymerase chain reaction (PCR). The DNA was amplified using primers whose sequences were previously reported, namely: (1) 5′-CGACGAAGCTTCCAAGGA-3′; and (2) 5′-ACAGTAAGGTAGGCACAGA-3′ (Ishikawa et al. 1998). Fifty ng of genomic DNA was amplified and an annealing temperature of 53°C for 30 s was used for each amplification cycle. Denaturation and elongation steps were carried out at 94°C and 72°C, respectively, for 30 s. This PCR motif was carried out for 30 cycles following an initial 94°C soak for 5 min. The 166-bp PCR product was digested with restriction enzyme *Pfl*MI (New England Biolabs, Beverly, MA, USA) at 37°C for 3 h. This enzyme cut the 166-bp fragment amplified from the *ADD1* W460 allele into 148- and 18-bp fragments, but did not cut the 166-bp fragment amplified from the *ADD1* G460 allele. The fragments were resolved on 10% polyacrylamide gels and visualized with ethidium bromide stain under UV light. As a genotypic control for subsequent analyses, we included genotypes for codon 235 of the angiotensinogen gene (*AGT*), which were determined as previously described (Hegele et al. 1997).

Statistical analysis

SAS (SAS Institute, 1995) was used for all statistical analyses. Normality for subsequent parametric statistical analyses was assessed using Wilk's W. The distributions of SBP and DBP were significantly non-normal in this data set. Therefore, for parametric analysis, the distribution of SBP was inverse transformed (1/SBP), and DBP was $\log_{10}$ transformed ($\log_{10}$(DBP)). The transformed variables were used for statistical analyses; however, the non-transformed data are presented in the tables. Analysis of variance (ANOVA) was performed using the general linear models procedure to determine the sources of variation for the clinical traits, with F tests computed from type III sums of squares (SAS Institute, 1995). Type III sums of squares analysis applies to unbalanced study designs, and takes into account the effects

of other independent variables included in the model. The dependent variables were transformed SBP and DBP. Since BP varies significantly by gender, age, and BMI (Hegele et al. 1997), we included these as independent covariates. Also, in order to account for differences in shared background genetic and environmental factors between families, we used a family identification covariate (designated as "family") as a classification variable. Finally, genotypes of *ADD1* codon 460 and *AGT* codon 235 were included as covariates, along with an interaction term of the *ADD1* and *AGT* genotypes. The general linear models procedure for least-squares means was used to test for significant differences in pairwise comparisons of BP between genotypic classes. Least-square means, also called "adjusted" or "population marginal" means, take into account all covariates included in the model. Also, the untransformed, unadjusted mean BP differences by genotype classification represented in the tables were compared using a *t*-test. χ^2 analysis was used to test for deviation of genotype frequencies from Hardy-Weinberg expectations. χ^2 analysis was also used to test for between-group differences in allele and genotype frequencies when hypertension was treated as a discrete trait, defined as SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg and/or medical treatment for hypertension.

Results

Baseline clinical features

The baseline clinical attributes of this study sample are shown in Table 1. This sample was remarkable for its youth and for the relatively low SBP and DBP. For analysis of "hypertension" as a discrete trait, the criteria included SBP $\geq$ 140 mmHg and/or DBP $\geq$ 90 mmHg and/or treatment for hypertension (usually prescription for angiotensinogen converting enzyme (ACE) inhibitors) (Hegele et al. 1997). In this sample, 37 subjects (7.7% of the total) had hypertension so defined, and 16 subjects (3.3% of the total) were taking antihypertensive medications.

Allele and genotype frequencies

The frequency of the *ADD1* W460 allele in this sample was 0.08, with observed genotype frequencies of 0.84 and 0.16 for G460/G460 and G460/W460, respectively. There were no W460/W460 homozygotes. Genotype frequencies did not deviate from those predicted by the Hardy-Weinberg law in this study sample ($\chi^2 = 3.54$; $P = 0.11$). The observed frequency of the W460 allele was significantly lower (data not shown) than that observed in Caucasians (Castellano et al. 1997; Kamitani et al. 1998) and Japanese subjects (Ishikawa et al. 1998; Iwai et al. 1997).

Table 1. Clinical characteristics of 481 non-diabetic Oji-Cree subjects

Age (years)	25.7 ± 13.0
Gender (% female)	54.3%
BMI (kg/m^2)	25.6 ± 5.7
SBP (mmHg)	113 ± 14
DBP (mmHg)	64.8 ± 11.3

BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure

Genetic association with variation in BP

Separate ANOVAs (Table 2) for transformed SBP and DBP showed that the *ADD1* codon 460 genotype was not associated with variation in BP. Since ANOVA takes multiple comparisons into account, we did not adjust the levels of nominal significance. As we previously reported, the *AGT* codon 235 genotype was significantly associated with SBP ($P = 0.016$), but not with DBP (Hegele et al. 1997). In addition, there was no interaction between the *ADD1* codon 460 and the *AGT* codon 235 loci, which was evaluated by the *ADD1*AGT* interaction term included in each ANOVA.

For SBP, between-genotype comparisons of *ADD1* codon 460 showed that there was no statistically significant difference in the unadjusted means (Table 3), nor was there a significant difference in the adjusted means, namely 119 ± 2 mmHg for G460/G460 homozygotes and 113 ± 5 mmHg for W460/G460 heterozygotes ($P = 0.27$). Also, the current analysis suggests that the *AGT* T235 allele has a dominant influence on SBP. Specifically, pairwise comparisons by genotype, using the least squared means from the present ANOVA, indicate that the SBP of *AGT* T235 homozygotes

Table 2. ANOVA for blood pressure in Sandy Lake Oji-Cree

	Inverse SBP		Log_{10} DBP	
Source of variation	F Value	$P > F$	F Value	$P > F$
Gender	39.8	<0.0001	12.6	0.0004
Age	28.2	<0.0001	8.99	0.0029
Family	1.09	NS (0.27)	1.13	NS (0.20)
BMI	46.2	<0.0001	41.85	<0.0001
Antihypertensive drug	12.3	0.0005	14	0.0002
AGT genotype	4.16	0.016	1.06	NS (0.35)
ADD1 genotype	1.26	NS (0.26)	0.84	NS (0.36)
*AGT*ADD1* genotypes	1.56	NS (0.21)	1.91	NS (0.15)

Abbreviations, as in Table 1, plus F Value, F test computed from type III sums of squares; P > F, random probability of a greater F than the one observed; NS, not significant with nominal $P < 0.05$; *AGT*, gene encoding angiotensinogen; *ADD1*, gene encoding α-adducin; BMI, body mass index

Table 3. Clinical characteristics according to *ADD1* codon 460 genotype

	G460/G460	W460/G460	*P*
Number	405	76	
Age (years)	25.7 ± 13.2	25.8 ± 12.1	NS (0.94)
BMI (kg/m^2)	25.6 ± 5.6	25.2 ± 5.4	NS (0.49)
SBP (mmHg)	114 ± 13	113 ± 12	NS (0.58)
DBP (mmHg)	64.8 ± 11.4	64.6 ± 10.6	NS (0.86)
Hypertension (%)	7.65%	7.89%	NS (0.86)

Abbreviations, as in Table 2

and heterozygotes, 121 ± 2 mmHg and 121 ± 3 mmHg, respectively, were each significantly higher than the SBP of 105 ± 7 mmHg observed for M235/M235 homozygotes (P = 0.021 and P = 0.020, respectively). DBP was not significantly associated with either genetic marker.

In this sample, 37 subjects (7.7% of the total) had hypertension according to defined criteria. The frequency of the *ADD1* W460 allele in this subset was 0.079, which was similar to the frequency of 0.081 observed in the normotensive subset. In this sample, 16 subjects (3.3% of the total) were taking antihypertensive medications. The frequency of the *ADD1* W460 allele was 0.13, which was not significantly different from the frequency of 0.07 observed in the rest of the sample (χ^2 = 0.42; NS).

Non-genetic factors associated with variation in BP

ANOVA revealed similar associations between both transformed SBP and DBP and gender, age, BMI, and treatment for hypertension (Table 2).

Discussion

In this sample of non-diabetic, predominantly normotensive Canadian Oji-Cree, our principal findings were: (1) a much lower allele frequency of the *ADD1* W460 variant compared with that observed in other populations (Ishikawa et al. 1998; Kamitani et al. 1998; Manunta et al. 1998; Castellano et al. 1997; Iwai et al. 1997); (2) absence of association of BP with *ADD1* codon 460 variation; (3) no significant difference in the *ADD1* W460 allele frequency between hypertensive and normotensive subsets; and (4) no significant difference in the *ADD1* W460 allele frequency between those subjects taking and not taking antihypertensive medications. Our findings indicated that the *ADD1* W460 allele was not significantly associated with higher BP in this sample, and are consistent with the lack of association reported in a growing number of studies (Ishikawa et al. 1998; Kamitani et al. 1998).

Several lines of experimentation in isolation indicate that α-adducin is an excellent candidate gene for determination of BP variation. For example, when compared with cells that have been transfected with normal α-adducin, the surface expression of Na^+ -K^+ pumps was increased in cells transfected with mutant α-adducin (Tripodi et al. 1996; Matsuoka et al. 1998), a change that may possibly lead to greater water retention and thus increased BP. It is thought that the observations regarding the influence of α-adducin genetic variation in rats may also apply to humans, because of the high homology between rat and human α-adducin (Bianchi et al. 1994). Despite these considerations, our study, and others, indicate that other factors, possibly including population stratification, differences in genetic background, gene X gene interactions and gene X environment interactions may affect the *ADD1* associations with BP in whole populations.

The absence of an association between the *ADD1* G460W polymorphism and either blood pressure or hypertension in the Oji-Cree could be due to a variety of factors. First, the association with BP may be a so-called "small effect", which our study may have been underpowered to detect due to the low frequency of the *ADD1* W460 allele. However, our results showed a trend opposite to the expected direction of the association: W460/G460 heterozygotes had a lower mean SBP than G460/G460 homozygotes (113 ± 12 *vs.*114 ± 13 mmHg). Second, it is possible that the association in other populations was related to linkage disequilibrium with another functional variant at *ADD1* or a nearby gene, and that such linkage disequilibrium was absent in the Oji-Cree. Third, it is possible that the association requires a longer exposure to environmental factors, such as diet and stress, and that the Oji-Cree had a low level of such exposure given their young mean age. Fourth, it is possible that the association requires a different genetic background from that of the Oji-Cree in order to be expressed. For example, differences in the genetic background may permit expression of a significant *ADD1*-BP association in other populations in which such associations have been detected. Finally, it is possible that a gene X gene interaction is necessary for the expression of an association between BP and *ADD1*. However, our data indicate that there is no interaction between *ADD1* and *AGT* in particular.

Archaeological evidence suggests that the Oji-Cree have inhabited the Sandy Lake region of Northern Ontario for about 6000 years (Hegele et al. 1998). This ancestry contributes to their fundamental genetic uniqueness. Furthermore, the contemporary inhabitants of the Sandy Lake region are mostly descended from one clan, who established the present reservation about 100 years ago (Hegele et al. 1997). The genetic architecture of the Oji-Cree is both similar and distinct from that observed in other populations. For example, Japanese control subjects had allele frequencies of 0.57 and 0.78 for the *ADD1* W460 and *AGT* T235 alleles, respectively (Ishikawa et al. 1998). In contrast, the Oji-Cree in our study sample had allele frequencies of 0.08 and 0.89 for the *ADD1* W460 and *AGT* T235 alleles (Hegele et al. 1997). Thus, the Oji-Cree and Japanese subjects have very similar *AGT* M235T allele frequencies, but very different *ADD1* G460W allele frequencies. This difference may be the result of founder effects, selection, and/or genetic drift, since the frequencies would otherwise be expected to be similar, given the common evolutionary ancestry that is postulated to link Japanese and native North American people (Szathmary 1993).

In summary, the *ADD1* W460 variant is relatively infrequent in the Sandy Lake Oji-Cree, and it is not associated with an increased BP. In addition, when treating hypertension as a discrete trait, we found that the hypertensive subset did not have a significantly higher frequency of the *ADD1* W460 allele compared with normal controls. It is possible that other phenotypes, such as the response to salt-loading or the effects on membrane transport kinetics, may more definitively define the role of the *ADD1* variation as a possible determinant of BP in the Sandy Lake Oji-Cree.

However, our results suggest that the *ADD1* polymorphism is not a significant determinant of BP variation in Oji-Cree.

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