Table 3. Genotype distribution in NTs and patients with EH

	rs2286245			rs3791124			rs2486001	
Genotype								
	NT	EH		NT	EH		NT	EH
TT	20 (0.028)	12 (0.016)	AA	45 (0.0631)	47 (0.0626)	AA	43 (0.060)	48 (0.064)
CT	168 (0.236)	153 (0.206)	GA	270 (0.3787)	242 (0.3222)	GA	277 (0.386)	248 (0.333)
TT	523 (0.736)	577 (0.778)	GG	398 (0.5582)	462 (0.6152)	GG	397 (0.554)	449 (0.603)
p		0.096	*p*		0.069	*p*		0.103
Dominant								
TT+CT	188 (0.264)	165 (0.221)	AA+GA	315 (0.4417)	289 (0.3848)	AA+GA	320 (0.446)	296 (0.397)
CC	523 (0.736)	577 (0.775)	GG	398 (0.5582)	462 (0.6152)	GG	397 (0.554)	449 (0.603)
OR		0.796	OR		0.79	OR		0.818
95%CI		0.626-1.012	95%CI		0.642-0.974	95%CI		0.664-1.007
p		0.062	*p*		0.027	*p*		0.058
Recessive								
TT	20 (0.028)	12 (0.016)	GG+GA	668 (0.9369)	704 (0.9374)	AA	43 (0.060)	48 (0.064)
CC+CT	691 (0.972)	730 (0.984)	AA	45 (0.0631)	47 (0.0626)	GG+AG	674 (0.940)	697 (0.936)
OR		0.568	OR		0.991	OR		1.079
95%CI		0.276-1.171	95%CI		0.650-1.512	95%CI		0.706-1.851
p		0.125	*p*		0.967	*p*		0.724
Allele								
T	208 (0.146)	177 (0.119)	A	360 (0.2525)	336 (0.2237)	A	363 (0.253)	344 (0.231)
C	1,214 (0.854)	1,307 (0.881)	G	1,066 (0.7476)	1,166 (0.7763)	G	1,071 (0.747)	1,146 (0.769)
OR		0.79	OR		0.853	OR		0.886
95%CI		0.637-0.980	95%CI		0.720-1.012	95%CI		0.748-1.049
p		0.032	*p*		0.068	*p*		0.16

NT: normotensive control, EH: essential hypertension

phism with schizophrenia[28, 29] or methamphetamine-use disorder[30] has been reported. There is a rapidly growing body of literature describing inhibitors of SLC6A9[20, 31]; however, the effect of these inhibitors on blood pressure has not been reported. The elevation of blood pressure in response to psychoemotional stimuli is well known by clinicians, and the SLC6A9 gene may affect blood pressure by altering the response to psychoemotional stimuli.

In summary, we examined polymorphisms of the SLC6A9 gene in a case-control study of the Japanese population. This finding needs further confirmation in a variety of ethnic groups, and functional studies are required to elucidate the mechanisms underlying the association of SLC6A9 polymorphisms with essential hypertension.

Acknowledgements

This work was supported in part by a 2006 Research Grant of the 60th 10 Anniversary Memorial Fund, Nihon University Medical Alumni Association (T.U.). We thank Professor Ktsushi Tokunaga and co-workers for partial SNP typings, and Akiko Tsunemi for excellent technical assistance.

References

1) Joffres MR, Hamet P, MacLean DR, L'italien GJ, Fodor G: Distribution of blood pressure and hypertension in Canada and the United States. Am J Hypertens, 2001; 14:1099-1105
2) Hitsumoto T, Takahashi M, Iizuka T, Shirai K: Relation-

ship between metabolic syndrome and early stage coronary atherosclerosis. J Atheroscler Thromb, 2007; 14:294-302
3) Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP: Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Engl J Med, 1990; 322:1561-1566
4) Kawamoto R, Tomita H, Ohtsuka N, Inoue A, Kamitani A: Metabolic syndrome, diabetes and subclinical atherosclerosis as assessed by carotid intima-media thickness. Journal of Atherosclerosis and Thrombosis, 2007; 14:78-85
5) Devereux RB, de Simone G, Ganau A, Roman MJ: Left ventricular hypertrophy and geometric remodeling in hypertension: stimuli, functional consequences and prognostic implications. J Hypertens Suppl, 1994; 12:S117-127
6) Dominiczak AF, Negrin DC, Clark JS, Brosnan MJ, McBride MW, Alexander MY: Genes and hypertension: from gene mapping in experimental models to vascular gene transfer strategies. Hypertension, 2000; 35:164-172
7) Brown DM, Provoost AP, Daly MJ, Lander ES, Jacob HJ: Renal disease susceptibility and hypertension are under independent genetic control in the fawn-hooded rat. Nat Genet, 1996; 12:44-51
8) Deng AY, Dene H, Pravenec M, Rapp JP: Genetic mapping of two new blood pressure quantitative trait loci in the rat by genotyping endothelin system genes. J Clin Invest, 1994; 93:2701-2709
9) Gu L, Dene H, Deng AY, Hoebee B, Bihoreau MT, James M, Rapp JP: Genetic mapping of two blood pressure quantitative trait loci on rat chromosome 1. J Clin Invest, 1996; 97:777-788
10) Jacob HJ, Lindpaintner K, Lincoln SE, Kusumi K, Bunker RK, Mao YP, Ganten D, Dzau VJ, Lander ES: Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. Cell, 1991; 67:213-224
11) Hilbert P, Lindpaintner K, Beckmann JS, Serikawa T, Soubrier F, Dubay C, Cartwright P, De Gouyon B, Julier C, Takahasi S, et al: Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. Nature, 1991; 353:521-529
12) Ueno T, Tremblay J, Kunes J, Zicha J, Dobesova Z, Pausova Z, Deng AY, Sun YL, Jacob HJ, Hamet P: Resolving the composite trait of hypertension into its pharmacogenetic determinants by acute pharmacological modulation of blood pressure regulatory systems. J Mol Med, 2003; 81:51-60
13) Amara SG, Kuhar MJ: Neurotransmitter transporters: recent progress. Annu Rev Neurosci, 1993; 16:73-93
14) Borowsky B, Hoffman BJ: Neurotransmitter transporters: molecular biology, function, and regulation. Int Rev Neurobiol, 1995; 38:139-199
15) Legendre P: The glycinergic inhibitory synapse. Cell Mol Life Sci, 2001; 58:760-793
16) Johnson JW, Ascher P: Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature, 1987; 325:529-531
17) Zahniser NR, Doolen S: Chronic and acute regulation of Na^+/Cl^- -dependent neurotransmitter transporters: drugs, substrates, presynaptic receptors, and signaling systems. Pharmacol Ther, 2001; 92:21-55
18) Borowsky B, Mezey E, Hoffman BJ: Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. Neuron, 1993; 10:851-863
19) Bergeron R, Meyer TM, Coyle JT, Greene RW: Modulation of N-methyl-D-aspartate receptor function by glycine transport. Proc Natl Acad Sci USA, 1998; 95:15730-15734
20) Kinney GG, Sur C, Burno M, Mallorga PJ, Williams JB, Figueroa DJ, Wittmann M, Lemaire W, Conn PJ: The glycine transporter type 1 inhibitor N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl] sarcosine potentiates NMDA receptor-mediated responses in vivo and produces an antipsychotic profile in rodent behavior. J Neurosci, 2003; 23:7586-7591
21) Chen L, Muhlhauser M, Yang CR: Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons in vitro and in vivo. J Neurophysiol, 2003; 89:691-703
22) Ueno T, Tremblay J, Kunes J, Zicha J, Dobesova Z, Pausova Z, Deng AY, Sun YL, Jacob HJ, Hamet P: Rat model of familial combined hyperlipidemia as a result of comparative mapping. Physiol Genomics, 2004; 17:38-47
23) Esler M, Jennings G, Lambert G, Meredith I, Horne M, Eisenhofer G: Overflow of catecholamine neurotransmitters to the circulation: source, fate, and functions. Physiol Rev, 1990; 70:963-985
24) Parsons CG, Danysz W, Hesselink M, Hartmann S, Lorenz B, Wollenburg C, Quack G: Modulation of NMDA receptors by glycine--introduction to some basic aspects and recent developments. Amino Acids, 1998; 14:207-216
25) Gomeza J, Hülsmann S, Ohno K, Eulenburg V, Szöke K, Richter D, Betz H: Inactivation of the glycine transporter 1 gene discloses vital role of glial glycine uptake in glycinergic inhibition. Neuron, 2003; 40:785-796
26) Supplisson S, Bergman C: Control of NMDA receptor activation by a glycine transporter co-expressed in Xenopus oocytes. J Neurosci, 1997; 17:4580-4590
27) Wood PL: The co-agonist concept: is the NMDA-associated glycine receptor saturated in vivo? Life Sci, 1995; 57:301-310
28) Tsai SJ, Cheng CY, Hong CJ, Liao DL, Liou YJ: Polymorphisms in glycine transporter with schizophrenia. Neuropsychopharmacol Hung, 2006; 8:17-21
29) Tsai SJ, Cheng CY, Hong CJ, Liao DL, Hou SJ, Yen FC, Liou YJ: Association study of polymorphisms in glycine transporter with schizophrenia. J Neural Transm, 2006; 113:1545-1549
30) Morita Y, Ujike H, Tanaka Y, Kishimoto M, Okahisa Y, Kotaka T, Harano M, Inada T, Komiyama T, Hori T, Yamada M, Sekine Y, Iwata N, Iyo M, Sora I, Ozaki N, Kuroda S: The glycine transporter 1 gene (GLYT1) is associated with methamphetamine-use disorder. Am J Med Genet B Neuropsychiatr Genet, 2008; 147B:54-58
31) Martina M, Gorfinkel Y, Halman S, Lowe JA, Periyalwar P, Schmidt CJ, Bergeron R: Glycine transporter type 1 blockade changes NMDA receptor-mediated responses and LTP in hippocampal CA1 pyramidal cells by altering extracellular glycine levels. J Physiol, 2004; 557:489-500

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.

© Med Sci Monit, 2009; 15(7): CR341-348
PMID: 19564823

www.MEDSCIMONIT.COM
Clinical Research

CR

Received: 2008.11.20
Accepted: 2009.02.09
Published: 2009.07.01

Influence of genetic polymorphisms in oxidative stress related genes and smoking on plasma MDA-LDL, soluble CD40 ligand, E-selectin and soluble ICAM1 levels in patients with coronary artery disease

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Takahiro Ueno[1ACDEFG], Hideyuki Watanabe[1CG], Noboru Fukuda[1G], Akiko Tsunemi[1G], Kazunobu Tahira[1D], Taro Matsumoto[1G], Tadateru Takayama[2D], Masaaki Chiku[2D], Satoshi Saito[2G], Yuichi Sato[3G], Atsushi Hirayama[2G], Koichi Matsumoto[1G], Masayoshi Soma[4G]

[1] Department of Medicine, Division of Nephrology, Hypertension and Endocrinology, Nihon University School of Medicine, Tokyo, Japan
[2] Department of Medicine, Division of Cardiology, Nihon University School of Medicine, Tokyo, Japan
[3] Department of Medicine, Division of Integrative Health Medicine, Nihon University School of Medicine, Tokyo, Japan
[4] Department of Medicine, Division of General Medicine, Nihon University School of Medicine, Tokyo, Japan

Source of support: Departmental sources

Summary

Background: Previous studies have shown that oxidative stress plays an important role in coronary heart disease. Polymorphisms in key enzymes that regulate oxidative stress may play a role in atherogenicity and were investigated in this study.

Material/Methods: One hundred and forty-three patients with angiographically proven coronary artery disease were studied. The effect of the C242T polymorphism of the p22phox gene, an essential component of the NADH/NADPH oxidase, and glutathione-S-transferase T1, M1 and P1 polymorphisms on plasma MDA-LDL, soluble CD40 ligand, E-selectin and soluble ICAM1 levels was determined. Genotyping of the p22 phox C242T polymorphism was performed by RFLP analysis, and GSTT1, GSTM1 and GSTP1 genotypes were determined using a multiplex PCR assay. The MDA-LDL, sCD40L, E-selectin and sICAM1 levels were determined using ELISA

Results: Patients with the TT or TC genotype of the p22 phox C242T polymorphism had significantly higher plasma MDA-LDL levels compared to those of the CC genotype.

Plasma E-selectin and soluble ICAM1 levels were significantly higher in the TT or TC genotype compared to that of the CC genotype. In GSTT1+ patients, plasma MDA-LDL levels were significantly higher than those of GSTT1– patients.

Conclusions: Genetic polymorphism of the p22 phox gene had a significant effect on plasma lipid peroxidation and endothelial function through oxidative stress. The results of this study confirm the effect of NADH/NADPH oxidase on atherogenecity.

key words: GST • p22phox • MDA-LDL • smoking • coronary artery disease

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=869708
Word count: 1935
Tables: 3
Figures: 2
References: 25

Author's address: Takahiro Ueno, Division of Nephrology, Hypertension and Endocrinology, Department of Medicine; Nihon University School of Medicine, Oyaguchi-kami 30-1, Itabashi-ku, Tokyo 173-8610, Japan, e-mail: tueno@med.nihon-u.ac.jp

Electronic PDF security powered by ISI Science.com

PERSONAL USE ONLY

BACKGROUND

Coronary artery disease (CAD) is a complex vascular disease which results from an interaction between genetic and environmental factors. Many genetic variants have been identified that impact upon incidence of CAD, including those involved in oxidative stress [1]. Oxidative stress has been proposed as one of the fundamental causes for the initiation and progression of CAD under the response to injury hypothesis [2]. While numerous genetic variantions have been observed to occur in patients with CAD, little work has been done to investigate the interaction of these genetic variants and oxidized products.

NAD(P)H oxidase is present in vascular smooth muscle cells and endothelial cells, and has been identified as a major source of superoxide production in animal models of vascular disease [3] and in human atherosclerosis [4,5]. P22 phox is an essential component of NADH/NADPH oxidase, and is expressed in human coronary arteries [6,7]. Oxidation of low density lipoprotein (LDL), an important process in early atherogenesis, is supposed to be associated with NADPH oxidase activity. Oxidative metabolites are involved in endothelial dysfunction, which is thought to be a major component of atherosclerosis [6]. The C242T polymorphism of the p22phox gene, which leads to an amino acid change, leads to alteration of potential heme binding sites on the protein, and might modulate its enzymatic activity. However, results from previous studies on the relationship of the C242T polymorphism and atherosclerotic disease conflict in their findings [8–11].

Glutathione-S-transferases (GSTs) are a class of phase II enzymes that catalyze the reaction between glutathione and various endogenous or exogenous reactive electrophilic substances. Many GSTs substrates are considered carcinogens, including dichloromethane and polycyclic aromatic hydrocarbons found in cigarette smoke. Other substrates are byproducts of free radical damage caused by reactive oxygen species, such as oxidized lipids. Smoking is believed to cause oxidative stress through several mechanisms, including direct damage by radical species and from the inflammatory response [12].

Investigation of the effect of p22 phox and GSTs gene polymorphisms on malondialdehyde-modified LDL (MDA-LDL), soluble CD40 ligand, E-selectin and soluble ICAM1 (intercellular adhesion molecule-1) in CAD patients, and the interaction of the combined effect with cigarette smoking may help elucidate their role in atherogenesis.

MATERIAL AND METHODS

Study population

Subjects were patients who underwent coronary angiography at Nihon University Itabashi Hospital, Japan, and had >75% diameter stenosis of at least one of the major coronary arteries. Among patients with significant coronary artery disease, sixty-two current smokers and seventy-eight patients without a history of smoking were recruited into the study after exclusion of patients suffering from kidney or liver disease. Written consent was obtained from every patient after full explanation of the study. These participants represented approximately 63% of hypertensive patients (n=90) and 48% of diabetic patients (n=69). Fasting plasma levels of total cholesterol, triglycerides, HDL-cholesterol, apolipoprotein AI, AII, B, CII, CIII, E, glucose, and HbA1c were determined before coronary angiography.

Determination of MDA-LDL, soluble CD40, E-selectin and soluble ICAM1

The ELISA method used to determine the plasma MDA-LDL level was based on the same principles as previously reported by Kotani et al. [13]. The ELISA was performed with a Bender Medsystems kit (Medsystem Diagnostics, Vienna, Austria) and plasma sCD40L, E-selectin and sICAM1 levels were quantitatively determined.

Genotyping

Peripheral blood samples were collected and genomic DNA isolated from leukocytes by standard methods.

Genotyping for the p22 phox C242T polymorphism was performed by RFLP analysis, as described previously [9]. Briefly, a 348bp DNA fragment, containing a C242T polymorphic site was amplified by PCR and digested with RsaI (New England Biolabs, Beverly, MA). The 242C allele remains uncut, while the 242T allele was cut into two fragments of 160 and 188bp.

The GSTT1, GSTM1 and GSTP1 genotypes were determined using a multiplex PCR assay as previously described [13–16]. Briefly, the presence of 215-bp and 480-bp amplicons correspond to individuals with intact GSTT1 and/or GSTM1 alleles. The absence of either of these amplicons corresponds to individuals homozygous for the null allele. The presence of a 175-bp amplicon from GSTmu2 was utilized as an internal control, confirming successful amplification.

The GSTP1 gene polymorphism at codon 105 was determined using the restriction fragment length polymorphism (RFLP) method described by Watson et al. [17]. Briefly, PCR products were digested with the restriction enzyme Alw26I (New England Biolab) which through restriction sites present on both the Ile allele and the Val allele allows determination of which allele/s are present. PCR-RFLP patterns resulted in a band of 113 bp in all samples, (a control cut that confirms proper digestion has occurred). In the wild-type (Ile/Ile), bands of 329 bp and 113 bp were generated, whereas in the homozygous mutant type (Val/Val), bands of 216 bp, 113 bp and 107 bp were produced. In the heterozygous mutant type (Ile/Val), all four bands were present.

Statistical analysis

Results are given as the mean ± standard deviation of the mean (S.D.). The significance of differences between the mean values was evaluated using Student's *t*-test for unpaired data. A $P<0.05$ was considered significant. Factors with $p<0.05$ in the univariate analysis were included in a forward stepwise logistic multivariate regression analysis.

RESULTS

Clinical characteristics of patients, divided into p22 phox, GSTT1, GSTM1 and GSTP1 genotypes are shown in Table 1A.

Electronic PDF security powered by IS-scientific.com

CR

Table 1A. Clinical characteristics of the subjects, categorized by GSTT1, GSTM1, GSTP1, and p22 phox polymorphisms.

	n	Genotypes		Age (y)	BW (kg)	Hight (cm)	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	A-I (mg/dl)	A-II (mg/dl)	B (mg/dl)	C-II (mg/dl)	C-III (mg/dl)
	143		Mean	63.4	67.6	162.8	188.0	151.1	42.0	107.3	22.9	92.5	4.9	8.9
			S.D.	9.3	11.0	7.6	40.7	102.5	15.7	23.3	4.9	26.0	6.9	8.5
p22phox	103	CC	Mean	63.3	67.6	163.1	182.3	143.9	42.5	107.1	22.8	88.6	5.1	9.0
			S.D.	8.8	10.9	7.5	35.1	98.7	16.3	22.9	5.0	22.3	7.9	9.8
	37	CT/TT	Mean	63.3	67.7	162.4	199.9	173.1	39.1	104.8	23.0	102.2	4.3	8.7
			S.D.	10.8	11.6	8.0	48.7	114.2	11.6	22.3	4.5	31.3	1.9	3.2
			p	0.99	0.94	0.65	0.06	0.18	0.18	0.62	0.89	0.06	0.32	0.78
GSTT1	74	T–	Mean	63.4	67.0	162.3	186.1	134.3	42.9	108.4	22.8	90.7	4.7	9.3
			S.D.	8.6	11.5	8.2	41.8	60.7	17.6	23.0	5.1	26.7	5.7	11.4
	69	T+	Mean	63.7	68.0	163.2	191.6	171.9	41.0	106.3	23.2	95.4	5.1	8.6
			S.D.	10.0	10.4	6.9	39.8	130.6	13.1	23.6	4.9	25.3	7.9	3.1
			p	0.87	0.61	0.48	0.42	0.05	0.48	0.60	0.63	0.29	0.77	0.65
GSTM1	76	M–	Mean	62.7	67.3	162.7	187.3	139.0	43.0	107.2	23.0	92.8	4.8	8.2
			S.D.	9.0	10.0	7.1	38.3	70.6	17.9	23.4	5.3	24.3	7.5	2.6
	67	M+	Mean	64.5	67.7	162.8	190.3	167.3	40.9	107.6	23.0	93.1	5.0	9.8
			S.D.	9.5	11.9	8.2	43.7	127.1	12.5	23.2	4.7	28.0	6.0	12.0
			p	0.24	0.83	0.94	0.66	0.11	0.42	0.93	0.95	0.95	0.85	0.27
GSRP1	113	AA	Mean	63.4	67.0	162.7	187.2	154.2	41.9	106.6	22.9	91.6	4.9	9.1
			S.D.	9.4	11.1	7.4	40.6	107.8	16.6	24.2	5.2	25.0	7.5	9.5
	29	AG	Mean	64.1	69.2	162.8	194.4	145.8	42.3	110.5	23.3	97.6	4.7	8.4
			S.D.	8.9	10.3	8.5	41.9	78.3	10.9	19.3	4.3	29.5	3.5	2.2
			p	0.71	0.33	0.96	0.41	0.63	0.88	0.36	0.67	0.32	0.82	0.46

Table 1B. Clinical characteristics of the subjects, categorized by smoking behavior.

	n		age (y)	BW (kg)	Hight (cm)	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	A-I (mg/dl)	A-II (mg/dl)	B (mg/dl)	C-II (mg/dl)	C-III (mg/dl)
Non-smokers	78	Mean	65.4	67.0	161.4	186.8	142.0	43.6	111.6	23.4	90.3	5.4	9.4
		S.D.	9.5	11.3	8.2	40.4	100.6	13.4	24.2	5.2	26.3	9.1	11.2
Smokers	62	Mean	60.9	68.3	164.5	189.5	162.5	40.0	101.9	22.3	95.1	4.2	8.3
		S.D.	8.3	10.6	6.6	41.4	104.6	18.0	21.1	4.5	25.6	1.9	2.9
		p	0.00	0.50	0.05	0.70	0.24	0.19	0.01	0.20	0.28	0.26	0.42

Unpaired Student's t-test was used for statistical comparisons between genotypes. BW – body weight; TC – total cholesterol; TG – triglycerides; Lp(a) – lipoprotein (a); RLP-C – remnant-like particle-cholesterol; FPG – fasting plasma glucose.

There were no significant differences observed for parameters including lipid and glucose metabolism between the two allelic groups for each gene.

Clinical characteristics for current smokers and non-smokers are shown in Table 1B. Serum apolipoprotein AI levels were significantly lower in current smokers compared to non-smokers.

Electronic PDF security powered by ISI Web of Knowledge

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.

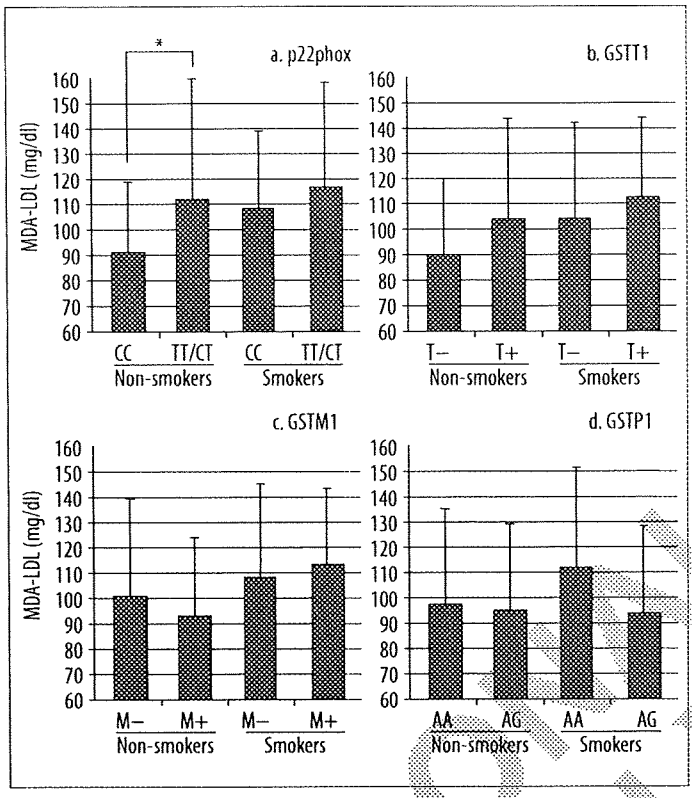

Figure 1. Plasma MDA-LDL levels in CAD patients categorized by p22 phox, GSTT1, GSTP1, GSTM1, and cigarette smoking. *$P<0.05$ by Student's t-test; GST: Glutathione-S-transferases; T−: GSTT1 null genotype; M−: GSTM1 null genotype.

The effect of each gene polymorphism on plasma MDA-LDL levels are shown in Figure 1. Patients with the TT or TC genotype of the p22phox C242T polymorphism showed significantly higher plasma MDA-LDL levels compared to that of patients with the CC genotype.

In the GSTT1+ subjects, plasma MDA-LDL levels were significantly higher in comparison with those in GSTT1-subjects. There were no significant differences observed in levels between the GSTM1 and GSTP1 polymorphism. Multivariate forward logistic regression analysis showed that the P22phox C242T polymorphism (F=4.5) and the GSTT1 polymorphism (F=2.9) were significant predictors of plasma MDA-LDL levels among the determined genotypes (p=0.005).

No significant effect on plasma soluble CD 40 levels was found to occur between the four examined polymorphisms. Plasma E-selectin levels were significantly higher in patients of the GSTT1+ genotype compared to those of the GSTT1− genotype group. Plasma E-selectin levels were also significantly higher in subjects with the CC and CT alleles of the p22 phox polymorphism and the AA genotype of the GSTP1 polymorphism (Table 2). Multivariate forward logistic regression analysis showed that P22phox C242T polymorphism (F=6.7) and GSTT1 polymorphism (F=3.4) were significant predictors of plasma E-selectin levels among the determined genotypes (p=0.001). Plasma soluble ICAM1 levels were significantly associated with the p22phox C242T polymorphism and the GSTM1 polymorphism (Table 2). Multivariate forward logistic regression analysis showed that the P22phox C242T polymorphism (F=11.6) and the GSTM1 polymorphism (F=6.4) were significant predictors of plasma sICAM1 levels among the determined genotypes (p=0.0003).

Comparison of plasma MDA-LDL levels in current smokers and non-smokers was made by dividing the patients into subgroups based on p22 phox, GSTT1, GSTM1 and GSTP1 polymorphism. With the p22 phox C242T polymorphism, plasma MDA-LDL levels were significantly lower only in non-smoking patients with CC genotypes (Figure 2). There were no significant differences observed between the GSTT1, GSTM1 and GSTP1 polymorphisms in smokers and non-smokers.

The plasma MDA-LDL, soluble CD40 ligand, E-selectin and soluble ICAM1 levels are shown in Table 3. Plasma soluble CD40 ligand levels were significantly higher in current smokers than those in non-smokers. There were no statistical differences identified in the plasma E-selectin and soluble ICAMI levels between smokers and non-smokers.

In non smokers the GSTT1 polymorphism affected plasma E-selectin levels, the GSTP1 polymorphism affected soluble CD40 ligand levels, and p22phox polymorphism affected plasma E-selectin and soluble ICAM1 levels. In smokers the GASTM1 polymorphism affected plasma soluble ICAM1 levels.

Discussion

The potential association of the C242T polymorphism of the p22phox gene and GSTs gene mutations with modified LDL and endothelial function was evaluated. The T allele

Electronic PDF security powered by IndexCopernicus.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.
FREE ONLY USE

Table 2. Serum MDA-LDL, soluble CD40 ligand, E-selectin, and soluble ICAM1 levels in participants categorized by p22 phox, GSTT1, GSTM1, and GSTP1 polymorphisms.

	n	Genotypes		MDA-LDL (U/l)	sCD40l (ng/ml)	ESELE (ng/ml)	sICAM1 (ng/ml)
CAD patients	143		Mean	103.2	9.2	21.5	234.5
			S.D.	34.9	4.3	19.8	124.0
p22phox	103	CC	Mean	98.9	9.1	17.8	218.1
			S.D.	30.0	4.2	12.3	117.4
	37	CT/TT	Mean	116.1	9.2	30.5	278.6
			S.D.	44.6	4.3	30.7	128.7
			p	0.04	0.97	0.02	0.02
GSTT1	74	T–	Mean	97.7	9.0	17.5	227.5
			S.D.	32.9	4.6	13.4	118.4
	69	T+	Mean	111.8	9.2	26.9	242.4
			S.D.	37.5	3.9	24.4	127.4
			p	0.02	0.74	0.01	0.47
GSTM1	76	M–	Mean	105.8	9.5	22.6	255.8
			S.D.	37.6	4.5	17.5	133.0
	67	M+	Mean	102.8	8.7	21.6	211.9
			S.D.	33.8	4.0	22.6	106.6
			p	0.62	0.25	0.77	0.03
GSRP1	113	AA	Mean	106.1	9.4	23.3	235.8
			S.D.	37.2	4.3	21.7	128.3
	29	AG	Mean	97.3	7.8	17.8	230.7
			S.D.	28.7	4.0	11.4	100.0
			p	0.18	0.07	0.07	0.82

Unpaired Student's *t*-test was used for statistical comparisons between genotypes. MDA-LDL – malondialdehyde-modified LDL; sCD40l – soluble CD40 ligand; ESELE, E-selectin; sICAM1 – soluble intercellular adhesion molecule-1.

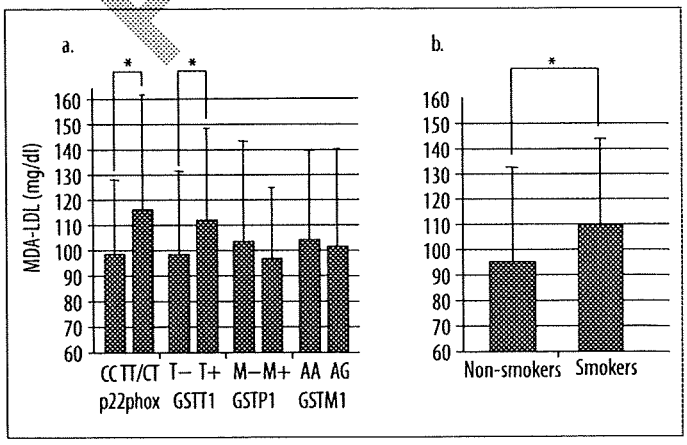

Figure 2. Plasma MDA-LDL levels in smoking and non-smoking CAD patients categorized by p22 phox, GSTT1, GSTP1, and GSTM1 polymorphisms. *$P<0.05$ by Student's *t*-test; GST: Glutathione-S-transferases; T–: GSTT1 null genotype; M–: GSTM1 null genotype.

of the P22 phox polymorphism showed a significant influence on lipid peroxidation *in vivo* as measured by MDA-LDL level from patients with CAD.

The C242T polymorphism results in a substitution of Tyr for His at residue 72 of p22 phox, leading to speculation that this polymorphism may modulate enzyme activity by affect-

Electronic PDF security powered by ISI Web of Knowledge

Table 3. Serum MDA-LDL, soluble CD40 ligand, E-selectin, and soluble ICAM1 levels in smoking and non-smoking subjects categorized by p22 phox, GSTT1, GSTM1, and GSTP1 polymorphisms.

	n		MDA-LDL (U/l)	sCD40l (ng/ml)	ESELE (ng/ml)	sICAM1 (ng/ml)
Non-smokers	78	Mean	97.5	8.5	20.6	218.9
		S.D.	34.9	4.3	17.2	119.3
Smokers	62	Mean	110.4	10.0	22.8	253.9
		S.D.	33.8	4.2	22.7	127.8
		p	0.03	0.04	0.54	0.10
p22phox						
Non-Smokers			MDALDL (U/l)	sCD40l (ng/ml)	ESELE (ng/ml)	sICAM1 (ng/ml)
CC	56	Mean	91.5	8.2	16.8	192.2
		S.D.	27.4	4.0	11.9	106.3
CC/CT	22	Mean	113.3	9.4	29.7	284.6
		S.D.	46.7	5.0	23.8	126.3
		p	0.014	0.278	0.003	0.002
Smokers						
CC	46	Mean	108.0	10.3	18.9	249.1
		S.D.	31.0	4.1	12.8	123.5
CC/CT	16	Mean	116.8	9.1	33.5	267.6
		S.D.	40.9	4.3	37.7	142.7
		p	0.901	0.357	0.075	0.389
GSTT1						
Non-Smokers	n		MDALDL (U/l)	sCD40l (ng/ml)	ESELE (ng/ml)	sICAM1 (ng/ml)
T1–	42	Mean	90.9	8.0	16.4	202.0
		S.D.	28.9	4.7	10.0	99.3
T+	36	Mean	105.7	9.1	25.1	237.8
		S.D.	40.0	3.9	21.8	137.2
		p	0.076	0.239	0.034	0.202
Smokers						
T–	30	Mean	106.8	10.4	18.4	260.3
		S.D.	36.8	4.3	16.8	135.6
T+	31	Mean	114.0	9.6	27.2	247.3
		S.D.	30.6	4.1	27.2	121.2
		p	0.413	0.450	0.135	0.695

ing heme binding [9]. Association of the T allele with CAD and cerebrovascular disease were previously reported in Japanese and Australian populations, but Li et al. [11] found no evidence of association of this polymorphism with CAD in the US based population studied. In this current study, a significant association found for the T allele of the polymorphism with plasma MDA-LDL levels in the Japanese CAD patients supports the results of previous reports from Japanese and Australian populations that showed a positive correlation of this mutation to the progress of atherosclerotic disease, and suggests that the impact of this mutation on atherosclerotic disease is through excessive lipid peroxidation.

Electronic PDF security powered by IndexCopernicus.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.

Table 3 continued. Serum MDA-LDL, soluble CD40 ligand, E-selectin, and soluble ICAM1 levels in smoking and non-smoking subjects categorized by p22 phox, GSTT1, GSTM1, and GSTP1 polymorphisms.

GSTM1						
Non-Smokers			**MDALDL (U/l)**	**sCD40l (ng/ml)**	**ESELE (ng/ml)**	**sICAM1 (ng/ml)**
M1–	39	Mean	101.8	9.5	21.2	222.2
		S.D.	37.4	4.8	16.6	132.6
M1+	39	Mean	93.3	7.5	20.0	215.9
		S.D.	32.1	3.7	17.9	106.8
		p	0.290	0.049	0.756	0.821
Smokers						
M1–	35	Mean	108.2	9.6	23.3	290.5
		S.D.	36.5	4.3	18.2	128.0
M1+	27	Mean	113.3	10.5	22.0	204.8
		S.D.	30.2	4.0	28.1	112.1
		p	0.558	0.402	0.843	0.007
GSTP1						
Non-Smokers			**MDALDL (U/l)**	**sCD40l (ng/ml)**	**ESELE (ng/ml)**	**sICAM1 (ng/ml)**
AA	64	Mean	97.6	9.1	21.0	222.8
		S.D.	35.7	4.4	17.9	126.4
AG	14	Mean	97.1	5.4	18.6	200.1
		S.D.	32.0	2.4	13.3	77.4
		p	0.955	0.001	0.596	0.403
Smokers						
AA	47	Mean	114.5	10.0	25.0	252.9
		S.D.	34.7	4.2	25.4	132.9
AG	15	Mean	96.8	10.0	15.9	257.2
		S.D.	27.5	4.1	9.2	115.0
		p	0.058	0.992	0.046	0.905

Unpaired Student's t-test was used for statistical comparisons between genotypes. MDA-LDL – malondialdehyde-modified LDL – sCD40l, soluble CD40 ligand; ESELE – E-selectin; sICAM1 – soluble intercellular adhesion molecule-1.

The results from this current study also indicated the influence of the C242T polymorphism of the p22phox gene on plasma E-selectin and soluble ICAM1 levels. E-selectin is a cell surface glycoprotein expressed on endothelial cells after activation by cytokines, and mediates adhesion of circulating monocytes and lymphocytes to endothelial cells. This adherence to activated arterial endothelium is one of the earliest detectable events in the pathogenesis of atherosclerosis[2]. The inflammatory process induces expression of adhesion molecules such as the intracellular adhesion molecule, which interacts with leukocyte integrins and promotes the atherothrombotic process at the surface of endothelial cells [18,19]. Expression of ICAM-1 can be rapidly upregulated several fold in atherosclerotic lesions by inflammatory mediators [20,21]. From these accumulated observations, the effect of the C242T polymorphism on E-selectin and soluble ICAM1 indicates induction of inflammation and endothelial dysfunction through oxidative stress in CAD patients.

Association of the GSTT1 and M1 null alleles with atherosclerotic disease has been reported previously. However, this data suggests an inability to provide any positive correlation of the GSTT1 or GSTM1 null allele with increased amount of plasma MDA-LDL, or increased plasma soluble CD40 ligand, E-selectin and soluble ICAM1 levels. These findings may suggest that the mechanism through which the GSTT1 and GSTM1 null alleles increase atherosclerot-

Electronic PDF security powered by ISI Science.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.

ic disease does not have any relation with lipid peroxidation or endothelial dysfunction.

Cigarette smoking has consistently been found to be a strong and independent risk factor for CAD. Studies have reported that GSTs can affect the body's ability either to detoxify or activate chemicals in cigarette smoke [22–25]. In this study, patients with the GSTT1 null allele (T–) showed significantly lower MDA-LDL levels compared to those with the wild type genotype. The GSTT1, GSTM1 and GSTP1 genotype did not show any significant effect on plasma MDA-LDL levels in current smokers. In patients without a smoking habit, those with the wild type allele of the GSTT1 genotype showed significantly higher E-selectin levels, and patients carrying the AG genotype of the GETP1 polymorphism showed significantly higher soluble CD40 ligand levels. Because GSTs function as a detoxifying enzyme, this data suggests an alternative mechanism for how GSTs act on modified lipids and endothelial function exists in non-smoking individuals. There is a possibility that some product from the reaction mediated by GSTs, which is an independent pathway from the detoxification of products from cigarette smoking, has an enhancing effect on oxidative stress and endothelial dysfunction.

Conclusions

In conclusion, this study shows that genetic polymorphism of the p22 phox gene had a statistically significant effect on plasma lipid peroxidation and endothelial function through oxidative stress. Results from this and previous studies confirm the effect of NADH/NADPH oxidase on atherogenecity.

References:

1. Mackness MI, Arrol S, Abbott C, Durrington PN: Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. Atherosclerosis, 1993; 104: 129–35
2. Ross R: Atherosclerosis – an inflammatory disease. N Engl J Med, 1999; 340: 115–26
3. Warnholtz A, Nickenig G, Schulz E et al: Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. Circulation, 1999; 99: 2027–33
4. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res, 2000; 86: 494–501
5. Guzik TJ, West NE, Black E et al: Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res, 2000; 86: E85–90
6. Azumi H, Inoue N, Takeshita S et al: Expression of NADH/NADPH oxidase p22phox in human coronary arteries. Circulation, 1999; 100: 1494–98
7. Cahilly C, Ballantyne CM, Lim DS et al: A variant of p22(phox), involved in generation of reactive oxygen species in the vessel wall, is associated with progression of coronary atherosclerosis. Circ Res, 2000; 86: 391–95
8. Cai H, Duarte N, Wilcken DE, Wang XL: NADH/NADPH oxidase p22 phox C242T polymorphism and coronary artery disease in the Australian population. Eur J Clin Invest, 1999; 29: 744–48
9. Inoue N, Kawashima S, Kanazawa K et al: Polymorphism of the NADH/NADPH oxidase p22 phox gene in patients with coronary artery disease. Circulation, 1998; 97: 135–37
10. Ito D, Murata M, Watanabe K et al: C242T polymorphism of NADPH oxidase p22 PHOX gene and ischemic cerebrovascular disease in the Japanese population. Stroke, 2000; 31: 936–39
11. Li A, Prasad A, Mincemoyer R et al: Relationship of the C242T p22phox gene polymorphism to angiographic coronary artery disease and endothelial function. Am J Med Genet, 1999; 84: 57–61
12. Bruno RS, Traber MG: Vitamin E biokinetics, oxidative stress and cigarette smoking. Pathophysiology, 2006; 13: 143–49
13. Kotani K, Maekawa M, Kanno T et al: Distribution of immunoreactive malondialdehyde-modified low-density lipoprotein in human serum. Biochim Biophys Acta, 1994; 1215: 121–25
14. Chen H, Sandler DP, Taylor JA et al: Increased risk for myelodysplastic syndromes in individuals with glutathione transferase theta 1 (GSTT1) gene defect. Lancet, 1996; 347: 295–97
15. Li R, Folsom AR, Sharrett AR et al: Interaction of the glutathione S-transferase genes and cigarette smoking on risk of lower extremity arterial disease: the Atherosclerosis Risk in Communities (ARIC) study. Atherosclerosis, 2001; 154: 729–38
16. Nedelcheva Kristensen V, Andersen TI, Erikstein B et al: Single tube multiplex polymerase chain reaction genotype analysis of GSTM1, GSTT1 and GSTP1: relation of genotypes to TP53 tumor status and clinicopathological variables in breast cancer patients. Pharmacogenetics, 1998; 8: 441–47
17. Watson MA, Stewart RK, Smith GB et al: Human glutathione S-transferase P1 polymorphisms: relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis, 1998; 19: 275–80
18. Luc G, Arveiler D, Evans A et al: Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and incident coronary heart disease: the PRIME Study. Atherosclerosis, 2003; 170: 169–76
19. Pancorbo D, Vazquez C, Fletcher MA: Vitamin C-lipid metabolites: uptake and retention and effect on plasma C-reactive protein and oxidized LDL levels in healthy volunteers. Med Sci Monit, 2008; 14(11): CR547–51
20. Dustin ML, Rothlein R, Bhan AK et al: Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J Immunol, 1986; 137: 245–54
21. Rubio-Guerra AF, Vargas-Robles H, Ayala GV, Escalante-Acosta BA: Correlation between circulating adhesion molecule levels and albuminuria in type 2 diabetic normotensive patients. Med Sci Monit, 2007; 13(8): CR349–52
22. Guengerich FP, Thier R, Persmark M et al: Conjugation of carcinogens by theta class glutathione s-transferases: mechanisms and relevance to variations in human risk. Pharmacogenetics, 1995; 5: S103–7
23. Meyer DJ, Coles B, Pemble SE et al: Theta, a new class of glutathione transferases purified from rat and man. Biochem J, 1991; 274(Pt 2): 409–14
24. Pemble S, Schroeder KR, Spencer SR et al: Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem J, 1994; 300(Pt 1): 271–76
25. Rebbeck TR: Molecular epidemiology of the human glutathione S-transferase genotypes GSTM1 and GSTT1 in cancer susceptibility. Cancer Epidemiol Biomarkers Prev, 1997; 6: 733–43

Electronic PDF security powered by ISI sciencedirect.com

Available online at www.sciencedirect.com
ScienceDirect
Clinical Biochemistry 42 (2009) 1493–1499
CLINICAL BIOCHEMISTRY

Haplotype-based case–control study between human apurinic/apyrimidinic endonuclease 1/redox effector factor-1 gene and cerebral infarction

Takahiro Naganuma [a,b], Tomohiro Nakayama [a,c,*], Naoyuki Sato [a], Zhenyan Fu [a], Mai Yamaguchi [a], Masayoshi Soma [d], Noriko Aoi [a], Ron Usami [b], Nobutaka Doba [e], Shigeaki Hinohara [e]

[a] *Division of Molecular Diagnostics, Department of Advanced Medical Science, Nihon University School of Medicine, Tokyo, Japan*
[b] *Department of Biological Applied Chemistry, Toyo University Graduate School of Engineering, Saitama, Japan*
[c] *Division of Laboratory Medicine, Department of Pathology and Microbiology, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30-1 Itabashi-ku, Tokyo 173-8610, Japan*
[d] *Division of General Medicine, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan*
[e] *Life Planning Center, Tokyo, Japan*

Received 28 February 2009; received in revised form 4 July 2009; accepted 14 July 2009
Available online 23 July 2009

Abstract

Objectives: The aim of this study was to investigate the relationship between cerebral infarction (CI) and the human apurinic/apyrimidinic endonuclease 1/redox effector factor-1 (APE1/REF-1) gene using single-nucleotide polymorphisms (SNPs) and a haplotype-based case–control study.

Design and methods: We selected 5 SNPs in the human APE1/REF1 gene (rs1760944, rs3136814, rs17111967, rs3136817 and rs1130409), and performed case–control studies in 177 CI patients and 309 control subjects.

Results: rs17111967 was found to have no heterogeneity in Japanese. The overall distribution of the haplotype-based case–control study constructed by rs1760944, rs3136814 and rs1130409 showed a significant difference. The frequency of the G-C-T haplotype was significantly higher in the CI group than in the control group (2.5% vs. 0.0%, $p>0.001$).

Conclusions: Based on the results of the haplotype-based case–control-study, the G-C-T haplotype may be a genetic marker of CI, and the APE1/REF-1 gene may be a CI susceptibility gene.
© 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Keywords: Cerebral infarction; Apurinic/apyrimidinic endonuclease 1/redox effector factor-1; Single-nucleotide polymorphism; Haplotype; Case–control study

Introduction

Cerebral infarction (CI) is the most common type of stroke, and often causes long-term disability [1]. In Japan, CI has recently become a major cause of death in the elderly population. CI is considered to be a multifactorial disease that results from interaction between genetic and environmental factors [2].

Apurinic/apyrimidinic endonuclease 1/redox effector factor-1 (APE1/REF-1) is a protein with multifunctional roles impacting a wide variety of important cellular functions [3]. APE1/REF-1 has 2 major functions: it acts as an apurinic/apyrimidinic endonuclease during the second step of the DNA base excision repair pathway, which is responsible for the repair of cellular oxidative DNA damage [4]; and it is also known as redox effector factor-1 (REF-1), which is important for the activation of transcription factors, such as activator protein 1 (AP1), p53 and nuclear factor kappaB (NF-kB) [5,6]. The human APE1/REF-1 gene is located on chromosome 14q11.2–q12, which consists of 5 exons and 4 introns spanning 2.64 kilobase pairs [7]. Human APE1/REF-1 is encoded by a gene is composed of 318 amino acids [8]. The apurinic/apyrimidinic endonuclease activity of APE1/REF-1 resides in the N-terminus, while the redox activity resides in the C-terminus [8,9].

Oxidative stress, or excess generation of reactive oxygen species (ROS), results in cellular damage and is thought to be a factor in various diseases, such as cancer, leukemia, CI,

* Corresponding author. Division of Laboratory Medicine, Department of Pathology and Microbiology, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30-1 Itabashi-ku, Tokyo 173-8610, Japan. Fax: +81 3 5375 8076.
E-mail address: tnakayam@med.nihon-u.ac.jp (T. Nakayama).

0009-9120/$ - see front matter © 2009 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.clinbiochem.2009.07.016

myocardial infarction (MI) and hypertension [10,11]. DNA is one of the biological molecules damaged by oxidative stress, and it has been reported that oxidative DNA damage is elevated in hypertension and cardiovascular diseases [12,13].

There have been no studies examining the association between the human APE1/REF-1 gene and cerebral infarction. However, it has been reported that mice heterozygous for the APE1/REF-1 allele (APE1/REF-1$^{+/-}$) were significantly more hypertensive than wild-type mice (APE1/REF-1$^{+/+}$) [9,14]. Therefore, hypertension is closely related to CI, and the APE1/REF-1 gene is thought to be a mediator in CI. Jeon et al. [9] showed that APE1/REF-1 regulated H-ras expression through its reducing function. Moreover, they placed H-ras upstream of phosphoinositide-3 kinase (PI3-K) and Akt kinase in the calcium sensitization of endothelial NO synthase by APE1/Ref1. The reducing function of APE1-REF1 may be associated with cerebral infarction.

The aim of this study was to investigate the relationship between CI and the human APE1/REF-1 gene using single-nucleotide polymorphisms (SNPs) and a haplotype-based case–control study.

Methods

Subjects

Participants in whom CI was diagnosed were recruited at Nihon University Itabashi Hospital in Tokyo, Japan, as well as neighboring hospitals, between 1993 and 2003. We selected 177 patients with CI diagnosed by neurologic examination and the findings of computed tomography (CT), magnetic resonance imaging (MRI) or both, and those who had neurologic deficit ratings greater than grade 3 on the modified Rankin Scale. Patients with hemorrhage stroke diagnosed by CT, MRI or both were excluded from this study group.

A total of 309 Japanese subjects were enrolled as control subjects. Control subjects were members of the New Elder Citizen Movement in Japan who lived in Tokyo, or in the suburbs of Tokyo, and who had vascular risk factors such as hypertension, hypercholesterolemia or diabetes mellitus, but no history of CI. They were confirmed as being grade 0 on the modified Rankin Scale. In this study group, participants with cancer or autoimmune diseases, including antiphospholipid antibody syndrome, were excluded. Patients with cerebral embolism caused by atrial fibrillation, diagnosed by anamnesis and findings of electrocardiography, echocardiography, CT or MRI were excluded. However patients in whom cerebral thrombosis was diagnosed were included. No participants had a history of peripheral arterial occlusive disease. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University [15,16].

Biochemical analysis

Blood samples were obtained from subjects in the morning, after resting in the sitting position for at least 30 min without eating. In the clinical laboratory department of our university hospital, these blood samples were used to measure plasma concentrations of total cholesterol and serum concentrations of creatinine [17].

Genotyping

As the detailed data for SNPs in the APE1/REF-1 gene on the Hap Map website were not clear, information on allelic

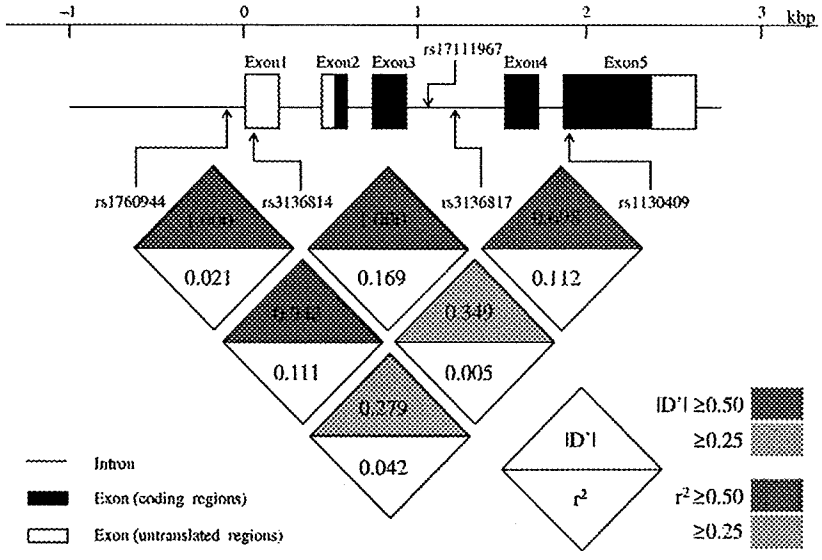

Fig. 1. Organization of the gene encoding human apurinic/apyrimidinic endonuclease-1/redox effector factor-1 (APE1/REF-1), locations of single-nucleotide polymorphisms (SNPs) used in present association study, and pair-wise LD in the APE1/REF-1 gene, as evaluated by $|D'|$ and r^2. Closed boxes indicate exons, and lines represent introns. Pair-wise LD among the 4 marker pairs studied in the human APE1/REF-1 gene were computed, upper triangles indicate $|D'|$ and lower triangles represent r^2. Pairs in LD ($|D'|$ or $r^2 \geq 0.5$) are shown as deep gray-shaded values, and pairs in LD ($|D'|$ or $r^2 \geq 0.25$) are shown as light gray-shaded values.

frequencies of SNPs registered on the National Center for Biotechnology Information (NCBI) website was used. We selected 5 SNPs in the human APE1/REF-1 gene. These SNPs have minor allele frequencies of >5%, and we selected at least 1 SNP from each of the promoter, intron and exon regions. We examined the relationship between CI and these 5 SNPs. All 5 SNPs were confirmed by information on the NCBI website. The accession numbers were rs1760944, rs3136814, rs17111967, rs3136817 and rs1130409 (Fig. 1). Genotypes were determined using Assays-on-Demand kits (Applied Biosystems, Branchburg, NJ) together with TaqMan polymerase chain reaction (PCR) (Applied Biosystems). TaqMan® SNP Genotyping Assay was performed using the method for Taq amplification.

On 5′-nuclease assay, discrimination occurs during PCR, as the allele-specific fluorogenic probes, when they are hybridized to the template, are cleaved by the 5′-nuclease activity of Taq polymerase. The probes contain a 3′-minor groove-binding group (MGB) that hybridizes to single-stranded targets with increased sequence-specificity when compared with ordinary DNA probes. This reduces nonspecific probe hybridization and results in low background fluorescence during the 5′-nuclease PCR assay (TaqMan®, ABI). Cleavage results in increased emission of a reporter dye. Each 5′-nuclease assay requires 2 unlabeled PCR primers and 2 allele-specific probes. Each probe was labeled with 2 reporter dyes at the 5′ end; in this study, VIC and FAM were used as reporter dyes. The primers and probes in the TaqMan® SNP Genotyping Assays (ABI) were chosen based on the information available on the ABI website (http://myscience.appliedbiosystems.com).

PCR amplification was performed using 2.5 μl of TaqMan® Universal Master Mix and No AmpErase® UNG (2×) (ABI) in 5-μl final reaction volumes, with 2 ng of DNA, 2.375 μl of ultrapure water, 0.079 μl of Tris-EDTA (TE) buffer (1×), 0.046 μl of TaqMan® SNP Genotyping Assay Mix (40×) containing final concentrations of 331.2 nM for primers, and 73.6 nM for probes. The thermal cycling conditions were 95 °C for 10 min, 50 cycles of 92 °C for 15 sec, and finally 60 °C for 1 min. Thermal cycling was performed using the GeneAmp 9700™ system [18].

Each 96-well plate contained 80 samples of an unknown genotype and 4 samples with reagents alone (control). The control samples without DNA are necessary in the Sequence Detection System (SDS) 7700™ for signal processing, as outlined in the TaqMan Allelic Discrimination Guide (ABI). Plates were read on the SDS 7700 instrument using the end-point analysis mode of the SDS version 1.6.3 software package (ABI). Genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X–Y scatter-plot of the SDS software. The genotypes were also determined automatically by the signal processing algorithms of the software. The results of each scoring method were saved in 2 separate output files for later comparison [19].

Statistical analysis

Data are shown as means±SD. Hardy–Weinberg equilibrium (HWE) was assessed using χ^2 analysis. The overall distribution of alleles was analyzed using 2×2 contingency tables, and the distribution of the genotypes between CI patients and control subjects was analyzed using a two-sided Fisher's exact test. Based on the genotype data of the genetic variations, linkage disequilibrium (LD) analysis and haplotype-based case–control study were performed using the expectation maximization (EM) algorithm of the SNPAlyze software program, version 3.2 (Dynacom Co., Ltd., Yokohama, Japan). Pair-wise LD analysis was performed using SNP pairs. $|D'|$ values of ≥0.5 were used to assign SNP locations to 1

Table 1
Characteristics of study participants.

	Total			Men			Women		
	Control	CI	p value	Control	CI	p value	Control	CI	p value
Number of subjects	309	177		150	106		159	71	
Age (years)	77.9±4.1	65.9±13.0	<0.001 *	78.2±4.5	64.2±11.9	<0.001 *	77.7±3.8	68.4±14.2	<0.001 *
BMI (kg/m^2)	22.6±2.9	23.2±3.5	0.067	22.8±2.8	23.1±3.0	0.397	22.4±3.0	23.4±4.4	0.103
SBP (mm Hg)	135.9±16.4	151.3±26.6	<0.001 *	136.0±15.2	148.6±26.2	<0.001 *	135.8±17.4	155.3±26.8	<0.001 *
DBP (mm Hg)	78.6±11.0	86.2±16.2	<0.001 *	78.8±10.3	86.9±16.4	<0.001 *	78.3±11.6	85.1±16.0	<0.001 *
Pulse (beats/min)	70.1±11.1	76.7±14.8	<0.001 *	68.9±11.7	75.5±14.3	<0.001 *	71.3±10.3	78.5±15.4	<0.001 *
Creatinine (mg/dL)	0.85±0.23	1.03±0.64	<0.001 *	0.96±0.22	1.13±0.57	<0.001 *	0.75±0.18	0.89±0.72	0.023 *
Total cholesterol (mg/dL)	218.0±43.0	194.8±52.1	<0.001 *	204.8±31.9	191.9±51.6	0.018 *	230.4±48.2	199.0±53.0	<0.001 *
Hypercholesterolemia (%)	19.7	26.6	0.083	10.0	22.6	0.006 *	28.9	32.4	0.597
Diabetes (%)	2.9	16.4	<0.001 *	4.0	14.2	0.004 *	1.9	19.7	<0.001 *
Drinking (%)	37.3	48.7	0.092	46.3	68.7	0.013 *	27.1	19.6	0.390
Smoking (%)	30.4	46.6	<0.001 *	46.2	65.3	0.034 *	14.0	17.4	0.648

CI, cerebral infarction; Control, non-cerebral infarction; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein.
Continuous variables were expressed as mean±standard deviation.
Categorical variables were expressed as percentages.
p values of continuous variables were calculated by Mann–Whitney U test.
p values of categorical variables were calculated by Fisher's exact test.
* $p<0.05$.

haplotype block. Tagged SNPs were selected by omitting 1 SNP from an SNP pair showing an r square of ≥ 0.25 for each haplotype block. In this haplotype-based case–control study, haplotypes with a frequency of <0.02 were excluded. The distribution of haplotype frequencies was calculated using χ^2 test. A probability level of $p<0.05$ was considered to indicate statistical significance. Differences in clinical data between the CI and control groups were assessed using analysis of variance followed by Fisher's protected least-significant-difference test [20,21].

Results

Table 1 shows the clinical features of the CI patients and control subjects. Significant differences in systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse, creatinine, total cholesterol, diabetes and smoking were observed between the CI and control groups. The age of subjects in the control group was significantly higher than in the CI group (77.9±4.1 years vs. 65.9±13.0 years, $p<0.001$), for both men (78.2±4.5 years vs. 64.2±11.9 years, $p<0.001$) and women (77.7±3.8 years vs. 68.4±14.2 years, $p<0.001$). Because the mean age of the control group was higher than that of the CI group, the control group was regarded as a super-control. Super-control groups, with age higher than case groups, are considered to be acceptable in case–control studies of late-onset diseases [20].

Table 2 shows the distribution of genotypic and allelic frequencies of the 5 SNPs in each group. rs1711967 was excluded because there was no heterogeneity; all of the participants were genotyped as C/C. The observed and expected genotypic frequencies of each SNP in the control group were in good agreement with predicted Hardy–Weinberg equilibrium values (data not shown). There were significant differences in

Table 2
Genotype and allele distributions in control subjects and patients with CI.

			Total			Men			Women		
			Control	CI	p value	Control	CI	p value	Control	CI	p value
Number of participants			309	177		150	106		159	71	
Variants											
rs1760944	Genotype	G/G	95 (0.307)	46 (0.260)	0.4244	42 (0.280)	32 (0.302)	0.8930	53 (0.333)	14 (0.197)	0.0851
		G/T	156 (0.505)	91 (0.514)		78 (0.520)	52 (0.491)		78 (0.491)	39 (0.549)	
		T/T	58 (0.188)	40 (0.226)		30 (0.200)	22 (0.208)		28 (0.176)	18 (0.254)	
		G/G and G/T	251 (0.812)	137 (0.774)	0.3144	120 (0.800)	84 (0.792)	0.8825	131 (0.824)	53 (0.746)	0.1751
		T/T	58 (0.188)	40 (0.226)		30 (0.200)	22 (0.208)		28 (0.176)	18 (0.254)	
		G/G	95 (0.307)	46 (0.260)	0.2663	42 (0.280)	32 (0.302)	0.7036	53 (0.333)	14 (0.197)	0.0358*
		G/T and T/T	214 (0.693)	131 (0.740)		108 (0.720)	74 (0.698)		106 (0.667)	57 (0.803)	
	Allele	G	346 (0.56)	183 (0.517)	0.1960	162 (0.540)	116 (0.547)	0.8736	184 (0.579)	67 (0.472)	0.0336*
		T	272 (0.44)	171 (0.483)		138 (0.460)	96 (0.453)		134 (0.421)	75 (0.528)	
rs3136814	Genotype	A/A	294 (0.951)	168 (0.949)	0.7238	142 (0.947)	101 (0.953)	0.7014	152 (0.956)	67 (0.944)	0.6860
		A/C	14 (0.045)	9 (0.051)		7 (0.047)	5 (0.047)		7 (0.044)	4 (0.056)	
		C/C	1 (0.003)	0 (0)		1 (0.007)	0 (0)		0 (0)	0 (0)	
		A/A and A/C	308 (0.997)	177 (1.000)	0.4487	149 (0.993)	106 (1.000)	0.3996	159 (1.000)	71 (1.000)	–
		C/C	1 (0.003)	0 (0)		1 (0.007)	0 (0)		0 (0)	0 (0)	
		A/A	294 (0.951)	168 (0.949)	0.9102	142 (0.947)	101 (0.953)	0.8249	152 (0.956)	67 (0.944)	0.6860
		A/C and C/C	15 (0.049)	9 (0.051)		8 (0.053)	5 (0.047)		7 (0.044)	4 (0.056)	
	Allele	A	602 (0.974)	345 (0.975)	0.9648	291 (0.970)	207 (0.976)	0.6611	311 (0.978)	138 (0.972)	0.6897
		C	16 (0.026)	9 (0.025)		9 (0.030)	5 (0.024)		7 (0.022)	4 (0.028)	
rs3136817	Genotype	C/C	232 (0.751)	134 (0.757)	0.6696	118 (0.787)	81 (0.764)	0.8494	114 (0.717)	53 (0.746)	0.7207
		C/T	70 (0.227)	41 (0.232)		30 (0.200)	24 (0.226)		40 (0.252)	17 (0.239)	
		T/T	7 (0.023)	2 (0.011)		2 (0.013)	1 (0.009)		5 (0.031)	1 (0.014)	
		C/C and C/T	302 (0.977)	175 (0.989)	0.3716	148 (0.987)	105 (0.991)	0.7752	154 (0.969)	70 (0.986)	0.4454
		T/T	7 (0.023)	2 (0.011)		2 (0.013)	1 (0.009)		5 (0.031)	1 (0.014)	
		C/C	232 (0.751)	134 (0.757)	0.8777	118 (0.787)	81 (0.764)	0.6697	114 (0.717)	53 (0.746)	0.6431
		C/T and T/T	77 (0.249)	43 (0.243)		32 (0.213)	25 (0.236)		45 (0.283)	18 (0.254)	
	Allele	C	534 (0.864)	309 (0.873)	0.6971	266 (0.887)	186 (0.877)	0.7470	268 (0.843)	123 (0.866)	0.5156
		T	84 (0.136)	45 (0.127)		34 (0.113)	26 (0.123)		50 (0.157)	19 (0.134)	
rs1130409	Genotype	G/G	56 (0.181)	21 (0.119)	0.1183	29 (0.193)	12 (0.113)	0.2267	27 (0.170)	9 (0.127)	0.3031
		G/T	139 (0.450)	78 (0.441)		60 (0.400)	47 (0.443)		79 (0.497)	31 (0.437)	
		T/T	114 (0.369)	78 (0.441)		61 (0.407)	47 (0.443)		53 (0.333)	31 (0.437)	
		G/G and G/T	195 (0.631)	99 (0.559)	0.1195	89 (0.593)	59 (0.557)	0.5578	106 (0.667)	40 (0.563)	0.1329
		T/T	114 (0.369)	78 (0.441)		61 (0.407)	47 (0.443)		53 (0.333)	31 (0.437)	
		G/G	56 (0.181)	21 (0.119)	0.0690	29 (0.193)	12 (0.113)	0.0851	27 (0.170)	9 (0.127)	0.4065
		G/T and T/T	253 (0.819)	156 (0.881)		121 (0.807)	94 (0.887)		132 (0.830)	62 (0.873)	
	Allele	G	251 (0.406)	120 (0.339)	0.0381*	118 (0.393)	71 (0.335)	0.1772	133 (0.418)	49 (0.345)	0.1382
		T	367 (0.594)	234 (0.661)		182 (0.607)	141 (0.665)		185 (0.582)	93 (0.655)	

CI, cerebral infarction; Control, non-cerebral infarction.
* $p<0.05$.

Table 3
Combinations of SNPs showing significant differences.

Combination of SNPs	Group	Number of participants		Overall distribution		Distribution of individual haplotypes				
		Control	CI	χ^2	p value	Haplotype	Control	CI	χ^2	p value
rs1760944–rs3136814–rs1130409	Total	309	177	19.98	0.001 *	G-A-T	0.278	0.255	0.59	0.441
						T-A-T	0.322	0.381	3.32	0.069
						G-C-T	0.000	0.025	15.09	<0.001 *
						G-A-G	0.268	0.237	1.14	0.285
						T-A-G	0.132	0.102	1.79	0.181
	Men	150	106	13.32	0.021 *	G-A-T	0.283	0.303	0.20	0.658
						T-A-T	0.319	0.338	0.27	0.601
						G-C-T	0.000	0.024	7.05	0.008 *
						G-A-G	0.231	0.220	0.05	0.831
						T-A-G	0.144	0.115	1.11	0.292
						G-T-G	0.024	0.000	5.08	0.024 *
	Women	159	71	17.79	0.001 *	G-A-T	0.265	0.184	3.69	0.055
						T-A-T	0.314	0.442	7.28	0.007 *
						G-C-T	0.000	0.028	8.64	0.003 *
						G-A-G	0.301	0.259	0.71	0.399
						T-A-G	0.121	0.086	1.37	0.242

CI, cerebral infarction; Control, non-cerebral infarction.
* $p<0.05$.

distribution of alleles for rs1130409 between the control and CI groups overall and in distribution of genotypes and/or alleles for rs1760944 in women.

LD patterns are shown with the $|D'|$ and r square values (Fig. 1). As most $|D'|$ values were large, all polymorphisms were located in 1 haplotype block, and because the r square of all pairs of 4 SNPs was <0.25, we constructed a haplotype-based association study using rs1760944, rs3136814, rs3136817 and rs1130409.

In the haplotype-based case–control study, 11 possible combinations of SNPs were predicted using these 4 SNPs. Table 3 shows the distribution of the individual haplotypes constructed with the rs1760944, rs3136814 and rs1130409. In this combination, significant differences between the control and CI groups were observed for 3 studies (total; $\chi^2=19.98$, $p=0.001$, men; $\chi^2=13.32$, $p=0.021$, women; $\chi^2=17.80$, $p=0.001$). The G-C-T haplotype constructed by rs17609443–rs3136814–rs1130409 in CI was significantly higher in all 3 studies (total; $\chi^2=15.09$, $p<0.001$, men; $\chi^2=7.05$, $p=0.008$, women; $\chi^2=8.64$, $p=0.003$).

Discussion

Although there have been some reports showing that the APE1/REF-1 gene is related to the pathophysiology of hypertension [9,14], there have been no association studies concerning CI and the APE1/REF-1 gene. Thus, this is the first time an association study between the human APE1/REF-1 gene and CI using a haplotype has been reported in the literature.

In the present study, we genotyped 5 SNPs of the APE1/REF-1 gene in Japanese subjects and assessed the association between this gene and cerebral infarction. In this association study, we used a super-control group, consistent with aged and unaffected subjects. Healthy elderly subjects are more suitable than young or middle-aged subjects for determining the phenotypes of cardiovascular and cerebrovascular diseases related to aging, because many such diseases occur late in life. CI is an age-influenced disease; therefore, it is better to use a super-control group than an age-matched control group. In this study, levels of total cholesterol were significantly higher in the control group. The all persons in the control groups live in healthy life. We could not collect data on total cholesterol levels in the CI group at their onset of CI. Therefore, total cholesterol levels in the control group may be higher than in the case group. Many control subjects had been diagnosed with hypercholesterolemia and were undergoing diet therapy without antihyperlipemic drugs.

Among SNPs used in the association study, rs1130409 is located in the amino acid coding region of the human APE1/REF-1 gene, is a nonsynonymous Asp148Glu (GAT→GAG) change, and has been studied previously [22–24]. Therefore, we selected rs1130409 as an important SNP in the human APE1/REF-1 gene for use in our association study. However, no previous studies have been conducted on the functions of the gene variants. Therefore, the functions of the nonsynonymous SNP and variant upstream region are unclear. The SNPs in introns may only serve as genetic markers, without directly affecting gene function.

In the present study, there were significant differences in overall distribution of genotypic and allelic frequencies of rs1130409 between the CI and control groups, and the haplotype-based case–control study based on rs1760944–rs3136814–rs1130409 showed a significant difference. Based on our results, the APE1/REF-1 gene is associated with both cancers [22–24] and vascular diseases. APE1/REF-1 is involved in the repair of DNA damage as well as in the reductive activation of transcriptional regulation of genes. Therefore, we questioned whether the endonuclease function, the reducing function, or both, play a role in cerebrovascular

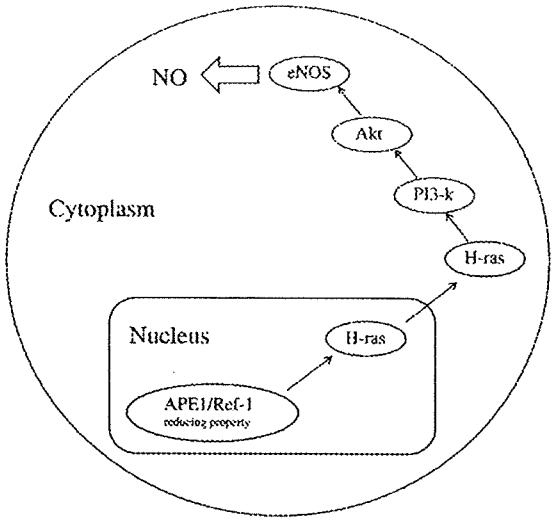

Fig. 2. Flow chart of APE1/Ref-1 on the H-ras-PI3-K/Akt signaling pathway, leading to endothelial NO production.

diseases. Jeon et al. [9] reported that expression of a redox-deficient mutant of APE1/REF-1 (cysteine to alanine mutations at codons 65 and 93) did not result in increased e-NOS-catalyzed NO production, in contrast to wild-type APE1/REF-1. They also showed that APE1/REF-1 regulated H-ras expression through its reducing function. Moreover, they placed H-ras upstream of phosphoinositide-3 kinase (PI3-K) and Akt kinase in the calcium sensitization of endothelial NO synthase by APE1/Ref1. Finally, they suggested a role for APE1/Ref1 in NO production (Fig. 2). NO is involved in maintaining the function of brain tissues, as it is associated with the regulation of brain and bloodstream metabolism. Therefore, it is thought that the reducing function of APE1-REF1 is associated with cerebral infarction. In their study, however, they did not experiment with APE1/REF-1 lacking the DNA repair function. It is therefore unknown whether the endonuclease function is involved in CI.

Fujimura et al. [25] discussed APE1/REF-1 expression after transient global CI in rats. In their report, APE1/REF-1 levels decreased in hippocampal CA1 neurons after transient CI. Kawase et al. [26] and Edwards et al. [27] also confirmed decreased APE1/REF-1 after transient CI in rats. These results suggest that decreases in APE1/REF-1 are associated with CI. In the present study, we performed an association study and a case–control study using the rs1760944–rs3136814–rs1130409 haplotype (G-C-T haplotype), and we found that the G-C-T haplotype was significantly more frequent in the CI group than in control group (total: 2.5% vs. 0.0%, $\chi^2=15.09$, $p<0.001$; men: 2.4% vs. 0.0%, $\chi^2=7.05$, $p=0.008$; women: 2.8% vs. 0.0%, $\chi^2=8.64$, $p=0.003$). These results suggest that this haplotype is a genetic marker for CI, irrespective of whether the haplotype has a functional role on arterial pressure, NO production and oxidative stress. CI may result from mutations linking this haplotype to decreased endonuclease activity, redox activity, or both in APE1/REF-1, thus affecting cerebrovascular function [28].

In genes with multiple susceptibility alleles, particularly when the LD between the polymorphisms is weak, a haplotype-based association study has advantages over analyses based on individual polymorphisms [29]. In the present study, the haplotype-based case–control study was based on rs1760944–rs3136814–rs1130409, which exhibited a significant difference in the haplotype analysis used to successfully localize susceptibility genes for multifactorial diseases [30,31]. Based on such findings, we hypothesized that haplotype analysis would be useful in assessing the association between haplotypes and CI, resulting in this attempt to use SNPs in order to establish the haplotypes of the APE1/REF-1 gene. We found that the low-frequency haplotype in the CI group exhibited a significant difference in the haplotype-based case–control study. As several studies have indicated that this phenomenon occurs frequently, we believe that our results confirm the usefulness of case–control studies in the examination of multifactorial diseases.

In conclusion, the G-C-T haplotype may be a genetic marker for CI, and the human APE1/REF-1 gene may be a susceptibility gene for CI. Further studies are needed in order to clarify causal/susceptibility mutations in the APE1/REF-1 gene and/or in neighboring genes in CI.

Acknowledgments

We would like to thank Kaoru Sugama for her technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports and Technology of Japan, Kissei Pharmaceutical Co., Ltd., the TORAY Conference, Japan, and Nihon University School of Medicine.

References

[1] Kubo M, Hata J, Ninomiya T, et al. A nonsynonymous SNP in PRKCH (protein kinase C eta) increases the risk of cerebral infarction. Nat Genet 2007;39:212–7.
[2] Dominiczak AF, Negrin DC, Clark JS, et al. Genes and hypertension from gene mapping in experimental models to vascular gene transfer strategies. Hypertension 2000;35:164–72.
[3] Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. Mutat Res 2000;461:83–108.
[4] Qu J, Liu GH, Huang B, et al. Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310. Nucleic Acids Res 2007;35:2522–32.
[5] Fritz G. Human APE/Ref-1 protein. Int J Biochem Cell Biol 2000;32(9): 925–9.
[6] Yang S, Irani K, Heffron SE, et al. Alterations in the expression of the apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/Ref-1) in human melanoma and identification of the therapeutic potential of resveratrol as an APE/Ref-1 inhibitor. Mol Cancer Ther 2005;4:1923–35.
[7] Robson CN, Hochhauser D, Craig R, et al. Structure of the human DNA repair gene HAP1 and its localisation to chromosome 14q 11.2–12. Nucleic Acids Res 1992;20:4417–21.
[8] Xanthoudakis S, Miao GG, Curran T. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. Proc Natl Acad Sci U S A 1994;91:23–7.
[9] Jeon BH, Gupta G, Park YC, et al. Apurinic/apyrimidinic endonuclease 1 regulates endothelial NO production and vascular tone. Circ Res 2004;95: 902–10.
[10] Umemoto S, Tanaka M, Kawahara S, et al. Calcium antagonist reduces oxidative stress by upregulating Cu/Zn superoxide dismutase in stroke-prone spontaneously hypertensive rats. Hypertens Res 2004;27:877–85.

[11] Tanaka M, Umemoto S, Kawahara S, et al. Angiotensin II type 1 receptor antagonist and angiotensin-converting enzyme inhibitor altered the activation of Cu/Zn-containing superoxide dismutase in the heart of stroke-prone spontaneously hypertensive rats. Hypertens Res 2005;28: 67–77.
[12] Volpe M, Alderman MH, Furberg CD, et al. Beyond hypertension toward guidelines for cardiovascular risk reduction. Am J Hyperens 2004;17: 1068–74.
[13] Negishi H, Ikeda K, Kuga S, et al. The relation of oxidative DNA damage to hypertension and other cardiovascular risk factors in Tanzania. J Hypertens 2001;19:529–33.
[14] Patterson C. Blood pressure control goes nuclear. Circ Res 2004;95: 849–51.
[15] Nakayama T, Soma M, Rehemudula D, et al. Association of 5′ upstream promoter region of prostacyclin synthase gene variant with cerebral infarction. Am J Hypertens 2000;13:1263–7.
[16] Baum L, Ng HK, Woo KS, et al. Paraoxonase 1 gene Q192R polymorphism affects stroke and myocardial infarction risk. Clin Biochem 2006;39:191–5.
[17] Sano M, Kuroi N, Nakayama T, et al. Association study of calcitonin-receptor-like receptor gene in essential hypertension. Am J Hypertens 2005;18:403–8.
[18] Saito K, Miyake S, Moriya H, et al. Detection of the four sequence variations of MDR1 gene using TaqMan MGB probe based real-time PCR and haplotype analysis in healthy Japanese subjects. Clin Biochem 2003;36:511–8.
[19] Morita A, Nakayama T, Soma M, et al. Association between the calcitonin-related peptide alpha (CALCA) gene and essential hypertension in Japanese subjects. Am J Hypertens 2007;20:527–32.
[20] Morita A, Nakayama T, Soma M. Association study between C-reactive protein genes and ischemic stroke in Japanese subjects. Am J Hypertens 2006;19:593–600.
[21] Yu Z, Chen J, Shi H, et al. Analysis of GABRB2 association with schizophrenia in German population with DNA sequencing and one-label extension method for SNP genotyping. Clin Biochem 2006;39:210–8.
[22] Chang-Claude J, Popanda O, Tan XL, et al. Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and acute side effects of radiotherapy in breast cancer patients. Clin Cancer Res 2005;11:4802–9.
[23] Su D, Ma S, Liu P, et al. Genetic polymorphisms and treatment response in advanced non-small cell lung cancer. Lung Cancer 2007;56:281–8.
[24] Pardini B, Naccarati A, Novotny J, et al. DNA repair genetic polymorphisms and risk of colorectal cancer in the Czech Republic. Mutat Res 2008;638:146–53.
[25] Fujimura M, Morita-Fujimura Y, Kawase M, et al. Early decrease of apurinic/apyrimidinic endonuclease expression after transient focal cerebral ischemia in mice. J Cereb Blood Flow Metab 1999;19:495–501.
[26] Kawase M, Fujimura M, Morita-Fujimura Y, et al. Reduction of apurinic/apyrimidinic endonuclease expression after transient global cerebral ischemia in rats: implication of the failure of DNA repair in neuronal apoptosis. Stroke 1999;30:441–8.
[27] Edwards M, Kent TA, Rea HC, et al. APE/Ref-1 responses to ischemia in rat brain. Neuroreport 1998;9:4015–8.
[28] Hopmeier P, Puehringer H, van Trotsenburg M, et al. Association of endothelial protein C receptor haplotypes, factor V Leiden and recurrent first trimester pregnancy loss. Clin Biochem 2008;41:1022–4.
[29] Morris RW, Kaplan NL. On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. Genet Epidemiol 2002;23:221–33.
[30] Sharma P, Hingorani A, Jia H, et al. Quantitative association between a newly identified molecular variant in the endothelin-2 gene and human essential hypertension. J Hypertens 1999;17:1281–7.
[31] Ombra MN, Forabosco P, Casula S, et al. Identification of a new candidate locus for uric acid nephrolithiasis. Am J Hum Genet 2001;68:1119–29.

© Med Sci Monit, 2009; 15(8): CR389-397
PMID: 19644414

www.MEDSCIMONIT.COM
Clinical Research

CR

Received: 2008.06.06
Accepted: 2009.02.03
Published: 2009.08.01

Relationship between haplotypes of KCNN4 gene and susceptibility to human vascular diseases in Japanese

Mai Yamaguchi[1,2ACDEFG], Tomohiro Nakayama[3ABCDEFG], Zhenyan Fu[2CD], Takahiro Naganuma[2CD], Naoyuki Sato[3CD], Masayoshi Soma[4EG], Nobutaka Doba[4B], Shigeaki Hinohara[5B], Akihiko Morita[3CDEF], Tomohiko Mizutani[1AG]

[1] Division of Neurology, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan
[2] Division of Molecular Diagnostics, Department Advanced Medical Science, Nihon University School of Medicine, Tokyo, Japan
[3] Division of Laboratory Medicine, Department of Pathology and Microbiology, Nihon University School of Medicine, Tokyo, Japan
[4] Division of General Medicine, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan
[5] Life Planning Center, Tokyo, Japan

Source of support: This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Nihon University School of Medicine, Kissei Pharmaceutical Co., Ltd., and Toray-Astellas Pharma Inc., Japan

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Background: Human vascular diseases such as myocardial infarction (MI) and cerebral infarction (CI) are thought to be affected by several environmental factors and genetic variants. It has been suggested that the expression of the KCNN4 calcium-activated potassium channel is associated with the developing vascular smooth-muscle cells of human neointimal hyperplasia. The aim of this study was to investigate the relationship between single-nucleotide polymorphisms (SNPs) in the human KCNN4 gene or haplotypes and the incidence of MI or CI in Japanese.

Material/Methods: Three hundred thirteen MI and 176 CI patients with 290 controls were enrolled in two independent case-control studies that examined the use of a haplotype-based case-control study involving five SNPs of the human KCNN4 gene (rs670950, rs2306801, rs2306799, rs347519, and rs3786954).

Results: There were significant differences between the MI and control groups in the overall distribution of genotypes and dominant or recessive models of rs670950, rs2306799, and rs3786954. Multiple logistic regression analyses revealed that even after adjusting for confounding factors (odds ratio: 1.96), the frequency of the G/G genotype of rs2306799 in the MI group was significantly higher than in the control group (p=0.005). Furthermore, the G-T-A haplotype of rs2306799-rs347519-rs3786954 was significantly more frequent in the MI (88.8%) than in the control group (83.6%).

Conclusions: The specific SNPs and haplotypes in the KCNN4 gene showed significant differences between MI and control patients. These results indicate that these polymorphisms and haplotypes could be genetic markers for MI.

key words: KCNN4 • myocardial infarction • cerebral infarction • case-control study • single-nucleotide polymorphism • haplotype

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=XXXXXX12882

Word count: 2691
Tables: 5
Figures: 2
References: 36

Author's address: Tomohiro Nakayama, Division of Molecular Diagnostics, Department Advanced Medical Science, Nihon University School of Medicine, Ooyaguchi-kamimachi, 30-1 Itabashi-ku, Tokyo 173-8610, Japan, e-mail: tnakayam@med.nihon-u.ac.jp

Electronic PDF security powered by IS-sciences.com

PERSONAL USE ONLY

This copy is for personal use only - distribution prohibited.

Background

Different types of calcium-activated potassium channels (KCa channels) are known to exist and they have been reported to have various functions. The three KCa channel groups are classified as large, intermediate, and small conductance channels, often referred to as the BK, IK, and SK channels, respectively. Four genes of the KCNN gene family are responsible for encoding the SK and IK channels, with the three SK channels encoded by KCNN1-3 and the IK channel by KCNN4. The KCNN4 channels were classified as IK by Ishii et al. [1] and are reported to have a unit conductance of 20-85 pS, which is an intermediate conductance that falls between the values reported for the BK and SK channels. There is a 40% homology for the KCNN4 channel polypeptides compared with the other SK channels [2].

It has been reported that the KCNN4 channels exist in various types of cells. KCNN4 channels have been reported in lymphocytes, erythrocytes, fibroblasts, proliferating vascular smooth-muscle cells (VSMCs), vascular endothelium, and intestinal and airway epithelia [3]. Therefore the KCNN4 channels are regarded as potential targets for various diseases that involve these tissues.

Recent studies have emphasized the involvement of inflammation in mediating all stages of atherosclerosis and that proliferation of VSMCs is the key process that must take place for atherosclerosis to occur [4–8]. Specific molecular requirements for the phenotypic modulation of VSMCs have been defined and it has been reported that there is an associated change in ion handling which involves specific effects that occur in relation to some of the ion channels [9–11]. Köhler et al. used a rat model to determine that the expression of KCNN4 might be responsible for promoting the neointimal proliferation of VSMCs and that the blockade of KCNN4 can prevent atherosclerosis and restenosis after angioplasty [12]. Other reports have also shown that the upregulated KCNN4 expression contributes to the proliferation of mitogen-stimulated fibroblasts and human T lymphocytes [13,14]. Cheong et al. reported that the gene encoding KCNN4 contains a functional repressor element 1-silencing transcription factor (REST) binding site and is repressed by REST. REST is expressed in the nuclei of human VSMCs and it has been shown to downregulate KCNN4 expression and neointimal VSMC proliferation [15].

Although it has been reported that the induction of KCNN4 that leads to the proliferation of VSMCs might be a key process in the etiology of atherosclerosis, there have been no reports that have examined the relationship between the KCNN4 gene and human vascular diseases. Myocardial infarction (MI) and cerebral infarction (CI) are thought to be multifactorial diseases that are affected by several environmental factors and genetic variants. Because these environmental and genetic factors lead to the development of multifactorial diseases, we hypothesized that specific genetic variances may contribute to the pathogenesis and risk of MI and CI.

The human KCNN4 gene is a single-copy gene that spans about 14 kilobase pairs (kb) and is composed of 9 exons that are interrupted by 8 introns (Figure 1). The gene is located on 19q13.2 [16]. The aim of the present study was to investigate the relationship between the single-nucleotide polymorphisms (SNPs) in the human KCNN4 gene or haplotypes and the occurrence of MI or CI in a Japanese population.

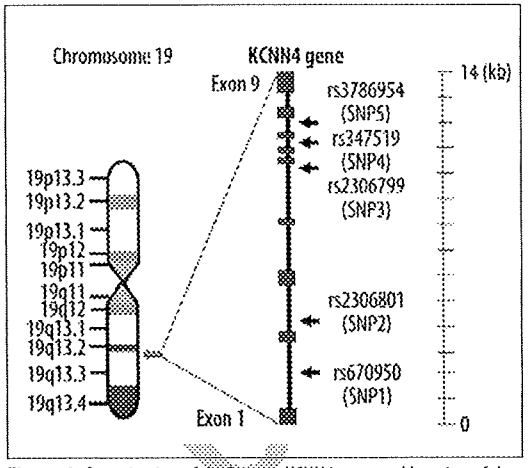

Figure 1. Organization of the human KCNN4 gene and location of the SNPs. Boxes indicate exons and lines indicate introns. The arrows show the locations of the five SNPs.

Material and Methods

Subjects

For this case-control study, all patients and control subjects were recruited from the northern area of Tokyo. The subjects were selected from patients who had been admitted to our hospital (Nihon University Hospital in Tokyo) or community hospitals (in Tokyo) between 1995 and April 2005 [17]. Control subjects were selected from outpatients who were present at the hospital during the same period. All subjects in the study groups were Japanese, with 313 patients diagnosed with MI (mean age: 61.8±10.1 years) and 176 patients diagnosed with CI (mean age: 65.8±13.0 years). MI diagnoses were made either by determination of cardiac enzyme increases or diagnostic changes on electrocardiograms. For the CI diagnosis, a board-certified neurologist evaluated new focal neurological deficits and symptoms using computed tomography (CT) or magnetic resonance imaging (MRI). Patients with potential sources of cardioembolism (e.g. recent MI, valvular heart disease, or arrhythmia including atrial fibrillation) were excluded from the study. Thus the CI group consisted of patients with non-cardioembolic CI, which included atherothrombotic and lacunar infarctions [18].

The control group consisted of 290 Japanese (mean age: 77.9±4.2 years). Because the mean age of the control group was higher than that of the MI and CI groups, the control group was regarded as a super-control group [19]. Because many cardiovascular and cerebrovascular diseases occur late in life, healthy elderly subjects are more suitable than young or middle-aged subjects for the determination of phenotypes of diseases related to aging. Since MI and stroke are age-influenced diseases, it was better to use a super-control group versus an age-matched control group. Informed con-

Electronic PDF security powered by IS-science.com

Table 1. Characteristics of the Japanese study participants.

	Control	MI	p value	CI	p value
Number of subjects (M/F)	290 (139/104)	313 (255/58)		176 (104/72)	
Age (years)	77.9±4.2	61.9±10.1		65.8±13.0	
BMI (kg/m²)	22.7±2.8	23.9±3.2	<0.001	23.3±3.5	0.091
SBP (mmHg)	135.6±16.6	135.3±22.6	0.416	151.9±26.0	<0.001
DBP (mmHg)	78.1±10.4	81.3±15.0	0.024	86.4±16.1	<0.001
Pulse (beats/min)	70.1±11.0	76.1±13.5	<0.001	76.2±13.9	<0.001
Creatinine (mg/dl)	0.85±0.23	0.95±0.38	0.033	0.97±0.37	0.003
Total cholesterol (mg/dl)	217.8±43.0	199.8±46.8	0.001	196.5±49.8	<0.001
HT (%)	10.7	17.3	0.020	42.0	<0.001
DM (%)	2.1	28.1	<0.001	15.9	<0.001
HL (%)	57.6	39.9	<0.001	27.3	<0.001

BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; HT – hypertension; DM – diabetes mellitus; HL – Hyperlipidemia; MI – myocardial infarction; CI – cerebral infarction. Values are the means ±SD. The p values were analyzed between the MI or CI groups and the control group.

sent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University.

Biochemical analysis

Plasma concentrations of total cholesterol and creatinine were measured using the methods of the Clinical Laboratory Department of Nihon University Hospital [20].

Genotyping

Based on the allelic frequency data for registered SNPs from the National Center for Biotechnology Information (NCBI) website and from the Applied Biosystems (Foster City, CA, USA) and Celera (Rockville, MD, USA) Discovery System, SNPs with minor allele frequencies >20% were chosen for study. This criterion was selected because SNPs with a high frequency of minor alleles are very useful genetic markers in genetic association studies. The accession numbers for the five selected SNPs in the human KCNN4 gene were as follows: rs670950 (C___8713531_10), rs2306801 (C___16194860_20), rs2306799 (C___16194819_10), rs347519 (C___8713313_10), and rs3786954 (C___11707105_10) (Figure 1). For the purposes of this study, the SNPs were designated as SNP1, SNP2, SNP3, SNP4, and SNP5, respectively. All SNPs were located in the intron region. Genotypes were determined using Assays-on-Demand kits (Applied Biosystems) together with TaqMan PCR, as has been previously described [21].

Linkage disequilibrium analysis and the haplotype-based case-control study

Based on the genotype data of the genetic variations, we performed a linkage disequilibrium (LD) analysis and a haplotype-based case control study by applying SNPAlyze version 3.2 (Dynacom Co., Ltd., Yokohama, Japan) that employed the expectation-maximization (EM) algorithm [22]. D' values >0.5 were used to assign SNP locations to one haplotype block.

Statistical analysis

Data are shown as the mean ±*SD*. Differences for the continuous variables in the clinical data between the MI or CI groups and control were assessed by the Mann-Whitney *U* test. Categorical variables were assessed with Fisher's exact test. The overall distributions of the genotypes and dominant or recessive models were examined with Fisher's exact test. Hardy-Weinberg equilibrium was assessed by chi-squared analysis. In addition, multiple logistic regression analyses were performed to assess the contribution of confounders. Statistical analyses were performed with SPSS™ software for Windows version 12 (SPSS Inc., Chicago, IL, USA). Statistical significance was established at $p<0.05$.

Pair-wise linkage disequilibrium (LD) patterns for the KCNN4 gene were evaluated using |D'| and r^2. The threshold value of the frequencies of the haplotypes included in the analysis was set to 1%. All haplotypes below the threshold value were excluded from the analysis. The overall distribution of the haplotypes was analyzed using 2 × m contingency tables, with a p value of <0.05 considered to indicate statistical significance. The p value of each haplotype was determined using chi-squared analysis, a permutation method, and SNPAlyze version 3.2 (Dynacom Co., Ltd.) [21,23].

Results

Table 1 shows the characteristics of the study participants. The control and MI or CI groups were used for association studies. The distributions of the genotypes and two kinds of dominant or recessive models of the five SNPs in the 313 MI and 176 CI patients and in the 290 control subjects are summarized in Table 2. Significant genotype distribution

Electronic PDF security powered by IndexCopernicus.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.