Association of the RYR3 gene polymorphisms with atherosclerosis in elderly Japanese population

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Abstract

Background: The Ryanodine receptor 3 gene (RYR3) encodes an intracellular calcium channel that mediates the efflux of Ca²⁺ from intracellular stores. Two single-nucleotide polymorphisms (SNPs) in the RYR3 gene have been shown to associate with stroke (rs877087) and carotid intima-media thickness (rs2229116) in two independent genome-wide association studies (GWAS) in Caucasian. We investigated the effect of these two SNPs as well as the 31.1 kilobases spanning region on atherosclerosis in Japanese population.

Methods: Atherosclerotic severity was assessed by carotid artery (n = 1374) and pathological atherosclerosis index (PAI) (n = 1262), which is a macroscopic examination of the luminal surfaces of 8 systemic arteries in consecutive autopsy samples. 4 tag SNPs in the 31.1 Kb region, rs877087, rs2132207, rs658750 and rs2229116, were genotyped and haplotypes were inferred to study the association with atherosclerotic indices.

Results: rs877087 and rs2229116 were associated with PAI (OR = 2.07 [1.04-4.12] (95% CI), p = 0.038; and OR = 1.38 [1.02-1.86], p = 0.035, respectively). rs2229116 was also associated with common carotid atherosclerosis (OR = 1.45 [1.13-1.86], p = 0.003). The risk allele of rs2229116 was opposite from the original report. The haplotype block of this 31.1 Kb region was different between Caucasian and Japanese. Haplotype analysis revealed that only TAGG haplotype was associated with PAI (OR = 0.67 [0.48-0.94], p = 0.020) and atherosclerosis of common carotid artery (OR = 0.75 [0.58-0.98], p = 0.034).

Conclusion: rs877087 and rs2229116 of RYR3 gene are associated with atherosclerosis severity in Japanese. The functional difference caused by rs2229116 needs to be investigated.

Keywords: Atherosclerosis, Polymorphism, Ryanodine receptor 3, Japanese

Background

Atherosclerosis is currently thought to be an inflammatory disease with deposits of cholesterol and inflammatory cells in the arterial wall [1-3]. In the later stages, thrombosis forms, and this leads to fatal events, like myocardial and cerebral infarctions [4,5]. The etiology of atherosclerosis is multi-factorial, in that it includes various genetic and environmental factors [6,7]. It is known that genetic factors play a pivotal role in atherosclerosis, but the influence of specific genes on susceptibility is not fully understood [8-10]. The discovery of novel risk factors for atherosclerosis may lead to efficient prevention and new therapeutic targets for this fatal disease.

Recently, two independent genome-wide association studies (GWAS) of atherosclerotic diseases in Caucasians pointed to two SNPs, closely resided in the 31.1 Kb (intron 14 - exon 19) region of the RYR3 gene [11,12]. RYR3 gene is located on chromosome 15q14-q15, and encode a large intracellular homotetrameric protein (> 2MDa) that comprises 4780 amino acids [13-15]; RYR3 protein resides on the sarcoplasmic reticulum membrane and releases Ca²⁺ from intracellular stores to regulates intracellular calcium concentration [16,17]. The RyR3-deficient mice lack calcium regulation properties in arterial smooth muscle cells, which lead to dysregulation of arterial tone [18]. In human arterial endothelial cells, Ca²⁺ release mediated by RyR3, but not by the RyR1 and RyR2, plays a
role in endothelial vasodilation [19]. Thus, it is conceivable that RYR3 is a good candidate for atherosclerosis susceptible gene. The rs877087 polymorphism, which is an intron SNP, was associated with stroke in a Caucasian population from 4 large cohorts \( n = 19,602 \) [11]. The rs2229116, which is an Ile to Val non-synonymous SNP, was associated with common carotid intima-media thickness (cIMT), a clinical parameter for subclinical atherosclerosis [20]. This was found in a specific group of Caucasian males infected with HIV and were treated with highly active antiretroviral therapy \( n = 177 \) [12]. Since rs877087 and rs2229116 on RYR3 gene is separated by 31.1 Kb, we hypothesize that this region might confer susceptibility to atherosclerosis, and thus studied this region in detail using tag SNPs and haplotypes.

**Methods**

**Subjects**

A total of 1536 consecutive autopsy cases of aged patients registered in the JG-SNP database were examined (http://www.tmghig.jp/jg-snp/english/E_top.html) which contains abundant pathological information on atherosclerosis [21]. The autopsies were performed from 1995 to 2004 at the Tokyo Metropolitan Geriatric Hospital in Tokyo, Japan. The distribution of age at death is 65 ~ 102 years old, and the mean is 80.21 ± 8.90 (mean ± SD). The major clinical diagnosis and direct cause of death in this Japanese population was described elsewhere [21]. The study was approved by the ethical committees of the Tokyo Metropolitan Geriatric Hospital and the Tokyo Medical and Dental University.

The methods for the pathological macroscopic evaluation of atherosclerosis severity are published previously [22,23]. Briefly, a total of eight arteries were examined, including the common carotid artery, subclavian artery, aorta, splenic artery, superior mesenteric artery, common iliac artery, external iliac artery, and femoral artery. Assessments were performed by macroscopic examinations of the luminal surface in formalin-fixed arteries. The degree of atherosclerosis had been scored according to the ratio of the atheroma area to the entire intimal area. The scale ranged from 0 ~ 8, where 0 = absent, or a ratio less than 1/20; 2 = minimal, or a ratio of 1/20 ~ 1/6; 4 = mild, or a ratio of 1/6 ~ 1/3; 6 = moderate, or a ratio of 1/3 ~ 2/3; and 8 = severe, or a ratio of 2/3 ~ 1. The pathological atherosclerotic index (PAI) was defined as the average degree of atherosclerotic severity in all eight arteries [23]. Since not all eight arteries were evaluated in some samples, the number of PAI records \( n = 1262 \) is less than the number of common carotid artery records \( n = 1374 \).

**Genetic analyses**

The RYR3 polymorphism (rs877087, rs2132207, rs658750 and rs2229116) was analyzed by the Taqman assay (Applied BioSystems) using specific primers and probes on a Light-Cycler 480 (Roche), according to previously described protocols [24]. The pathological assessments and genotyping were performed in different institutions in a double-blind fashion.

**Statistical analyses**

Statistical analyses were performed with the IBM SPSS 19.0.0 statistical software. The associations of genotypes with atherosclerosis severity were tested by multiple logistic regression analyses with adjustment for conventional risk factors. All continuous and discrete variables of atherosclerotic severity were categorized by the cut-point of 75% severity, and divided subjects into Ath(+) group with top 25% severity, and Ath(−) group with severity lower than that, as we did previously [22]. The conventional risk factors were also categorized as follows: gender (male vs. female), hypertension, diabetes mellitus, hyperlipidemia, and history of smoking or drinking (absent vs. present were classified as 0 vs. 1, respectively). P-values less than 0.05 were considered statistically significant, and correction for multiple testing was not considered in this study. Haplotype frequency estimation and haplotype-based associations were performed using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) [25]. Hardy–Weinberg equilibrium (HWE) was determined with Fisher’s exact test. Linkage disequilibrium among SNPs was assessed using Haploview software [26].

**Results**

**Genetic structure data**

rs877087 and rs2229116 is 31.1 Kb apart and the D’ and \( r^2 \) values of this susceptibility region in Japanese and Caucasian were shown in Figure 1. The trends of LD value showed that distinct difference of LD values could be found in several SNP sites between two the populations (Figure 1). In order to investigate this region, two tag SNPs (rs2132207 and rs658750, both are intron SNPs) between rs877087 and rs2229116, were selected by using Haplovie software (pairwise tagging, \( r^2 \) threshold ≥ 0.1). Genotype and haplotype data of the four RYR3 polymorphisms are summarized in Table 1. Five major haplotypes (TGGA, TAGA, TAAA, TAGG and TGGG) from the 4 SNPs could explain near 90% of the genetic diversity constituted by these 4 SNPs in this region (Table 1).

**Relationship between RYR3 polymorphisms and atherosclerosis severity**

Comparisons of atherosclerosis severity in each artery for rs877087 and rs2229116 genotypes were performed using Fisher’s exact test, and followed by logistic regression analysis in dominate or recessive models, adjusted...
by gender, history of hyperlipidemia, hypertension, diabetes mellitus, drinking and smoking (Table 2). rs877087 T allele persisted significant association with PAI (OR = 2.07, 95% CI = 1.04-4.12, \( p = 0.038 \)) after adjustment for conventional risk factors. Also, rs2229116 A allele present significant associations with PAI (OR = 1.38, 95% CI = 1.02-1.86, \( p = 0.035 \)) and atherosclerosis in common carotid arteries (OR = 1.45, 95% CI = 1.13-1.86, \( p = 0.003 \)) after adjustment. The two tag SNPs, rs2132207 and rs658750, are not significantly associated with PAI and atherosclerosis in common carotid arteries (Additional file 1: Table S1). Subgroup analysis of sex (male/female) for rs877087 and rs2229116 showed that, rs2229116 A allele present significant associations with PAI and common carotid arteries only in males (Table 2). Additional age subgroup analysis for rs877087 and rs2229116 showed that, rs877087 T allele persisted significant association with PAI only in subgroup age above 80; rs2229116 A allele present significant associations with PAI and common carotid arteries only in males (Table 2). Additional age subgroup analysis for rs877087 and rs2229116 showed that, rs877087 T allele persisted significant association with PAI only in subgroup age above 80; rs2229116 A allele present significant associations with PAI in subgroup age below 80 and with common carotid arteries in subgroup age above 80 (Additional file 2: Table S2).

Atherosclerosis degrees in each rs2229116 genotype

The tendency from the means of atherosclerosis degree in three genotypes of rs2229116, showed that risk allele is the A allele in both PAI and common carotid arteries in our samples (Table 3). In common carotid arteries, statistically significant \( p \)-values were observed from ANOVA test (\( p = 0.030 \)) and from linear regression test (\( p = 0.010 \)), indicating that there is significant linear relationship between degree of atherosclerosis and the rs2229116 polymorphism. Also, in PAI, the same trend was seen with regard to the genotype but it was not statistically significant (\( p = 0.060 \)) (Table 3).

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**Table 1 Genotype and haplotype distribution of the SNPs**

| rsSNPs       | Genotypes, n(%) | P-value for HWE test |
|--------------|-----------------|----------------------|
| rs877087     | TT 1279(94.2)   | 0.102                |
|              | CT 76(5.6)      |                      |
|              | CC 3(0.2)       |                      |
| rs2132207    | GG 463(34.2)    | 0.443                |
|              | AG 668(49.4)    |                      |
|              | AA 221(16.4)    |                      |
| rs658750     | GG 978(72.2)    | 0.629                |
|              | AG 349(25.8)    |                      |
|              | AA 28(2.0)      |                      |
| rs2229116    | AA 923(66.4)    | 0.191                |
|              | AG 411(29.5)    |                      |
|              | GG 57(4.1)      |                      |

**Haplotypes Estimated frequencies and number of samples**

| Haplotypes | Estimated frequencies and number of samples |
|------------|---------------------------------------------|
| TGGA       | 0.504, n = 681                              |
| TAGA       | 0.158, n = 214                              |
| TAAA       | 0.121, n = 164                              |
| TAGG       | 0.102, n = 138                              |
| TGGG       | 0.057, n = 77                               |

*Frequencies of haplotypes were estimated using PLINK software.*
Relationships between RYR3 haplotypes and atherosclerosis severity

From haplotype linear regression test, positive beta values were observed in the TGGG haplotype group in common carotid arteries (beta = 0.34, p = 0.032) and observed in the TAAA haplotype group in PAI (beta = 0.50, p = 0.048). Also, a negative beta value was observed in TGGG haplotype group in common carotid arteries (beta = -0.70, p = 0.007) (Table 4). Logistic regression test of haplotype association was performed for atherosclerosis severity >75%. In TGGG haplotype group, significant preventive odds ratio were observed in common carotid arteries (p = 0.034, OR = 0.75; 95% CI, 0.58-0.98), and in PAI (p = 0.020, OR = 0.67; 95% CI, 0.48-0.94) (Table 4).

Discussion

The previous GWAS showed that the RYR3 gene polymorphisms rs877087 and rs2229116 was associated with stroke and cIMT, respectively [11,12]. In this study, we investigated these two SNPs on RYR3 gene and the 31.1 Kb region in between employing elderly Japanese. We observed that both rs877087 and rs2229116 was associated with PAI. Our study suggested that the atherosclerosis risk allele is T for rs877087, as the same risk allele from the GWAS of stroke. It should be noted that the minor allele frequency of rs877087 is 0.035 for C allele in Japanese, and 0.42 for T allele in Caucasian according to the HapMap data. rs2229116 was associated with PAI and atherosclerosis severity in common carotid after adjusted for conventional risk factors (Table 2). However, our risk allele was opposite from the original paper [12], in that, the GWAS for cIMT the risk allele was G, but in our study it was A (Table 3). The LD patterns between rs877087 and rs2229116 from HapMap data showed great differences between CEU and JPT (Figure 1 and Additional file 3: Figure S1 and S2). As reference to rs2229116, the LD values of two populations showed large difference (ΔD’ > 0.5) in several SNP sites, including the 2 tag SNPs (rs2132207 and rs658750), suggesting that the haplotypes within this region may be different between CEU and JPT (Figure 1). Therefore, different haplotypes might account for the different observation between Japanese and European. In order to further investigate the region between the two reported SNPs, we further selected 2 tag SNPs (rs2132207 and rs658750) and build haplotypes (Table 1). Haplotype linear regression analyses revealed

Table 3 Means of atherosclerosis degrees in rs2229116 genotype groups

| Arteries     | rs2229116 genotypes, mean(n) | p^p | p° | p^p |
|--------------|------------------------------|-----|----|-----|
|              | AA                          | AG  | GG | Total |
| Common carotid | 7.18 (913)                  | 6.63 (404) | 6.42 (57) | 6.99 (1374) | 0.030 | 0.010 |
| PAI          | 4.29 (834)                  | 4.15 (376) | 3.97 (52) | 4.23 (1262) | 0.171 | 0.060 |

^p value from ANOVA test for means of three genotype groups.

^p value from linear regression test for means of three genotype groups.

p-values less than 0.05 were marked in bold.

Table 2 Relationship between RYR3 polymorphisms and atherosclerosis severity >75%

| Arteries     | rs877087 genotypes, n(%) | rs2229116 genotypes, n(%) | p^p | OR(95% CI) | p° | p^p |
|--------------|--------------------------|---------------------------|-----|------------|----|-----|
|              | TT                       | CT                        | CC  | TT vs. CT + CC | p^p | p° | p^p |
| PAI          | +                        | 319(96.4%)                | 11(3.3%) | 10(3.3%) | 0.023 | 2.07 | 0.038 |
| (Total)      | -                        | 840(93.2%)                | 59(6.6%) | 20(2.2%) | (1.04–4.12) | 593(64.1%) | 292(31.6%) | 40(4.3%) | (1.02–1.86) | 0.008 | 1.38 | 0.035 |
| Common carotid | +                       | 666(94.7%)                | 35(5.0%) | 20(3.0%) | 0.214 | 1.31 | 0.294 |
| (Total)      | -                        | 597(93.5%)                | 40(6.3%) | 10(2.0%) | (0.79–2.15) | 412(62.7%) | 214(32.6%) | 31(4.7%) | (1.13–1.86) | 0.003 | 1.45 | 0.003 |

^p value from Fisher’s exact test, 1-sided.

^p value from logistic regression test after adjusted by gender, history of hyperlipidemia, hypertension, diabetes mellitus, drinking, and smoking. (Parameter of gender was excluded for adjustment in Male and Female subgroups).

p-values less than 0.05 were marked in bold.
that the carriers of TAGG haplotype had a decrease trend of atherosclerosis severity in both common carotid arteries and PAI. Also, an increase trend was observed in TGGA haplotype in common carotid arteries and TAAA haplotypes in PAI, respectively (Table 4). In haplotype logistic regression model for atherosclerosis severity trait, we observed TAGG haplotype had a preventive effect in both common carotid arteries and PAI (Table 4). Although we tried to explain the opposite effect in Japanese and Caucasian, using trans-ethnic fine mapping, and selected these two tag SNPs which gave a significant difference in LD value between two ethnics, we could not explain the association with either of the SNP. Nevertheless, taken together the results of haplotype analysis, it is likely that the non-synonymous polymorphism rs2229116 (Val/Ile) itself may be the primary determinant for the observed associations (Table 4).

The rs2229116 SNP causes amino acid substitution (Ile to Val) at the 731st amino acid site of RYR3. However, the functional consequence has not yet been investigated. RYR3 have a major role in calcium signaling in the vasculature and thus is a good candidate gene in atherosclerosis pathogenesis.

Along with the function of RYR3 in vascular cells, recent studies have found that RYR3 have function in T cells, which is involved in the inflammation process of atherosclerotic lesions [27,28]. RYR3 appear to regulate Ca2+ signaling in T lymphocytes [29], in which, calcium signaling is essential for its activation and differentiation [30]. The association of T cell activation markers and carotid arterial stiffness was also reported in a group of HIV-infected women [31]. These lines of evidence suggest that, intracellular calcium mobilization mediated by RYR3 may be involved in atherosclerosis process through its common participants, such as arterial smooth muscle cells, endothelial cells and T lymphocytes.

Our study has some limitations. The subjects of the present study were consecutive autopsy cases of patients in a geriatric hospital. Therefore, the selection bias and whether the subjects can represent the demographics of Japan should be evaluated. The autopsy rate of the hospital was kept around 40% and those who died suddenly outside the hospital and medicolegal cases were not autopsied for this study. The leading mortality probability at the age of 75 years according the census data in ‘Abridged Life Tables For Japan 2010’ by the Ministry of Health, Labor and Welfare of Japan (http://www.mhlw.go.jp/english/database/db-hw/lifetb10/index.html) were as follows; 25.9% men vs. 16.5% women by malignant neoplasms, 15.2% men vs. 20.3% women by heart diseases, 10.4% men vs. 12.1% women by cerebrovascular diseases, and 15.3% men vs. 12.4% women by pneumonia. Almost all death rates for major diseases at 75 years of age in Tokyo were consistent with our autopsy data, except for the frequency of malignant neoplasms is a little higher and that of cerebrovascular diseases is lower in our autopsy subjects. Since the national health insurance covers the whole population of Japan and in Japan most death occurred in hospitals, the selection bias from socioeconomic difference of the subjects seems minimal. Altogether, the subjects in our study did not differ greatly from the standard elderly residents in Tokyo, and may represent the general population, including young residents, in Japan rather than a selected hospitalized population.

Conclusions

RYR3 gene rs877087 and rs2229116 polymorphisms are associated with atherosclerosis in elderly Japanese. Functional studies are still needed to identify the causal variation.

Additional files

- Additional file 1: Table S1. Relationship between RYR3 polymorphisms (rs2132207 and rs658750) and atherosclerosis severity >75%.
- Additional file 2: Table S2. Relationship between RYR3 polymorphisms and atherosclerosis severity >75% in age subgroups.
- Additional file 3: Figure S1. The LD (D’) heatmap of the 31.1 Kb region in CEU.
- Additional file 4: Figure S2. The LD (D’) heatmap of the 31.1 Kb region in JPT.

Abbreviations

RYR3: Ryanodine receptor 3 gene; SNP: Single nucleotide polymorphism; GWAS: Genome-wide association studies; PAI: Pathological atherosclerosis
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CZ and MM designed the study and wrote the paper. CZ and SI carried out the laboratory experiments and analyzed the data. CZ, NS and MM interpreted the results. TA, MNM and MS were responsible for sample collection, pathological data acquisition, and evaluation. All authors have contributed to, read and approved the final manuscript.

Author’s contributions

Competing interests
The authors declare that they have no competing interests.

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