Original Article

Association of COMT Gene Polymorphisms with Systemic Atherosclerosis in Elderly Japanese

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Aim: Atherosclerotic disease is a major health problem among the elderly, which arises from a complex interaction between genetic and environmental factors. The catechol-O-methyltransferase (COMT) gene encodes an enzyme that degrades catecholamines and estrogens to less active metabolites. The objective of this study was to examine whether polymorphisms of the COMT gene affected the severity of atherosclerotic disease in a Japanese elderly population.

Method: A total of 1536 autopsy cases of hospital deaths were assessed for the degree of pathological atherosclerotic index (PAI), coronary stenotic index (CSI) and intracranial stenotic index (ICAI), which were obtained by macroscopic examination of the luminal surface of formalin-fixed arteries. Two single nucleotide polymorphisms (SNPs) in the COMT gene, rs4633 (C/T) and rs4680 (G/A) were genotyped. The rs4680 (G/A) corresponds to a functional SNP with the substitution of valine to methionine.

Result: The CC genotype of rs4633 (C/T) and the GG genotype of rs4680 (G/A) showed a significantly higher degree of PAI and the association remained positive after adjustment for age, hypertension, diabetes, smoking and drinking ($p=0.035$ and $p=0.031$, respectively). There were no significant associations between COMT genotypes and CSI or ICAI. When male and female subjects were analyzed separately, the association was observed only in female subjects ($p=0.012$ and $p=0.027$) after adjustment for age, hypertension, diabetes, smoking and drinking.

Conclusion: The functional SNP in the COMT gene associated with the severity of atherosclerosis in a Japanese elderly population, whereby the influence of the genotype appears to be stronger in females than in males.

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Key words; Single nucleotide polymorphism, Pathological atherosclerosis, Coronary stenosis, Autopsy

Introduction

Atherosclerosis is the main cause of cardiovascular diseases that initiates and evokes morbidity, disability and mortality¹. Atherosclerotic lesions are formed by asymmetric focal thickenings in the intima, the

innermost layer of the arterial wall, due to the collection of cellular debris and lipid accumulation. Chronic inflammation is one of the causes of the disease, involving pro- and anti-inflammatory cytokines². While diabetes, hypertension, and obesity are confirmed risk factors for the development of disease, disease etiology is also believed to involve genetic factors³. Also, the incidence of atherosclerotic disease reportedly varies with age and sex differences¹, but the detailed mechanism is still unclear. One study indicated that the genetic aspect of intima-media thickness in atherosclerotic disease is approximately

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38%⁴); however, details of such genetic components are still largely unknown.

Catechol-O-methyltransferase (COMT) is an enzyme that degrades estrogens and catecholamines, such as noradrenalin, adrenalin and dopamine, by the transfer of a methyl group from S-adenosylmethionine⁵. The COMT gene is located in the 22q11 region, and contains a non-synonymous single nucleotide polymorphism (rs4680G/A) in the fourth exon, which causes a substitution of valine (G allele) to methionine (A allele) at 158 codon. This polymorphism is known to affect the biochemical activity of the enzyme⁶. Previous studies have shown that Val/Val genotype carriers have three-to-four times higher enzymatic activity than Met/Met genotype carriers^{5,7}. Furthermore, one study demonstrated that female met/met genotype carriers have a higher level of estrogen (estradiol) than val/val genotype carriers⁸. COMT also assists in catalyzing the synthesis of homocysteine⁹, which enables cells to methylate constituent compounds such as DNA, lipids, proteins and neurotransmitters¹⁰. Homocysteine is also known to be an independent risk factor for atherosclerosis by damaging the vascular endothelium⁹. COMT-mediated molecular mechanisms for neurological disorders such as schizophrenia and depression have been postulated, but the putative modulation of the inflammatory etiology in vascular atherosclerosis and cardiovascular risk remains to be investigated.

The primary objective of this study was to investigate the effects of two highly linked polymorphisms in the COMT gene on systemic atherosclerosis in the elderly Japanese population. As a secondary objective, we aimed to evaluate whether there is a difference in the effect of these polymorphisms among males and females.

Materials and Methods

Study Subjects

A total of 1503 consecutive autopsy cases (male: 696; female: 807) in Tokyo Metropolitan Geriatric Hospital from 1995 to 2003 were included in this study. All the subjects were Japanese. The details of the autopsy cases are described elsewhere¹¹, and can be referred through the Internet-based database, "The Japanese SNP database for geriatric research (JG-SNP)" at (http://www.tmgh.metro.tokyo.jp/jg-snp/english/E_top.html). The ethics committees of Tokyo Metropolitan Geriatric Hospital, Japan and Tokyo Medical and Dental University, Japan approved the study. Regarding the clinical parameters, hypertension was diagnosed as repeatedly elevated systolic blood

pressure >140 mmHg or diastolic blood pressure >90 mmHg. Diabetes was diagnosed according to the guidelines issued by the Japan Diabetes Society in 1998, i.e., fasting plasma glucose >126 mg/dL or a casual plasma glucose >200 mg/dL, or if the two-hour blood glucose level in the 75-g oral glucose tolerance test was ≥ 200 mg/dL. BMI ≥ 25 was diagnosed as obesity according to the Japan Society for the Study of Obesity (JASSO). Lifestyle habits of smoking and drinking were obtained from the history of the patients.

Pathological Assessment of Atherosclerosis

Three different criteria were utilized, thereby giving extensive coverage to the body vascular map. Moreover, due to the critical pathophysiological roles played by the coronary and cerebral arteries, specific criteria were also used for these vessels. The severity of atherosclerosis assessment has been previously reported elsewhere¹². Briefly, eight arteries including the common carotid artery, subclavian artery, aorta, splenic artery, superior mesenteric artery, common iliac artery, external iliac artery, and femoral artery were evaluated by macroscopic examination of the luminal surface in the formalin-fixed arteries. The degree of atherosclerosis was scored according to the ratio of the occupying atheroma to the entire intimal area: 0-8: [0 (absent, <1/20 of the intimal areas occupied by the atheroma), 2 (minimal, 1/20-1/6), 4 (mild, 1/6-1/3), 6 (moderate, 1/3-2/3) to 8 (severe, 2/3-1)]. The pathological atherosclerotic index (PAI) was defined as the average atherosclerotic degree of these eight arteries. The coronary stenotic index (CSI) was examined by using transverse sections at 5 mm intervals¹³. The degree of coronary stenosis was scored from 0-5: [(0: no sclerosis, 1: slight stenosis, 2: 25% stenosis, 3: 50%, 4: 75%, and 5: 100% obstructions)]. CSI was the sum of the stenotic scores of the three branches: left anterior descending branch, left circumflex branch, and right coronary artery. The intracranial atherosclerotic index (ICAI) was established by observing the cut sections of the intracranial arteries and scoring the degree of stenosis from 0-3: [(0: no stenosis, 0.5: cases with only fatty streaks, 1: <50% stenosis, 2: 50%-90% stenosis, 3: 90% stenosis to occlusion)]. The intracranial atherosclerotic index (ICAI) is the sum of the stenotic scores of the left and right middle cerebral arteries and basilar arteries¹⁴.

Genotyping

DNA samples were extracted from the renal cortex by the phenol/chloroform method. The PCR primers and hybridization probes were ordered

Table 1. Basic characteristics of the study subjects

Characteristics	Total <i>n</i> = 1412	Male <i>n</i> = 763	Female <i>n</i> = 649	<i>p</i>
Age at death (years)	80.27 ± 8.97	78.92 ± 8.37	81.85 ± 9.39	<0.001*
PAI	4.24 ± 1.58	4.36 ± 1.50	4.09 ± 1.65	0.002*
CSI	8.21 ± 3.69	8.59 ± 3.50	7.75 ± 3.86	<0.001*
ICAI	2.63 ± 2.07	2.45 ± 1.94	2.85 ± 2.17	0.001*
Total Cholesterol	165.63 ± 44.42	157.07 ± 42.23	176.66 ± 44.79	<0.001*
HDL	42.47 ± 15.64	41.25 ± 15.09	44.02 ± 16.20	0.003*
Hypertension (%)	415 (29.39)	219 (28.70)	196 (30.20)	0.538 [†]
Diabetes (%)	208 (14.73)	112 (14.67)	96 (14.79)	0.953 [†]
Obesity (%)	46 (3.27)	22 (2.89)	24 (3.71)	0.392 [†]
Smoking (%)	675 (51.56)	533 (74.13)	143 (24.23)	<0.001 [†]
Drinking (%)	472 (36.25)	400 (55.78)	72 (12.30)	<0.001 [†]

PAI: pathological atherosclerotic index, CSI: coronary stenotic index, ICAI: Intracranial stenotic index.

Numerical data were analyzed by *Student's *t*-test, described as the mean ± S.D and categorized data were analyzed by

[†]Fisher's exact test, described as numbers and percentage (%) of prevalence.

p: statistical differences between males and females.

through Applied Biosystems (Foster City, CA, USA). Briefly, putative SNPs in the COMT genes were identified from the literature and SNP databases (<http://www.ncbi.nlm.nih.gov>). The thermal cycling conditions were 95°C for 10 min (DNA melting temperature), and then 40 cycles at 92°C for 15 sec, 60°C for 1 min (strand melting, extension and re-annealing temperatures respectively). The amplification cycle was performed on a GeneAmp PCR System 9700 (Applied Biosystems). After finishing the PCR amplification, the genotypes of two SNPs (rs4633 and rs4680) in COMT genes were determined by TaqMan allelic discrimination assay with a commercial kit (Light Cycler, Roche, Penzberg, Germany) according to the manufacturer's instructions.

Statistical Analysis

Data for continuous variables were analyzed by Student's *t*-test and expressed as the mean ± S.D. (standard deviation). Fisher's exact test was used to compare categorical variables, expressed as the mean and percentage of cases. The distribution for the genotyping result was tested by Hardy-Weinberg equilibrium. To account for multiple risk factors that affect the level of atherosclerosis, regression analysis was performed with adjustment for age, hypertension, diabetes, smoking and drinking. *P* < 0.05 was considered significant for all analysis performed by the Statistical Package for the Social Sciences for Windows, version 19.0 (IBM SPSS Statistics Desktop for Japan). Linkage disequilibrium between the SNPs was calculated by HaploView 4.2 (Broad Institute, MA, USA).

Results

Initially, 1503 elderly autopsy subjects were included in this study. We subsequently excluded 104 subjects for rs4633 and 91 subjects for rs4680 because of non-available clinical history, unclear atherosclerotic assessments, inadequate DNA samples and undetermined genotyping results. Finally, 1399 subjects for rs4633 and 1412 subjects for rs4680 were included in this study.

Table 1 shows the basal characteristics of the total study subjects separated into male and female groups. There were significant differences in mean age at death, PAI, CSI, ICAI, total cholesterol, HDL, frequency of smoking and drinking. PAI and CSI were significantly higher in males but ICAI was significantly higher in females. The two SNPs, rs4633 and rs4680, in the COMT gene were in high linkage disequilibrium (LD) with *D'* = 1 and *r*² = 0.916. Both rs4633 and rs4680 in this study were under Hardy-Weinberg equilibrium (*p* = 0.2995 and *p* = 0.0839 respectively), as shown in **Table 2**.

As demonstrated in **Table 3**, the CC genotype of rs4633 (C/T) and the GG genotype of rs4680 (G/A) showed a significantly higher level in PAI (*p* = 0.026 and *p* = 0.040, respectively), in the minor allele dominant model. After adjustment for age, hypertension, diabetes, smoking and drinking, PAI was significantly associated with both rs4633 and rs4680 (*p* = 0.035 and *p* = 0.031, respectively). There was no significant association of CSI and ICAI with either of the two genotypes. We then analyzed the effect of the genotypes on

Table 2. COMT genotyping results of the study subjects

COMT	rs4633C/T				Minor allele Frequency (T)	p for H-W
	Total	CC	CT	TT		
Total subjects	1399	665	611	123	0.31	0.2995
Male subjects	750	370	317	63	0.30	0.6716
Female subjects	649	295	294	60	0.32	0.2764

COMT	rs4680A/G				Minor allele Frequency (A)	p for H-W
	Total	GG	AG	AA		
Total subjects	1412	642	641	129	0.32	0.0839
Male subjects	763	357	341	65	0.31	0.1922
Female subjects	649	285	300	64	0.33	0.2437

COMT: Catechol-O-methyltransferase, H-W: Hardy-Weinberg equilibrium

Table 3. Minor allele dominant model (rs4633 & rs4680)

Characteristics	rs4633 (<i>n</i> = 1399)				P	P1
	CC		CT + TT			
	N	Mean ± S.D	N	Mean ± S.D		
PAI	614	4.33 ± 1.60	658	4.13 ± 1.56	0.026 [*]	0.035 ^{**}
CSI	660	8.28 ± 3.54	729	8.15 ± 3.80	0.490 [*]	0.295 ^{**}
ICAI	537	2.66 ± 2.09	576	2.65 ± 2.08	0.931 [*]	0.740 ^{**}
Characteristics	rs4680 (<i>n</i> = 1412)				P	P1
	GG		AG + AA			
	N	Mean ± S.D	N	Mean ± S.D		
PAI	593	4.33 ± 1.58	686	4.15 ± 1.57	0.040 [*]	0.031 ^{**}
CSI	636	8.32 ± 3.59	763	8.11 ± 3.71	0.280 [*]	0.129 ^{**}
ICAI	516	2.61 ± 2.09	610	2.65 ± 2.06	0.730 [*]	0.465 ^{**}

PAI: pathological atherosclerotic index, CSI: coronary stenotic index, ICAI: intracranial stenotic index.

Data were analyzed by Student's *t*-test, described as the mean ± S.D.P: statistical differences between CC and CT+TT in rs4633 and GG and AG+AA in rs4680 by *Student's *t*-test. P1: statistical association by **linear regression analysis after adjustment for age, hypertension, diabetes, smoking and drinking.

PAI in each sex. We found a significant association of PAI in female subjects in both rs4633 and rs4680, $p=0.012$ and $p=0.027$, respectively, in the same minor allele dominant model after adjustment for age, hypertension, diabetes, smoking and drinking, shown in **Fig. 1**.

Discussion

We have shown that COMT polymorphisms, rs4633 and rs4680, are significantly associated with systemic atherosclerotic disease, measured by PAI in an elderly Japanese population. The effect of COMT

polymorphism was more apparent in females than in males, suggesting the presence of gender-related differences.

COMT is a protein with pleiotropic effects and analysis of the COMT pathway may offer insights into the development of atherosclerosis. COMT supports the production of homocysteine⁹⁾, predisposing subjects to coronary artery disease¹⁵⁾, internal carotid artery stenosis and occlusion¹⁶⁾, by thickening the arterial wall and narrowing the vascular radius¹⁷⁾. Thus, it is conceivable that higher levels of COMT activity may result in higher total plasma homocysteine (tHcy) levels. This is consistent with the results of

Fig. 1. Comparison of PAI between males and females in the two SNPs.

(A) and (B) show the mean value of PAI in different genotypes of rs4633 and rs4680, respectively, comparing male and female subjects. Black and gray bars in panel A indicate CC genotype and CT + TT genotype of rs4633, respectively. Black and gray bars in panel B indicate GG genotype and AG + AA genotype of rs4680, respectively.

P: association by linear regression analysis after adjustment for age, hypertension, diabetes mellitus, smoking and drinking.

Error bar: standard deviation.

PAI: pathological atherosclerotic index

SNPs: single nucleotide polymorphisms

our model that higher enzymatic activity could occur in homozygotes (who carry the val/val genotype), predisposing them to a higher level of systemic atherosclerosis than others possessing val/met + met/met genotypes (model presented in **Table 3**).

However, the sex difference observed in our study cannot be readily explained. To account for this, elucidating the role of COMT in estrogen metabolism might be relevant. Estrogens play an important role in the protection of the cardiovascular system, affecting endothelial and smooth muscle cells¹⁸). Through genomic and non-genomic effects, estrogens regulate vascular tone and growth, with advantages to the vasculature¹⁸⁻²⁰). Indeed, it has been shown that female val/val carriers have lower estradiol concentration levels than met/met genotype carriers⁸). These findings are in line with our current result that higher enzymatic activity in val allele carriers is more susceptible to a higher degree of atherosclerosis, and probably

accounts for the sex difference. It is of note that there may be crosstalk between catecholamine and estrogen metabolism, i.e., increased level of catecholamine may abolish the vascular-stabilizing effect of estradiol by competing with COMT and disrupting the alteration of catecholsteroids and methoxyestradiols²¹).

The functional polymorphism of the COMT gene has been related to phenotypes of metabolic syndrome, such as diabetes²²) and hypertension^{23, 24}), suggesting that the effect of COMT polymorphism on systemic atherosclerosis may be an indirect effect. However, our analysis was conducted by adjusting of diabetes, hypertension, smoking and drinking; thus, we think that there is an independent effect of this polymorphism on atherosclerosis. Nevertheless, due to the highly complex nature of atherosclerotic disease etiology, we should not overlook the effect on these dependent factors.

In our study, we had adequate samples of male

and female subjects and then performed sex-based subgroup analysis in order to investigate whether there was a difference due to COMT gene polymorphisms affecting atherosclerotic disease among males and females. We could postulate that COMT gene polymorphisms are associated with PAI in female subjects but no significant association was found with CSI or ICAI. PAI assessed the large systemic, peripheral arteries, while CSI and ICAI evaluated atherosclerotic disease in the heart and brain vasculature, respectively. Due to the nature of our results, we suggest that different single nucleotide polymorphisms in different genes may account for different sites of atherosclerotic disease in the body. This is consistent with our previous result that different genetic polymorphisms associated with different arteries, i.e., the -1031C allele of TNF- α has a protective effect against atherogenesis in carotid, femoral and intracranial arteries, whereas the -511T allele of IL-1 β and the +29T allele of TGF- β 1 have pro-atherogenic risk effects in subclavian and intracranial arteries¹¹. Progressive development of atherosclerosis is affected by various factors, such as the anatomy of the vessels, blood flow and shear stress^{25, 26}. We speculate that such artery-specific-atherogenic factors interact with gene polymorphisms to affect the severity of atherosclerotic disease. Also, currently utilized scientific methods are still not robust enough to explain the sex differences accounting for higher values of ICAI in females, whereas PAI and CSI values were higher in males in our study (**Table 1**).

There are some limitations of the study. Due to the nature of the samples, we could not obtain detailed clinical information, such as tHcy, estrogen level and hyperlipidemia. Mortality due to severe atherosclerosis at an early age may result in non-inclusion amongst our study population and predispose a survival bias in our study. Also, tag SNP and haplotype analysis may give more detailed information on the effect of the COMT gene.

In conclusion, we found that a functional polymorphism in the COMT gene associates with atherosclerosis in elderly women but not in men. Our observation warrants future investigation of the function of this gene in the molecular mechanisms contributing to atherosclerotic disease.

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