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A gene variant in the Atp10d gene associates with atherosclerotic indices in Japanese elderly population

James Tumaini Kengia [a, b, 1], Kyi Chan Ko [a, 1], Shinobu Ikeda [a], Atsuko Hiraishi [a], Makiko Mieno-Naka [c], Tomio Arai [d], Noriko Sato [a], Masaaki Muramatsu [a, *], Motoji Sawabe [d]

[a] Department of Molecular Epidemiology, Medical Research Institute, Tokyo Medical and Dental University, Japan
[b] Health Care Economics, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Japan
[c] Department of Medical Informatics, Center of Information, Jichi Medical University, Japan
[d] Department of Pathology, Tokyo Metropolitan Geriatric Hospital, Institute of Gerontology, Japan

ARTICLE INFO

Article history:
Received 27 March 2013
Received in revised form
27 August 2013
Accepted 28 August 2013
Available online 5 September 2013

Keywords:
Atp10d
Single nucleotide polymorphism (SNP)
Cardiac stenotic index
Intracranial artherosclerotic index
Pathological atherosclerotic index
High density lipoprotein

ABSTRACT

Background: ATP10D belongs to a subfamily of P-type ATPases implicated in phospholipids translocation from the exoplasmic to the cytoplasmic leaflet of cellular biological membrane. Previous genome-wide association study (GWAS) identified that a variant in Atp10d gene (rs2351791) associates with serum lipid profile and myocardial infarction. The objective of this study is to assess the effect of this variant on atherosclerosis in Japanese elderly population.
Method: Consecutive autopsy cases registered in JG-SNP study were recruited ($n = 1536$). The samples were pathologically assessed for atherosclerosis using macroscopic examination of the formalin-fixed arteries, and coronary stenotic index (CSI), intracranial atherosclerotic index (ICAI) and pathological atherosclerotic index (PAI), which represent systemic arteries were calculated. The variant rs2351791 (G/T) in Atp10d gene was genotyped by Taqman genotyping assay and association determined.
Result: Both CSI and ICAI were significantly higher in GG genotype than GT genotype and TT genotype ($p = 0.003$ and $p = 0.001$, respectively). Both associations remained significant in minor allele dominant model after adjusting for age, hypertension, diabetes, HDL, smoking and drinking ($p = 0.001$ and $p = 0.001$, respectively). PAI was not associated with this variant. Consistent with the previous report, plasma HDL cholesterol level was lower in GG genotype compared to GT + TT genotypes ($p = 0.001$).
Conclusion: The rs2351791 SNP in the Atp10d gene affects the susceptibility for cardiac and intracranial vascular stenosis in the elderly Japanese population.
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1. Introduction

According to WHO estimates, ischemic heart diseases and cerebrovascular diseases are the two leading causes of mortality worldwide [1]. The disease is caused by atherosclerosis which is formed by vascular stiffening initiated by focal asymmetrical thickening and hardening in the intima, from plaque due to cell debris and lipid accumulation [1]. Chronic inflammation is one of the causes of disease formation that produces pro-inflammatory cytokines enhancing the plaque enlargement [2]. The disease is also associated with certain lifestyle characteristics and to date a large body of scientific literature has established that tobacco use, unhealthy diet, physical inactivity, raised blood pressure, raised blood cholesterol, obesity, diabetes and psychosocial factors [3]. Besides these risk factors, genetic role had also been strongly implicated in the pathogenesis of vascular diseases [4], which accounts for 38% of the disease development [5]. Lipid metabolism especially LDL level elevation and conversely low level of HDL concentration are mainly involved in disease development and progression [6].

ATP10D is a member of P4-ATPases (subfamily IV P-type ATPases), a specialized subfamily of P-type ATPases implicated in

Abbreviations: Atp10d, ATPase, class V, type 10D,; CSI, cardiac stenotic index; ICAI, intracranial atherosclerotic index; PAI, pathological atherosclerotic index; HDL, high density lipoprotein.

* Corresponding author. Department of Molecular Epidemiology, Medical Research Institute, Tokyo Medical and Dental University, MRI Bldg. F-2, 3-10 Kanda Surugadai 2, Chiyoda-ku, Tokyo 101-0062, Japan. Tel.: +81 3 5280 8060; fax: +81 3 5280 8061.
E-mail address: muramatsu.epi@mri.tmd.ac.jp (M. Muramatsu).
[1] First two authors contributed equally.

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http://dx.doi.org/10.1016/j.atherosclerosis.2013.08.034

phospholipids translocation from the exoplasmic to the cytoplasmic leaflet of biological membrane. This function is essential for maintenance of lipid imbalance in biological membrane structures and this group of translocases belongs to a group of proteins, called aminophospholipid translocases (APLTs), or Flippases [7]. Though not exactly, known APLTs activity is thought to be associated with the formation of asymmetric membrane structure through pumping phosphatidylethanolamine (PE) and phosphatidylserine (PS) from the external/luminal leaflet of the plasma membrane to the inner/cytosolic leaflet in an ATP dependent manner [7–9], and therefore along with phospholipid scramblases and floppases enhances membrane stability [8]. Furthermore, this attribute of P-type ATPases facilitates the formation of exocytic and endocytic vesicles linked to protein transport [9]. In additional, multiple studies of simple and complex multicellular organisms had shown that P-type ATPases facilitate growth in permissive temperature, sterol metabolism [8,9] and ribosome assembly and function [8,10]. Allowing to a conserved role through evolution as such it may have critical roles in maintaining cellular homeostasis.

Studies in mice for Atp10d; C57BL/6J strain were shown to carry a constitutive stop codon in the sequence of Atp10d exon 12 [11]. Interestingly, C57BL/6J mice developed obesity, hyperglycemia, and hypertension when fed a high fat diet [12,13]. Also Atp10d was reported as a candidate gene that modulates plasma HDL cholesterol level [14]. In humans GWAS reports had identified Atp10d variants among other genes significantly associated with circulating sphingolipids (Glucosylceramides and ceramides) species levels and HDL. The SNP rs2351791 located in intronic region of Atp10d gene in chromosome 4p12 [15] was also shown significantly associated with myocardial infarction in German population. Atp10d gene and its role in the etiology of cardiovascular diseases had been studied [15], but more still needed to be investigated due to the highly complex nature of genetic risks and cardiovascular disease pathogenesis.

The main objective of our study is to assess the effect of Atp10d gene polymorphism on coronary and intracranial vascular stenosis in the elderly Japanese population. Secondly, we aimed to explore whether the Atp10d gene polymorphism associate with plasma HDL cholesterol level or not in the population.

2. Materials and methods

2.1. Study subjects

Total subjects of 1536 autopsy cases obtained from 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 elsewhere [16–18], and referred as "The Japanese SNP database for geriatric research (JG-SNP)". JG-SNP was created by adding the data of genetic polymorphism, based on the clinical and pathological information of the autopsy database, performed in Tokyo Metropolitan Geriatric Hospital. The JG-SNP contains clinical diagnoses for 26 geriatric diseases, 42 pathological diagnoses and genetic polymorphism. The JG-SNP database was performed to investigate the roles of genetic polymorphisms in geriatric diseases.

The clinical parameters were obtained retrospectively from medical charts at the time of either admission or in the outpatient clinic. Hypertension was diagnosed based on usual medical practice definition as repeated elevation of systolic blood pressure $>$ 140 mmHg or diastolic blood pressure $>$ 90 mmHg. Diabetes was diagnosed as, fasting plasma glucose $>$ 126 mg/dL, or a casual plasma glucose $>$ 200 mg/dL or if the 2 h blood glucose level in the 75-g oral glucose tolerance test was $\geq$200 mg/dL, according to the guidelines issued by the Japan Diabetes Society in 1998. BMI was obtained by direct measurement of height and weight. Obesity was diagnosed as BMI $\geq$ 25 according to the Japan Society for the Study of Obesity (JASSO). HDL cholesterol level was measured by conventional precipitation method and total cholesterol level measured by enzymatic cholesterol assay method. Information on drinking and smoking status was obtained from the medical records. In order to input in the regression analysis, we prepared dichotomous variable for smokers and drinkers as 1, otherwise 0. The study had been approved by the ethical committees of Tokyo Metropolitan Geriatric Hospital, Japan and Tokyo Medical and Dental University, Japan.

2.2. Pathological assessment of the atherosclerosis

We measured the atherosclerotic severity of the whole body by dividing into three indices, termed coronary stenotic index (CSI), intracranial atherosclerotic index (ICAI) and pathological atherosclerotic index (PAI). To assess the vascular stenotic level near the coronary area, the coronary stenotic index (CSI) was measured. CSI was examined by using transverse sections at 5 mm intervals [19]. The degree of coronary stenosis was scored from 0 to 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 measured for vascular stenotic level near intracranial part. ICAI was established by observing the cut sections of the intracranial arteries and scoring the degree of stenosis from 0 to 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 arteries [20].

The severity of atherosclerosis assessment has been previously reported elsewhere [17]. To determine the systemic atherosclerosis, eight large arteries were measured. They are the common carotid artery, subclavian artery, aorta, splenic artery, superior mesenteric artery, common iliac artery, external iliac artery, and femoral artery. Each artery was 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.

In order to ensure our accuracy and validity in pathological assessments, all internal organs were extirpated, examined and fixed in 10% formalin. Weekly conferences were held and the macroscopic re-examinations of extirpated organs were performed. With the results of microscopic examinations, all cases were presented and discussed in details with clinicians at weekly conferences. All initial data were taken from the gross and microscopic autopsy reports and entered into the free-text type autopsy database [18].

2.3. Genotyping

DNA samples were extracted from the renal cortex by the phenol/chloroform method. The PCR primers and hybridization probes were obtained by ordering through Applied Biosystems (Foster City, CA, USA). Putative SNPs in the Atp10d genes were briefly identified from the literatures and SNP databases (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 s, 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 SNP (rs2351791) in Atp10d gene were determined by TaqMan allelic discrimination assay with a commercial kit (Light Cycler, Roche, Penzberg, Germany) according to the manufacturer's instructions. SNP call rates more than 95% were used in allelic discrimination procedure in this study.

2.4. Statistical analysis

Data were analyzed by Student's *t*-test and analysis of variance (ANOVA), expressed as the mean ± SD (standard deviation). Hardy–Weinberg equilibrium was performed to test the distribution for genotyping result. To account for multiple risk factors that affect the level of atherosclerosis, regression analysis was performed with adjustment for age, hypertension, diabetes, plasma HDL cholesterol level, all the categories of smoking and drinking. Bonferroni's correction was tested for multiple comparisons. All the analysis were performed by the Statistical Package for the Social Sciences for Windows, version 19.0 (IBM SPSS Statistics Desktop for Japan). $p < 0.05$ was considered statistically significant.

3. Results

Initially, the total subjects in the study include of 1536. We excluded 139 subjects because of not available adequate clinical history ($n = 2$), blood parameter ($n = 13$), pathological assessment ($n = 34$) and lack of DNA or undetermined genotyping ($n = 90$). Finally, 1397 subjects were included in our study.

Table 1 shows the clinical–pathological assessment of the study subjects according to the genotype status. Age, plasma HDL cholesterol level, CSI, ICAI and PAI are statistically significant among the three genotype groups ($p = 0.037$, $p = 0.001$, $p = 0.003$, $p = 0.001$ and $p = 0.037$ respectively). GG genotype possessing subjects have lower plasma HDL cholesterol level, subsequently higher in both CSI and ICAI than GT and TT genotype groups (Table 1). The distribution for the genotyping result was calculated by chi-square test for deviation of Hardy–Weinberg equilibrium. The distribution for genotyping result was under Hardy–Weinberg equilibrium with $p = 0.1377$ shown in Table 2. In the minor allele dominant model (GG vs GT + TT), CSI and ICAI were significantly higher in GG genotype group ($p = 0.001$ ($\beta = -0.115$) and $p = 0.001$ ($\beta = -0.140$) respectively) performed by linear regression analysis adjusted for age, hypertension, diabetes, plasma HDL cholesterol level, smoking and drinking (Table 3). But, conversely, PAI is significantly lower in GG genotype group in the same model in our study ($p = 0.032$, $\beta = 0.058$). In addition, when we applied Bonferroni's correction for multiple testing of three separate phenotypes; CSI and ICAI remain positive ($p = 0.003$) and ($p < 0.001$ respectively), while PAI ($p = 0.037$), no longer remained significant.

Further, we tested for plasma HDL cholesterol level and the mean plasma HDL cholesterol level showed significantly lower in GG genotype group (40.48 ± 14.73) than GT + TT genotype group (45.30 ± 17.03) with ($p = 0.001$) performed by students' *t* test as shown in Fig. 1.

4. Discussion

Atherosclerotic diseases in particular coronary and cerebral vascular diseases are major causes of death around the world [1]. Genetic association studies provide opportunities to be wider in understanding of genetic risk factors associated with the development of complex traits of interest [21]. In this article, we provide evidence of genetic association of Atp10d gene polymorphism with plasma HDL cholesterol level and atherosclerotic disease measured in (CSI, ICAI and PAI) in Japanese elderly population.

Atp10d belongs to the P4 ATPase family of phospholipid translocases with a role in human diseases [7]. Previously, genome wide association study revealed significant association of Atp10d polymorphisms (rs10938494 and rs2351791) with circulating ceramide, glucosylceramide species levels and myocardial infarction [15]. Therefore, Atp10d is a ceramide inward translocase at the biological membrane and functional changes of Atp10d activity may result in enhanced cytosolic ceramide concentrations leading to accelerated ceramide glucosylation and elevated serum glucosylceramide [22].

Sphingolipids species (ceramide and glucosylceramide) are known to be pathogenic lipids in human and have been linked to play a role in the pathogenesis of atherosclerosis. Sphingolipids promote atherosclerosis and thrombosis in a number of different ways. Firstly, ceramides have a strong tendency to self-aggregate, that may contribute to the amalgamation of ceramide-enriched LDL (low density lipoproteins). Secondly, ceramides can induce apoptosis in vascular cell wall, thus contributing to plaque erosion that can induce thrombosis. Thirdly, by blocking access to apoE (apolipoprotein E) and lipoprotein lipase, sphingomyelin may block LDL (low density lipoproteins) uptake. Fourthly, S1P (sphingosine1-phosphate) stimulates endothelial and smooth muscle cell proliferation, thus contributing to thickening of the vascular wall and plaque stabilization. Fifthly, ceramide may regulate the synthesis of PAI-1 (plasminogen activator inhibitor 1), which contributes to atherosclerosis and thrombosis [23]. In direct experiments, both in vitro and animals models demonstrated involvements of ceramide in ischemic death of myocardial cells [24]. It is therefore,

Table 1
Clinico-pathological assessment of the study subjects.

Atp10d, rs2351791	GG		GT		TT		*p*
	n	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	
Age (year)	837	80.63 ± 8.82	501	79.38 ± 8.92	59	80.78 ± 8.32	0.037
BMI (kg/m^2)	833	17.13 ± 3.68	499	17.24 ± 3.59	59	17.01 ± 3.69	0.815
TC (mg/dL)	658	164.40 ± 43.79	414	166.84 ± 45.44	46	166.00 ± 50.26	0.681
HDL (mg/dL)	630	40.48 ± 14.73	409	45.40 ± 17.12	43	44.37 ± 16.29	<0.001
CSI	831	8.54 ± 3.42	496	7.84 ± 3.95	58	8.13 ± 3.53	0.003
ICAI	699	2.98 ± 2.12	381	2.37 ± 2.04	47	1.86 ± 2.02	<0.001
PAI	779	4.12 ± 1.60	444	4.36 ± 1.56	51	4.10 ± 1.65	0.037
Hypertension	837	202 (24.13%)	501	176 (35.12%)	59	13 (22.03%)	0.001
Diabetes	837	111 (13.26%)	501	79 (15.76%)	59	11 (18.64%)	0.286
History of smoking	764	374 (48.95%)	474	255 (54.48%)	54	29 (53.70%)	0.361
History of drinking	764	271 (35.47%)	468	168 (35.89%)	52	23 (44.23%)	0.654

Continuous variables are analyzed by analysis of variance (ANOVA) test, expressed as mean ± standard deviation.
Categorical variables are analyzed by Chi-squared test, expressed as number and percentage (%) of disease.

Table 2
Genotyping result.

Atp10d, rs2351791	rs2351791(G/T)				MAF (T) our study	*p* for H–W
	Total	GG	GT	TT		
Total subjects	1397	837 (59.91)	501 (35.86)	59 (4.22)	0.22	0.1377
Male subjects	755	429 (56.82)	293 (38.80)	33 (4.37)	0.23	0.0520
Female subjects	642	408 (63.55)	208 (32.39)	26 (4.04)	0.20	0.9372

MAF, minor allele frequency.

Table 3
Minor allele dominant model.

Atp10d, rs2351791	GG		GT + TT		p^*	p^{**}	$p^{\dagger}$
	n	Mean ± SD	*n*	Mean ± SD			
CSI	831	8.54 ± 3.42	554	7.87 ± 3.91	0.001	0.003	0.001 ($\beta = -0.115$)
ICAI	699	2.98 ± 2.12	428	2.32 ± 2.04	0.001	0.001	0.001 ($\beta = -0.140$)
PAI	779	4.12 ± 1.60	495	4.33 ± 1.57	0.019	0.037	0.032 ($\beta = 0.058$)

*Value by students' *t* test.
**Value by multiple testing (Bonferroni's correction).
†Value by liner regression test adjusting for age, hypertension, diabetes, plasma HDL cholesterol level, smoking & drinking.

tempting to speculate that Atp10d polymorphism gives rise into atherosclerotic diseases through modulation of cytosolic pathogenic syphingolipids species. Our findings in Atp10d genetic polymorphism with atherosclerotic disease (Tables 1 and 3) could therefore be explained by this proposed role of Atp10d modulation of cytosolic pathogenic syphingolipids species.

Since Atp10d gene was proposed as a marker for regulation of plasma HDL cholesterol level [14], therefore, it is considered to be a marker for atherosclerosis through indirect effects. However, each sample in our study has been measured for plasma HDL cholesterol level and our analysis was performed by adjusting age, hypertension, diabetes, HDL, smoking and drinking; thus, it is conceivable that there is an independent effect of Atp10d polymorphism on atherosclerosis. Nevertheless, we should not look over the effect of these dependent factors, due to undiscovered and complex nature of etiology of atherosclerosis.

Our findings in the minor allele dominant model however, in which ICAI and CSI were significantly higher in GG genotype while PAI is significantly lower in the same genotype that suggests site specificity of the effect of genetic polymorphism in the pathogenesis of atherosclerotic diseases. Indeed, in our previous study, we had demonstrated a differential effect of genetic polymorphisms in different arteries. Therefore, our finding is consistent with previous research and postulated that different polymorphisms account for different site of atherosclerotic disease [16]. However, the development of atherosclerosis disease is affected by a number of factors, for instance, the blood flow, anatomy of the vessels and shear stress [25,26]. We therefore hypothesize that site specific gene polymorphism interacts with artery-specific factors to affect a sequelae of atherosclerotic disease.

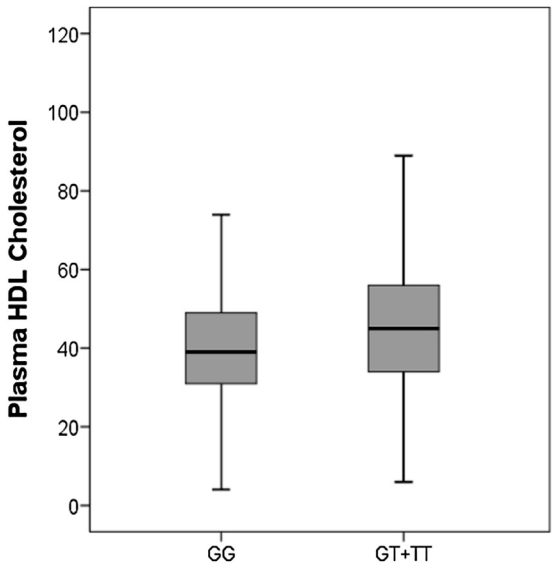

Fig. 1. Comparison of plasma HDL cholesterol level (dominant model).

We have also shown that Atp10d polymorphism is significantly associated with HDL concentration. This finding is consistent with previous studies [15], and also in mice it has been shown that Atp10d is located in a genomic region associated with HDL modulation [27]. Low HDL and conversely high LDL had been implicated in the literature to increase risks of atherosclerosis diseases [28]. Our findings in the association of Atp10d polymorphism with low plasma HDL cholesterol level (Table 1 & Fig. 1) and previous studies that implicated Atp10d polymorphism with circulating syphingolipids species levels suggest that Atp10d polymorphism leads into atherosclerosis through multiple complex pathways involving Atp10d SNPs modulation of intermediate phenotypes.

There are some limitations in our study. Due to the characteristics of our sample, we could not get sufficient clinical data related to circulating sphingolipids (ceramides and glucosylceramides) in supporting of our conceived hypothesis that Atp10d polymorphism lead into atherosclerosis through modulation of cytosolic pathogenic sphingolipids species levels. Death related to severe atherosclerosis at early age might result in non-inclusion amongst our study subjects and predispose to survival bias, and also we performed experiments for only one type of SNP. Nevertheless, our study used autopsy cases that provide a unique opportunity for investigating the effect of genetic polymorphism in undiagnosed conditions [18].

In conclusion we have shown that rs2351791 SNP in Atp10d gene is associated with cardiac and intracranial vascular stenosis in the Japanese elderly population and also, it was associated with reduced plasma HDL cholesterol level. Atp10d appears to be a good candidate for investigation of vascular diseases and it is especially interesting to investigate Atp10d polymorphism as a biomarker in detecting coronary and cerebrovascular diseases.

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