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The Case-Control Studies Between The Single Nucleotide Polymorphisms of the Human SA and MTHFR Gene and Hypertension in Korean Population

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ABSTRACT: **The role of the kidney in initiating hypertension has been much debated. The SA gene is expressed in the kidney and is association with hypertension in man and in experimental animal models. Also, increased plasma concentrations of homocysteine have been found in patients with coronary artery disease (CAD) and hypertension. The genetic variation of methlene tetrahydrofolate reductase (MTHFR) gene is related to its enzyme activity and to the plasma homocysteine concentration. In view of the effect of SA and MTHFR as risk factor for cardiovascular diseases, we investigated the *Pst* I RFLP of the SA gene and C667T mutation of the MTHFR gene in the Korean patients with hypertension. There were no significant differences in the allele and genotype frequencies of these polymorphisms between normotensive and hypertensive subjects. Therefore, our results do not support a possible role of these genes on hypertension in Korean population.**

Keywords: Allele, Genotype, Hypertension, MTHFR and SA

Introduction

Hypertension is a common polygenic disorder affecting approximately 20% of adult population. The number of genes involved in this disorder is unknown, but several genes have been identified using a candidate gene approach. Among them, SA and methylene tetrahydrofolate reductase (MTHFR) genes are two candidates that may be implicated in the pathogenesis of hypertension.

SA is a gene of unknown function identified in screening for genes with increased expression in the kidney of the spontaneously hypertensive rat (SHR) compared with the kidney of the normotensive Wistar-Kyoto rat at the early stage (Iwai and Inagami, 1991). Also, these hypertensive rat strains have shown many pathophysiological characteristics of the human disease, and the SA gene locus cosegregates with elevation in blood pressure, accounting for up 25% of the genetic variance in systolic and diastolic blood pressure in F_2 populations involving SHR (Iwai *et al.*, 1992; Iwai and Inagami, 1992; Samani *et al.*, 1993), stroke-prone SHR (Lindpaintner *et al.*, 1993) and Dahl salt-sensitive genetically hypertensive rat strains (Harris *et al.*, 1993). These observations by independent groups suggest that SA gene is a likely candidate for a causative gene of hypertension in some models of hereditary hypertension in the rat. Since these hypertensive rat strains share many pathophysiological characteristics of the human disease (Yamori, 1983), it is of interest to examine the possible relation of the SA gene to hypertension of human. The human SA gene is on chromosome 16p13.11-12.3 (Iwai *et al.*, 1994; Nabika *et al.*, 1995; Szpirer *et al.*, 1993; Samini *et al.*, 1994) and displays several restriction fragment length polymorphisms (RFLPs) one of which involves *Pst* I RFLP (Iwai *et al.*, 1994).

As MTHFR is highly expressed in the kidney, and impairment of intra-renal homocysteine metabolism may contribute to the development of hyperhomocysteinemia. 5,-10-methylenetetrahydrofolate reductase (MTHFR) is one of the enzymes responsible for hyperhomocysteinemia and provides the methyl group for methionine synthase to methylate homocysteine to methionine. This variants consisting of cytosine (C) to thymine (T) transition at nucleotide position

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677 leading to the exchange of a highly conserved alanine to valine in the mature protein, has been associated with reduced activity and increased thermolability of this enzyme on lymphocyte extracts (Frosst *et al.*, 1995). MTHFR activity in the CC genotype has been found to be reduced and homocysteine significantly elevated compared with the CT and TT genotypes. This mutation is relatively common and has the potential for increasing plasma homocysteine levels. Homocysteine levels are influenced by environmental as well as genetic factors. A high plasma concentration of homocysteine, derived from the demethylation of dietary methionine, may predispose to atherosclerosis by injuring the vascular endothelium, which results in endothelial dysfunction (Boushey *et al.*, 1995). An elevated level of plasma total homocysteine is independent, graded and strong risk factor for cardiovascular disease, which shows a strong interactive effect with conventional risk factors. (Boushey *et al.*, 1995). The C667T mutation of the MTHFR gene has been associated with increase risk for cardiovascular disease in some, but not all studies (Jee *et al.*, 2000).

In view of the important role of the SA and MTHFR genes in kidney function and homocysteine metabolism, respectively, and lack of association study for Korean population, it is important to investigate the relationship between the single nucleotide polymorphisms (SNPs) of the SA and MTHFR genes and hypertension in Korean population. To clarify whether the SNPs of the SA and MTHFR genes are genetic risk factor for hypertension in Korean population, the present study examined a possible role of the two polymorphisms, *Pst* I RFLP in the SA gene and C677T mutation in the MTHFR gene in Korean patients with hypertension.

Materials and Methods

Study subjects

A total of 200 unrelated individuals were randomly chosen from the Seoul Hygiene Hospital, Seoul, Korea. We studied 100 subjects with hypertension. Patients were classified as having hypertension if they had systolic blood pressures above 140 mmHg and diastolic blood pressure above 90 mmHg on at least three separate occasions, and had no clinical signs, symptoms and laboratory findings suggestive of secondary hypertension. In additon, a randomly selected normal population (100 individuals) was analysed as the control groups (blood pressure value, <140/90 mmHg). The clinical data were described in Table 1. There was statistically significant difference in age distribution, serum HDL-cholesterol level and serum apolipoprotein AI (ApoAI) concentration between normotensives and hypertensives, respectively (P<0.05).

Table 1. Clinical details of the study subjects

Variables	Subjects		P[1]
	Normotensives	Hypertensives	
[a]Age(year)	56.4 ± 9.5[9]	62.8 ± 11.8	<0.05
BMI(kg/m^2)[2]	23.4 ± 2.4	24.0 ± 2.5	
TG(mg/dl)[3]	123.0 ± 83.2	133.2 ± 65.4	
TC(mg/dl)[4]	149.5 ± 38.3	151.9 ± 32.0	
LDL-chol(mg/dl)[5]	96.4 ± 38.5	100.1 ± 31.3	
[b]HDL-chole(mg/dl)[6]	28.3 ± 9.5	25.1 ± 9.3	<0.05
Lp(a)(mg/dl)[7]	14.8 ± 10.9	17.8 ± 12.3	
[c]ApoAI(mg/dl)[8]	70.8 ± 20.5	116 ± 32.1	<0.05

[1]Probability of significant difference between normotensives and hypertensives.
[2]Body Mass Index, [3]Triglyceride, [4]Total cholesterol, [5]LDL-cholesterol, [6]HDL-cholesterol, [7]Lipoprotein(a) and [8]Apolipoprotein AI. [9]Values are means ± standard deviations.
[a]Statistically significant difference (Student's t-test, P<0.0001).
[b]Statistically significant difference(Students't-test, P=0.040).
[c]Statistically significant difference(Students't-test, P<0.0001).

Determination of serum lipid levels

Blood samples were obtained in EDTA tubes from individuals who had been fasting for 12-16 hr. Concentration of serum total cholesterol (TC) and triglyceride were measured by enzymatic colorimetry methods with commercial kit (Boehringer Mannheim, Germany) and chemistry analyzer. Serum HDL-cholesterol level was determined by measuring cholesterol in the supernatant after precipitation of the serum with $MgCl_2$ and dextran sulfate, with a Gilford Impact 400E automated analyzer with reagents and calibrators from Boehringer Mannheim. Serum lipoprotein(a) (LP(a)) level was measured by the immunoprecipitation method (SPQ Test System, INCSTAR Corporation, Stillwater, Minnesota, USA) and serum ApoAI concentration was determied by immunoturbidimetric method (COBAS INTEGRA, ROCHE Diagnostics, USA). Also, serum low density lipoprotein (LDL)-cholesterol level was calculated by Friedewald's equation (Friedwald *et al.*, 1972).

DNA analysis

Genomic DNA was isolated from buffy coat by the method of Sambrook *et al.* (1989) with slight modification. Polymerase Chain Reaction (PCR) techniques were used for *Pst* I RFLP of SA gene (Zee *et al.*, 1997) and C667T mutation of MTHFR gene (Frosst *et al.*, 1995). Briefly, total 50 μl of the reaction mixture contained 200-400 *ng* of genomic DNA, 100 *ng* of each primer, 200 μl of each dNTP, and buffers recommended by the manufacturer. The sequences of the primers for two polymorphisms studied were:

(a) *Pst* I RFLP in the SA gene; sense, 5'-GTC ACA

CAT TAG GGC AGC TGC ACA C-3' and nonsense, 5'-GCC AGG CAT GGT GAT GCA ATC CTG-3' (Zee *et al*, 1997); (b) C677T polymorhism in the MTHFR; sense 5'-TGA AGG AGA AGG TGT ATG AGG GA-3' and nonsense, 5'-AGG ACG GTG CGG TGA GAG TG-3' (Frosst *et al.*, 1995). Amplification for the *Pst* I RFLP in the SA gene was carried out in a Perkin-Elmer DNA thermocycler, in which, after an initial denaturation step at 95°C for 5 min, there were 30 cycles of 94°C, 62°C, and 72°C for 1 min each. For the detection of C677T mutation in the MTHFR gene, samples were ampilified for 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 10 sec, and extension at 72°C for 60 sec, followed by a final extention step at 72°C of 10 min. And the nucleotide 677 mutation creates restriction site for *Hinf* I. Ten μl of each PCR product was restriction-digested overnight with 5 unit of each enzyme at 37°C. Digested product were size-fractionated after 2% agarose gel electrophoresis in TBE buffer for 40 min along with molecular markers. Ethidium bromide was incorporated into the gel. The gels were directly photographed on an UV transilluminator and genotyped. The *Pst* I RFLP in the SA gene was characterized by two bands, one of 315 bp (designated allele P1) and the other of 230 bp (allele P2) (Fig. 1). The C667T mutation in the MTHFR gene was also characterized by two bands, one of 198 bp (designated allele Ala) and the other of 175 bp (allele Val) (Fig. 2).

Statistical anaysis

Alleic frequencies were estimated by the gene counting method. Deviation in genotype distribution from that expected for Hardy-Weinberg equilibrium was estimated by χ^2-fitness test. The heterozygosity and polymorphism information content (PIC) was estimated by the methods of Bostein *et al*., (1980). The significance of differences in allele frequencies between groups was also estimated by χ^2-independence test. The relative risk of essential hypertension associated with allelic variation was expressed in terms of an odds ratio (OR) with 95% confidence interval (CI). One-way ANOVA test was performed to compare the mean levels of biochemical parameters among different genotypes. Statistical significance was accepted at the P=0.05 level. All statistical analyses were performed by the computer program of SPSSWIN (version 8.0).

Results

Genotype distribution

In the present study, we attempted to clarify the distribution of two polymorphisms in the SA and MTHFR genes in Korean population. Tables 2 and 3 display each data presenting the gene frequencies and the values of heterozygosity and PIC for *Pst* I RFLP of the SA gene and C677T mutation of the MTHFR gene in Korean normotensives and hypertensive groups, respectively. In the case of *Pst*I RFLPs, the genotype and allele frequencies were not significantly different between normotensives and hypertensives. The observed genotype distributions of this polymorphism were not significantly different from those expected for Hardy-Weinberg equilibrium. The frequencies of P1P1, P1P2 and P2P2 genotypes were 54, 43 and 3% in normotensives, and 56, 38 and 6% in hypertensives, respectively. The heterozygosity and PIC values of *Pst*I RFLP of the SA gene represented the values of 0.3700 and 0.3015 for

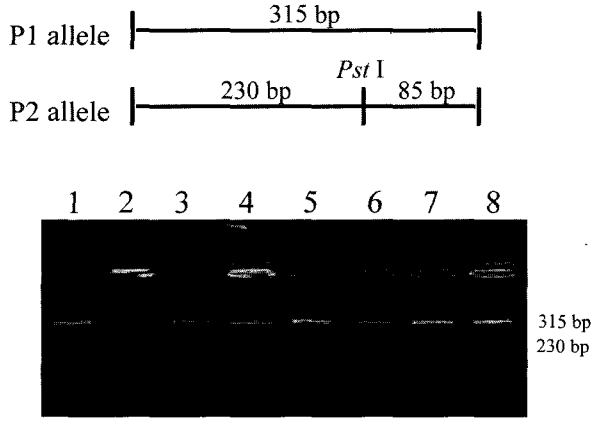

Fig. 1. The Pst I RFLP of SA gene. Lane 1, 3 ~5, 7 and 8, P1P1 genotypes; lane 2 and 6, P1P2 genotypes.

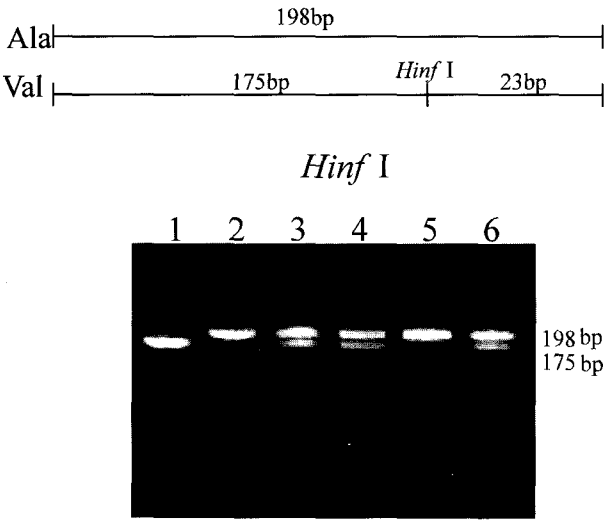

Fig. 2. C667T polymorphism of MTHFR gene. Lane 1, Val/Val homozygote; lane 2 and 5, Ala/Ala homozygotes; lane 3, 4 and 6, Ala/Val heterogotes.

normotensives, and 0.3750 and 0.3047 for hypertensives, respectively.

For C667T mutation of the MTHFR gene, there were also no significant differences in allele and genotype frequencies between two groups. The frequencies of Ala/Ala, Ala/Val and Val/Val genotypes were 37, 46 and 17% in normotensives, and 32, 47 and 21% in essential hypertensives, respectively. The observed genotype distribution was not in Hardy-Weinberg equilibrium ($P<0.05$). The heterozygosity and PIC values of C667T mutation represented the values of 0.4796 and 0.3646 for normotensives, and 0.4940 and 0.3720 for hypertensives, respectively. According to the heterozygosity and PIC values, C677T mutation of the MTHFR gene showed a relatively high degree of polymorphism in the both groups compared with the *Pst* I RFLP in the SA gene.

Association with biochemical parameters

Table 4 presented the comparision of anthropometric data and intermediate phenotypes across *Pst* I RFLP in the SA gene. The *Pst* I RFLP in the SA gene was not significantly associated with any anthropometrical parameters or serum lipid levels. The comparison of the anthropometric data and serum lipid levels across C667T mutation in the MTHFR gene is shown in Table 5. Likewise SA gene, The C667T mutation in the MTHFR gene was not also significantly associated with any anthropometrical parameters or serum lipid levels.

Discussion

Hypertension is one of the most common disease in civilized contries. It is currently seen as a "complex" genetic trait caused by multiple susceptibility genes which are modulated by gene-environment and gene-gene interactions. Also, their etiology is complex with substantial environmental components. There is a strong indication that multiple genes are implicated. Specific candidate genes have been tested for linkage and association with a blood pressure or the diagnosis of hypertension. Nevertheless, the genetic alterations responsible for inherited hypertention remain largely unknown, and the success to date in identifying susceptibility genes has been very limited. Depending on the genetic factors of human hypertension, it appears that SNPs in the candidate genes may play a significant role as useful genetic markers in the association study (Schrader *et al.*, 1996).

In the present study, we failed to demonstrate the significant association between the *Pst* I RFLP of the SA gene and hypertension or other cardiovascular risk factors

Table 2. Genotype and allele frequencies of *Pst* I RFLP of the SA gene in normotensives and essential hypertensives

	Genotype No. (%)			Allele No. (%)		H^1	PIC^2
	P1P1	P1P2	P2P2	P1	P2		
Normotensives	54(54)	43(43)	3(3)	151(76)	49(25)	0.3700	0.3015
Hypertensives	56(56)	38(38)	6(6)	150(75)	50(25)	0.3750	0.3047
χ^2		1.3450			0.0134		
P		0.5104			0.9078		
Odds ratio(CI)[3]			1.03(0.65-1.62)				

[1]Heterozygosity, [2]Polymorphism Information Content, [3]95% Confidence Interval.
Frequency is given as a percentage in parenthesis.

Table 3. Genotype and allele frequencies of C667T mutation of the MTHFR gene in normotensives and essential hypertensives

	Genotype No. (%)			Allele No. (%)		H^1	PIC^2
	Ala/Ala	Val/Ala	Val/Val	Ala	Val		
Normotensives	37(37)	45(46)	17(17)	119(60)	79(40)	0.4796	0.3646
Hypertensives	32(32)	47(47)	21(21)	111(55)	89(45)	0.4940	0.3720
χ^2		0.8220			0.8630		
P		0.6630			0.3530		
Odds ratio(CI)[3]			1.21(0.81-1.80)				

[1]Heterozygosity, [2]Polymorphism Information Content, [3]95% Confidence Interval.
Frequency is given as a percentage in parenthesis.
The observed genotype distribution was in Hardy-weinberg equilibrium (For normotensives, $\chi^2=0.2700$, df=1, P=0.6033; for essential hypertensives, $\chi^2=0.2350$, df=1, P=0.6278; for essential hypertensives).

Table 4. Clinical characteristics of subjects according to genotypes of Pst I RFLP at the SA gene

Variables	Genotypes		
	P1P1(No.)[8]	P1P2(No.)	P2P2(No.)
Age (year)	60.1 ± 10.6(109)	58.7 ± 12.0(80)	62.0 ± 9.9(9)
BMI (kg/m^2)[1]	23.7 ± 2.3(103)	23.7 ± 2.7(70)	23.8 ± 1.7(8)
Tg (mg/dl)[2]	132.3 ± 75.7(81)	120.9 ± 78.7(64)	130.4 ± 55.2(8)
TC (mg/dl)[3]	149.5 ± 36.8(81)	149.4 ± 34.4(64)	170.8 ± 30.7(8)
LDL-chol (mg/dl)[4]	95.5 ± 35.9(81)	99.1 ± 35.5(64)	114.2 ± 29.2(8)
HDL-chol (mg/dl)[5]	27.5 ± 10.2(81)	25.8 ± 8.9(64)	30.5 ± 5.3(8)
Lp(a) (mg/dl)[6]	15.7 ± 10.5(90)	15.9 ± 13.3(61)	18.3 ± 8.8(8)
Apo AI (mg/dl)[7]	98.7 ± 35.2 (29)	112.0 ± 38.5(17)	96.0 ± 0.0(1)

[1]Body Mass Index, [2]Triglyceride, [3]Total cholesterol, [4]LDL-cholesterol, [5]HDL-cholesterol, [6]lipoprotein (a), [7]apolipoprotein AI and [8]Number. Values are mean ± SD (Standard Deviation).

Table 5. Clinical characteristics according to genotypes of C667T mutation at the MTHFR gene

Variables	Genotypes		
	Ala/Ala(No.)[8]	Val/Ala(No.)	Val/Val(No.)
Age (year)	59.3 ± 11.6(69)	59.0 ± 11.0(88)	61.34 ± 11.1(38)
BMI (kg/m^2)[1]	23.8 ± 2.4(63)	23.6 ± 2.2(82)	23.6 ± 3.3(34)
Tg (mg/dl)[2]	134.3 ± 79.2(49)	130.0 ± 81.4(75)	111.1 ± 51.5(27)
TC (mg/dl)[3]	147.6 ± 31.2(49)	152.7 ± 39.0(75)	150.5 ± 35.2(27)
LDL-chol (mg/dl)[4]	93.0 ± 30.5(49)	101.5 ± 38.0(75)	98.0 ± 37.8(27)
HDL-chol (mg/dl)[5]	27.8 ± 9.6(49)	25.6 ± 8.7(75)	28.5 ± 11.3(27)
Lp(a) (mg/dl)[6]	15.4 ± 9.9(58)	16.1 ± 12.0(67)	16.8 ± 13.6(32)
Apo AI (mg/dl)[7]	106.42 ± 38.55(20)	94.9 ± 31.2(17)	103.32 ± 33.45(20)

[1]Body Mass Index, [2]Triglyceride, [3]Total cholesterol, [4]LDL-cholesterol, [5]HDL-cholesterol, [6]lipoprotein (a), [7]apolipoprotein AI and [8]Number. Values are mean ± SD (Standard Deviation).

Table 6. Comparison of allele frequencies of *Pst* I RFLP in the SA gene from various ethnic groups

Populations	Sample number	Allele frequencies		P[1]	Reference
		P1	P2		
(Normotensive)					
Caucasian	96	0.93	0.07	<0.05	Zee *et al.*, 1997
Japanese	81	0.91	0.09	<0.05	Iwai *et al.*, 1994
Korean	100	0.76	0.25		Present study
(Hypertensive)					
Caucasian	106	0.89	0.11	<0.05	Zee *et al.*, 1997
Japanese	86	0.73	0.27	NS[2]	Iwai *et al.*, 1994
Korean	100	0.75	0.25		Present study

[1]Probability, [2]Not significant.

Table 7. Comparison of allele frequencies of C667T mutation in the MTHFR gene from Asian populations

Populations	Sample number	Allele frequencies		P[1]	Reference
		Ala	Val		
(Normotensive)					
Japanese	184	0.58	0.42	NS[2]	Nakata *et al.*, 1998
Korean	99	0.60	0.40		Present study
(Hypertensive)					
Japanese	173	0.63	0.37	NS[2]	Nakata *et al.*, 1998
Korean	100	0.55	0.45		Present study

[1]Probability, [2]Not significant.

in Koreans. Therefore, it is unlikely that this SNP may influence the etiology of hypertension or other cardiovascular diseases in our subjects. In normotensives, P2 allele frequency of Korean (0.25) was higher than those of Caucasian (0.07) and Japanese (0.09) (Table 6), while in hypertensives, allele frequency of Korean (0.25) similar to that of Japanese (0.27), but higher that of Caucasian (0.11). As a possible explanation for this phenomenon, the difference in genetic background or sampling bias could be considered. It seems to be important for carefully designed studies to minimize the ethnic heterogeneity of the case and control populations (Pollak *et al.*, 2000).

In the case of C667T mutation in the MTHFR gene, A significant deviation from Hardy-Weinberg equilibrium has been observed. This suggests a probable recent origin of this polymorphism. Nakata *et al.*, (1998) reported that this mutation was associated with hypertension in Japanese population. However, there was also no significant association with any cardiovascular risk factors as well as hypertension in Korean population. This inconsistency may not be explained by the difference in genetic background, because the Val allele frequency of MTHFR gene in Korean was similar to that of Japanese in the both groups (Table 7). The sampling bias or the difference in environmental condition could be considered as a probable explanation for this difference. This phenomenon was already detected in case-control association studies between Glu298Asp polymorphism of endothelial nitric oxide synthase gene and hypertension in Japanese populations (Kato *et al.*, 1999). To our knowledge, the studies for the association between C667T mutation of MTHFR gene and hypertension were only performed in Asian population (Nakata *et al.*, 1998). Thus, studies in other racial or ethnic groups will be of great interest.

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