



Autopsy and postmortem examination case study on genetic risk factors for cardiac death: polymorphisms of endothelial nitric oxide synthase gene Glu298Asp variant and T-786C mutation, human paraoxonase 1 (PON1) gene and $\alpha 2\beta$ -adrenergic receptor gene

Autopsijsko i postmortem određivanje genetskih faktora rizika za smrt od srčanih oboljenja – polimorfizmi gena za: endotelnu azot-oksidi sintazu (Glu298Asp varijanta i T-786C mutacija), humanu paraoksanazu 1 (PON1) i $\alpha 2\beta$ -adrenergički receptor

Kiyoshi Ameno*, Setsuko Ameno*, Hiroshi Kinoshita[†], Mostofa Jamal*,
Weihsuan Wang *, Mitsuru Kumihashi*, Ikuo Uekita*, Iwao Ijiri*

Faculty of Medicine, Kagawa University, *Department of Forensic Medicine, Kagawa,
Japan; Hyogo College of Medicine, [†]Department of Legal Medicine, Hyogo, Japan

Abstract

Background/Aim. The Glu298Asp variant in exon 7 and T-786C mutation in the 5'-flanking region of the endothelial nitric oxide synthase (eNOS) gene, paraoxonase I gene (PON1), and $\alpha 2\beta$ -adrenergic receptor gene ($\alpha 2\beta$ -AR) have been reported to be genetic risk factors for coronary heart disease (CHD). The aim of this study was to investigate the effects of these four genetic polymorphisms on the probability of death due to CHD, using data obtained from medico-legal autopsies. **Methods.** Blood samples from three groups: healthy controls, dead cases with CHD and without CHD (the latter as a control for dead cases) were used. After DNA extraction, genotyping was performed by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) test. **Results.** The frequency of the T allele in Glu298Asp variant in the dead cases with CHD was significantly higher than that in the healthy control ($p < 0.001$, OR = 4.47) and that in the dead cases without CHD ($p < 0.001$, OR = 7.62). The gene frequency of PON1 was significantly different ($p = 0.007$) between dead cases with and without CHD, and was also significantly different ($p = 0.025$) between the healthy control and dead cases without CHD. The gene frequency of PON1 was not significantly different ($p = 0.401$) between the healthy controls and dead cases with CHD. Hence this gene was not associated with death due to CHD. The other polymorphisms (T-786C mutation, $\alpha 2\beta$ -AR) also showed no effect on death due to CHD. **Conclusion.** The polymorphism of Glu298Asp eNOS gene in dead cases may be useful for determining the cause of death in CHD cases in the Japanese population.

Key words:
forensic medicine; death, sudden, cardiac; coronary disease; risk factors; polymorphism, genetic.

Apstrakt

Uvod/Cilj. Kao genetski faktori rizika za koronarna srčana oboljenja (KSO) navedeni su varijanta Glu298Asp u egzonu 7 i T-786C mutacija u 5'-flankirajućoj zoni endotelnog azot-oksidi sintaza (eNOS) gena, paraoksanaza 1 gena (PON1), i $\alpha 2\beta$ -adrenergički receptor gena ($\alpha 2\beta$ -AR). Cilj ovog istraživanja bio je da se ispituju uticaji ova četiri genetska polimorfizma na mogućnost smrti od KSO uz korišćenje podataka dobijenih na kliničkim autopsijama. **Metode.** Uzeti su uzorci krvi od tri grupe: zdravi kontrolni, smrtni slučajevi sa i bez KSO (ovi drugi kao kontrolni za umrle slučajeve). Posle ekstrakcije DNK izvršeno je određivanje genotipa pomoću *polymerase chain reaction – restriction fragment length polymorphism* (PCR-RFLP) testa. **Rezultati.** Učestalost T alela u Glu298Asp varijanti kod umrlih sa KSO bila je značajno veća nego kod zdravih kontrolnih ($p < 0.001$, OR = 4,47) i umrlih slučajeva bez KSO ($p < 0.001$, OR = 7,62). Genska učestalost PON1 razlikovala se značajno ($p = 0,007$) između smrtnih slučajeva sa i bez KSO, a razlikovala se takođe značajno ($p = 0,025$) između zdravih osoba i umrlih bez KSO. Genska učestalost PON1 nije se značajno razlikovala ($p = 0,401$) između zdravih osoba i umrlih sa KSO. Ovaj gen nije se, zbog toga, dovođio u vezu sa smrću od KSO. Drugi polimorfizmi (T-786C mutacija, $\alpha 2\beta$ -AR) takođe nisu uticali na smrt od KSO. **Zaključak.** Polimorfizam Glu298Asp eNOS gena kod umrlih može da se iskoristiti za određivanje uzroka smrti kod osoba sa KSO u japanskoj populaciji.

Ključne reči:
medicina, sudska; smrt, iznenadna srčana; koronarna bolest; faktori rizika; polimorfizam, genetički.

Introduction

Many of the causes of sudden unexpected natural death are attributable to circulatory organ diseases. The most frequently detected of these is ischemic heart disease, whose main cause are coronary organic stenosis, coronary artery spasm and stenosis of coronary artery by thrombosis. In fatal causes of coronary spasm, there is no evidence of pathological abnormalities of the heart at medico-legal autopsy, and the mechanism underlying this type of coronary spasm is unclear. The prevalence of coronary spasm is higher in the Japanese population than in Caucasians, suggesting that genetic factors may be related to heart disease^{1,2}. It has been reported that the Glu298Asp variant and T-786C mutation in the endothelial nitric oxide synthase (eNOS) gene³⁻¹¹, the PON1 gene¹²⁻¹⁶ and the $\alpha 2\beta$ -adrenergic receptor gene ($\alpha 2\beta$ -AR)¹⁷⁻¹⁹ are associated with cardiac heart disease (CHD). Therefore, we examined the relationship between the polymorphisms of these genes and fatal CHD in medico-legal autopsy cases and postmortem examination cases.

Nitric oxide (NO), produced by the NO synthase enzyme, plays an important role in the regulation of blood pressure and regional blood flow. Two polymorphisms of the eNOS gene were found to be associated with CHD. One was the Glu298Asp variant in exon 7 of the eNOS gene, which has been reported to be a risk factor for acute myocardial infarction^{3,4}, coronary spasm⁵, coronary artery disease⁶, and essential hypertension⁶⁻⁹ in Japanese. The other was a T to C conversion at nucleotide position -786 in the 5'-flanking region of the eNOS gene (T-786C); this conversion was associated with coronary spasm^{5,7}.

Furthermore, oxidative stress may contribute to the pathogenesis of coronary spasm¹². Lower serum PON1 activity is associated with oxidative stress²⁰. PON1 is expressed as alleles Q and R. The Q allele codes for glutamine and the R allele for arginine at position 192 of the paraoxonase enzyme¹³. Compared to the PON1-192Q allele, the PON1-129R allele in PON1 is less able to protect high density lipoprotein by inhibiting the accumulation of lipid peroxidase^{21,22}. It has been reported that the Gln192Arg (Q192R) polymorphism of PON1 was associated with the risk for coronary artery spasm¹², coronary heart disease, and atherosclerotic disease^{14,15}.

An insertion/deletion polymorphism in $\alpha 2\beta$ -AR was also a novel genetic risk factor for acute coronary events such as acute myocardial infarction and sudden cardiac death^{17,18}. Vasoconstriction has been suggested to trigger acute coronary events, and the $\alpha 2\beta$ -adrenergic receptor mediates coronary vasoconstriction in humans²³. It was also demonstrated that the activation of $\alpha 2\beta$ -adrenergic receptor gene reduces coronary blood flow in both normal and atherosclerotic arteries¹⁹.

The aim of the present study was to clarify whether any of four polymorphisms - eNOS Glu298Asp, eNOS T-786C, PON1, and $\alpha 2\beta$ -AR - were risk factors for CHD cases in medico-legal autopsies.

Methods

Samples

Blood samples were collected from healthy adults and from dead cases with or without CHD by medico-legal

autopsies and postmortem examination. Healthy control blood samples were obtained from Kagawa University students (ranging in age from 21–27 years) who were genetically unrelated to each other. The numbers of blood samples tested were as follows: eNOS, Glu298Asp: n = 249, eNOS, T-786C: n = 215, PON1: n = 84, $\alpha 2\beta$ -AR: n = 90.

Blood samples were also collected from medico-legal autopsy and postmortem examination cases with or without CHD. CHD was classified as follows: coronary atherosclerotic heart disease plus chronic ischemic heart disease (17 cases), myocardial infarction (7 cases), hypertensive heart disease (11 cases), and acute cardiac death (3 cases). Of the dead cases without CHD, the causes of death were accidents, suicides, murders, or diseases other than heart disease.

Extraction of genomic DNA

DNA was isolated from blood samples using a PURE-GENETM DNA purification kit (Gentra Systems, USA) according to the manufacturer's protocol.

Genotyping

The genotypes of Glu298Asp in eNOS, PON1, and $\alpha 2\beta$ -AR were determined according to the previously published protocols^{6,13,17}. We investigated the genotype of the T-786C mutation in eNOS by PCR-RFLP method. The primer set and PCR conditions were as follows: sense primer, 5'-ATGCTCCCACCAGGGCATCA-3; antisense primer, 5'-GTCCTTGAGTCTGACATTAGGG-3'¹⁰; the PCR cycling conditions were 30 cycles at 94 °C for 1min, 59 °C for 1min and 72 °C for 1min. The 236 bp PCR product was digested by the Nae I, restriction enzyme (TAKARA SHUZO Co., LTD, Japan). The restriction digest products were 236 bp in the T allele, and 202 and 34 bp in the C allele.

The restriction digest products of the eNOS and PON1 genes were separated by electrophoresis on 4% GTG agarose gel (NuSieve GTG: SeaKem GTG = 3:1, BMA, USA). That of the $\alpha 2\beta$ -AR gene was separated on 3.5% MethaPhor agarose gel (BMA, USA) and visualized by using ethidium bromide.

Statistical analyses

Significant differences in the allelic frequencies among the three study subject groups (healthy controls, autopsy cases with CHD, and autopsy cases without CHD) were analyzed using Fisher's exact test. A *p* value of less than 0.05 was taken to be significant. Odds ratios (approximating relative risk) were calculated as an index of the association of each gene genotype (normal homozygote, heterozygote, abnormal homozygote) with various heart diseases. The effect of the mutant allele was assumed to be additive (T allele vs. G allele for Glu298Asp, C allele vs. T allele for T-786C mutation, R allele vs. Q allele for PON1, and D allele vs. I allele for $\alpha 2\beta$ -AR).

Results

Polymerase chain reaction – Restriction fragment length polymorphism (PCR-RFLP) analyses of the Glu298Asp variant of exon7 in the eNOS gene, PON1, and

$\alpha 2\beta$ -AR were reported previously. The genotyping of the T-786C mutation of 5'-flanking region in the eNOS gene has been performed by hybridizing using the allele specific oligonucleotide method with 32P-radiolabeled oligonucleotide T-786 probe and C-786 probe¹⁰. We performed a PCR-RFLP analysis of the T-786C mutation without RI. The PCR products of the heterozygote (T/C) and homozygote (C/C) determined by our PCR-RFLP method were sequenced. It was confirmed that our genotyping method was appropriate.

Table 1 shows the genotype distributions, allele frequencies, and odds ratios of the eNOS gene missense

ence between the healthy control group and dead cases without CHD ($p = 0.025$, OR = 1.88), and no differences were found in the R allele frequency between the healthy control and dead cases with CHD. This suggests that there is no association between the mutation of PON1 and cardiac death. Suehiro et al.¹⁶ also reported no association between the PON1 gene and dead cases with CHD.

There were no significant differences among the three groups in the allele frequencies of T-786C and $\alpha 2\beta$ -AR genes.

Table 2 shows the genotype distributions and allele frequencies for the polymorphisms of eNOS Glu298Asp, eNOS

Table 1

Genotype distributions and allele frequencies for polymorphisms of eNOS Glu298Asp, eNOS T-786C, PON1 and $\alpha 2\beta$ -AR in study subject

		Control (I) (n = 249)	Dead cases with CHD (II) (n = 36)	Dead cases without CHD (III) (n = 23)	Significance	OR (95% CI)
eNOS, Glu298Asp					(I) vs (II) $p < 0.001$	4.47 (4.0–7.6)
Genotype	GG	200 (80.3%)	16 (44.4%)	21 (91.3%)	(II) vs (III) $p < 0.001$	7.62 (2.5–23.7)
	GT	45 (18.1%)	15 (41.7%)	1 (4.35%)	(I) vs (III) $p = 0.610$	infinite
	TT	4 (1.6%)	5 (13.9%)	1 (4.35%)		
Allele frequency	G	0.894	0.653	0.935		
	T	0.106	0.341	0.065		
eNOS, T-786C						
Genotype	TT	181 (87.9%)	33 (89.2%)	17 (77.3%)	(I) vs (II) $p = 1$	infinite
	TC	24 (11.6%)	4 (10.8%)	5 (22.7%)	(II) vs (III) $p = 0.291$	infinite
	CC	4 (1.6%)	0 (0.0%)	0 (0.0%)	(I) vs (III) $p = 0.206$	infinite
Allele frequency	T	0.907	0.946	0.886		
	C	0.093	0.054	0.114		
PON1, Glu192Arg						
Genotype	QQ	22 (26.2%)	7 (18.9%)	19 (43.2%)	(I) vs (II) $p = 0.401$	infinite
	QR	35 (41.7%)	16 (43.3%)	17 (38.6%)	(II) vs (III) $p = 0.007$	2.44 (1.30–4.6)
	RR	27 (32.1%)	14 (37.8%)	8 (18.2%)	(I) vs (III) $p = 0.025$	1.88 (1.11–3.18)
Allele frequency	Q	0.470	0.405	0.625		
	R	0.530	0.595	0.375		
$\alpha 2\beta$ -AR						
Genotype	II	36 (41.1%)	14 (38.9%)	19 (43.2%)	(I) vs (II) $p = 0.314$	infinite
	ID	41 (45.6%)	13 (36.1%)	20 (45.5%)	(II) vs (III) $p = 0.257$	infinite
	DD	12 (13.3%)	9 (25.0%)	5 (11.3%)	(I) vs (III) $p = 0.786$	infinite
Allele frequency	I	0.639	0.569	0.659		
	D	0.361	0.431	0.341		

Glu298Asp and T-786C mutation, PON1 (Q192R), and $\alpha 2\beta$ -AR. In the Glu298Asp gene, in which the G to T conversion at nucleotide position 894 in exon 7 of the eNOS gene results in the replacement of glutamic acid by aspartic acid, the T allele frequency in dead cases with CHD was significantly higher than that in the healthy controls and that in the dead cases without heart disease ($p < 0.001$, OR = 4.47). The frequency of this allele differed significantly between the control group and dead cases with CHD group ($p < 0.001$, OR = 7.62) and between without and with CHD groups ($p < 0.001$). There was no significant difference between the healthy control and dead cases without CHD. These results suggested that the Glu298Asp gene is associated with CHD.

Significant differences were found in PON1 between dead cases with and dead cases without CHD ($p = 0.007$, OR = 2.44) as shown in Table 1. The frequency of the R allele in dead cases with CHD was higher than that in the dead cases without CHD. However, there was a significant differ-

T-786C, PON1, and $\alpha 2\beta$ -AR in the characterization of cardiac death and controls. CHD was classified into 4 groups: 1) coronary atherosclerotic heart disease + chronic ischemic heart disease (these heart diseases had similar characteristics of cardiac death and were classified as one group), 2) myocardial infarction, 3) hypertensive heart disease, and 4) acute cardiac death. As the number of subjects in each group was very small, statistical analysis could not be performed. However, based on the small quantity of data that we do have from these four groups, we dare to discuss the relationships between these four genes and allelic distributions.

Discussion

The frequency of the T allele of the Glu298Asp gene in the myocardial infarction group was higher than in the other groups, except for the acute cardiac death group, which had only 2 subjects. The distribution in T/T of myocardial in-

Table 2

Genotype distributions and allele frequencies for polymorphisms of eNOS Glu298Asp, eNOS T-786C, PON1 and α 2B-AR in the characterization of CHD and controls

		Gene types											
		eNOS, Glu298Asp		eNOS, T-786 C			PON1 (Glu192Arg)				α2β-AR		
		GG	GT	TT	TT	TC	CC	QQ	QR	RR	II	ID	DD
Coronary atherosclerotic heart disease + Chronic ischemic heart disease		8	7	2	15	2	0	6	5	6	5	6	5
	Allele frequencies	G=0.676		T=0.324	T=0.941		C=0.058	Q=0.50		R=0.50	I=0.050		D=0.50
Myocardial infarction		3	2	2	5	1	0	0	4	3	2	2	3
	Allele frequencies	G=0.571		T=0.428	T=0.917		C=0.083	Q=0.286		R=0.71	I=0.429		D=0.571
Hypertensive heart disease		5	4	1	10	1	0	1	6	4	5	5	1
	Allele frequencies	G=0.70		T=0.30	T=0.954		C=0.054	Q=0.364		R=0.63	I=0.682		D=0.318
Acute cardiac disease		0	2	0	3	0	0	0	1	1	2	0	0
	Allele frequencies	G=0.50		T=0.50	T=1.0		C=0	Q=0.25		R=0.75	I=1.0		D=0
Controls withoutCHD	Allele freq.	G=0.935		T=0.065	T=0.886		C=0.114	Q=0.625		R=0.375	I=0.659		D=0.341
Healthy	Allele freq.	G=0.895		T=0.106	T=0.907		C=0.093	Q=0.470		R=0.530	I=0.639		D=0.361

fraction in our data was 28.6%, and those of the healthy controls and the dead cases without heart disease were 1.6% and 4.3%, respectively. Hibi et al.⁶ reported also that the rate of T/T (Asp/Asp) of Glu298Asp in acute myocardial infarction was 2.2%, versus 0.1% in a control group, and suggested an association between homozygous mutation (T/T) and acute myocardial infarction.

There have been two opposing reports on an association between hypertensive heart disease and Glu298Asp. Miyamoto et al.⁹ reported that the distribution of G/T plus T/T in hypertensive heart disease was 21.7% against 10.6% in a control. In contrast, Tsujita et al.¹¹ reported no association between Glu298Asp and hypertension. In our experiment, the distributions of G/T plus T/T in the dead cases with hypertension, healthy control, and dead cases without CHD were 50%, 19.7%, and 8.7%, respectively. The mutation may affect somewhat the cause of death by these CHD.

The frequency of the C allele in eNOS T-786C in the four disease groups of dead cases with CHD (C = 0-0.083) was lower than that in the healthy control group (C = 0.093) and that in the dead cases without CHD (C = 0.114). The C/C genotype was not found in any of the dead cases. No association between eNOS T-786C and CHD was found in this study. Kajiyama et al.⁸ also reported no association of T-786C polymorphism with hypertension. There was no association between the healthy control and CHD in PON1, as shown in Table 1. But the frequency of the R allele was higher in myocardial infarction (R = 0.714), hypertensive heart disease (R = 0.636), and acute cardiac death (R = 0.75) than healthy control (R = 0.530) and dead cases without CHD (R = 0.530).

Although the frequency in the D allele of α 2 β -AR did not differ significantly among the three subject groups (healthy control, dead cases with CHD, and dead cases without CHD) as shown in Table 1, the frequency of D allele in

two of the disease classification in Table 2 - myocardial infarction in (D = 0.50) and coronary atherosclerotic heart disease plus chronic ischemic heart disease (D = 0.571) - was high, suggesting an association between the D allele and death in those two groups. Snapir et al.¹⁸ reported that the DD genotype gene was a risk factor for prehospital fatal myocardial infarction and sudden cardiac death, and that the risk was especially high in middle-aged (under 55 years) men from eastern Finland. The DD genotype group had a three fold greater risk for an acute coronary event than the ID group¹⁸.

The differences between the average age of the healthy control group and those of the two groups of dead cases were large, whereas the difference between the two groups of dead cases was not significant. The healthy control subjects ranged from 21–27 years old, whereas the average ages of the dead cases with and without CHD was 73.8 $\pm$ 10.8 and 63.2 $\pm$ 15.3 years, respectively. There were several reports on the distributions of these genes in Japanese^{5, 6, 10, 14, 16, 22} and the frequency of each allele in the control groups (age range 40–70 years) in those reports was almost the same as in our healthy control group. So, we used our healthy subjects as controls even though they were younger than the dead cases. We did not study the effects of other risk factors, such as cigarette smoking, cholesterol value, blood pressure, or diabetes mellitus affecting NO producing or paraoxonase activity. The body mass index (BMI) did not differ significantly between the two groups of dead cases: 22.8 $\pm$ 4.12 for those with CHD and 22.9 $\pm$ 7.5 for those without.

Recently, Polisecki et al.²⁴ reported a possible association between mtDNA 4977 bp common deletion and sudden natural death (SND). This information may be a useful tool for obtaining additional information to clarify investigations into complex SND.

Conclusion

The present study implies that the mutation of the Glu298Asp gene of eNOS may be linked to deaths due to

CHD in the Japanese population. It is considered that the Glu298Asp variant of the eNOS gene may be one of the risk factors for cases with CHD in medico-legal autopsies.

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