Association of non-synonymous variants in WIPF3 and LIPA genes with abdominal aortic aneurysm: an autopsy study

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Abstract

Background Abdominal aortic aneurysm (AAA) is a multifactorial disease with strong genetic components. Various genetic loci have been associated with clinical AAA, but few studies have investigated pathological AAA, an intermediate phenotype of the disease. Methods We examined 2263 consecutive autopsies of older Japanese subjects from a study on geriatric diseases in Japanese individuals (The JG-SNP study). The presence of AAA was determined with a pathological diagnosis during autopsy. Single nucleotide variants (SNVs) associated with AAA were determined with an Illumina HumanExome Beadchip array. Logistic regression analyses were performed to determine genetic associations. Age, gender, and other risk factors of AAA were analyzed as covariates. Results 118 subjects with AAA and 2145 subjects without AAA were analyzed in a case-control setting. No variants reached significance after applying the Bonferroni correction ($P < 2.05 \times 10^{-6}$). The strongest associations were found with rs3750092 (p.E321G, OR: 0.36, 95% CI: 0.24–0.56, $P = 6.09 \times 10^{-6}$), a variant in the WAS/WASL interacting protein family 3 (WIPF3), and with rs1051338 (p.T16P, OR: 2.50, 95% CI: 1.70–3.66, $P = 2.79 \times 10^{-6}$) and rs2246942 (intronic, OR: 2.32, 95% CI: 1.58–3.41, $P = 1.61 \times 10^{-5}$), variants in the lysosomal acid lipase A (LIPA). LIPA is essential for macrophage cholesterol metabolism. Immunohistological analyses of WIPF3 protein in AAA samples from three subjects revealed that WIPF3 was expressed in macrophages of atheromatous plaques. Conclusions This study suggests that WIPF3 and LIPA, both of which are expressed in the macrophages are involved in pathological AAA. These results should be regarded as hypothesis-generating; thus, replication study is warranted.


Keywords: Abdominal aortic aneurysm; Lysosomal acid lipase A; WAS/WASL interacting protein family 3

1 Introduction

Abdominal aortic aneurysm (AAA) is a serious condition of the aorta; a ruptured AAA may lead to death in older individuals. Vascular aging in large arteries, such as the aorta, leads to aneurismal dilation and aortic dissection.$^{[1]}$ The prevalence of AAA is approximately 5% among white males aged 65–75 years.$^{[2]}$ A recent report from Japan showed that AAA was present in 4.1% of patients with hypertension that were over 60 years old.$^{[3]}$ Based on a meta-analysis that assessed the prevalence of AAA in the general population, the overall pooled prevalence of AAA was 4.8%. However, stratified analyses showed different prevalence in different geographic areas, as follows: America 2.2%, Europe 2.5%, Australia 6.7%, and Asia 0.5%.$^{[4]}$ The prevalence of AAA also differed among ethnic groups; it was more common in Caucasian individuals compared to African-American individuals.$^{[5,6]}$ Thus, a study conducted in older Japanese individuals may provide valuable information.

Various risk factors contribute to the development and
progression of AAA, including male sex, older age (over 65 years old), smoking, high blood pressure, high cholesterol, atherosclerosis, and a family history of AAA.\textsuperscript{5,6} In addition, multiple gene variants are thought to be related to AAA. To date, more than 100 genetic association studies have investigated single nucleotide variants (SNVs) to determine biologically-relevant genes associated with AAA.\textsuperscript{7} Those studies investigated SNVs of genes involved in the extracellular matrix, cardiovascular system, immune system, and signaling pathways. Many of those SNVs were associated with AAA. More recently, a GWAS identified genes that conferred AAA susceptibility, such as \textit{CNTN-3}, \textit{DAB2IP}, and \textit{LRP1}.\textsuperscript{7–9} Because AAA is a multifactorial disease, identification of novel candidate genes may increase our understanding of its pathogenesis.

Here, we aimed to identify novel SNVs associated with AAA by conducting an exome-wide association study in older Japanese subjects.

2 Methods

2.1 Study population

This study included 2343 consecutive autopsies of older Japanese subjects. All subjects had been enrolled in the “The Japanese SNP database for geriatric research (JG-SNP)”\textsuperscript{10} All autopsies were performed at Tokyo Metropolitan Geriatric Hospital in Tokyo, Japan, between 1995 and 2012. We performed genotyping on DNA arrays with DNA samples from 2336 cases. We excluded 45 cases, due to heterozygosity, and 28 cases that had blood relationships with each other, or they were outliers from the stratified population. We included 2263 cases in the final association analyses of risk factors for AAA.

The presence or absence of AAA was determined in a pathological examination performed at autopsy, based on the appearance of the aorta. In addition, we collected other clinical and pathological findings from the medical charts.

2.2 Genotyping and quality control

All samples were genotyped with an Illumina Infinium Human Exome Bead Chip, Version 1.1 (Illumina, San Diego, CA) in an iScan system, in accordance with the Illumina protocols. Genotype calling was performed for all samples as a single project, with the Genotyping Module (version 1.9) of the GenomeStudio data analysis software package. Initial genotype clustering was performed with the default Illumina cluster file (HumanExome 12v1-1_A.egt) and the manifest file (HumanExome-12v1-1_A.bpm), analyzed with the Gen-Train2 clustering algorithm.

The BeadChip included 247,451 markers. Variants were excluded from analyses when the genotyping rate was < 0.99, the minor allele frequency (MAF) was < 0.05, or the variant deviated from Hardy-Weinberg equilibrium with a \( P \)-value < 1.00 \( \times \) 10\(^{-5} \) in any group. After these exclusions, the final analysis data set contained 24,423 SNVs.

2.3 Assessment of clinical and pathological findings

The clinical data were dichotomized according to the presence and absence of a smoking history, hypertension, atherosclerosis obliterans, diabetes, and hyperlipidemia. For pathological findings of arteriosclerosis, we used three measurements, including the aortic atherosclerosis index (AAI), the pathological arteriosclerotic index (PAI), and the coronary stenosis index (CSI). The degrees of AAI and PAI were determined based on a microscopic examination, and they reflected the ratio between the atheroma-occupied area and the entire surface area. Then, the AAI and PAI were scored semi-quantitatively on a scale of 0–8. The PAI was defined as the average value of the arteriosclerotic scores for eight arteries. The CSI was defined as the sum of stenotic scores obtained in three branches of the coronary arteries. Details of the methods for defining the PAI and CSI were previously reported.\textsuperscript{11} The upper 25\textsuperscript{th} percentile of the AAI, PAI, and CSI distributions were used as cut off values for separating samples into those with the presence and those with an absence of pathological findings.

2.4 Statistical analysis

We investigated 24,423 SNVs; thus, statistical significance was defined as \( P < 2.05 \times 10^{-6} \), according to the Bonferroni correction. For ascending order of the \( P \)-values, we selected the top three SNVs for subsequent statistical analysis. For each SNV, stratified by the presence or absence of AAA, we estimated an odds ratio (OR) and 95% confidence interval (CI) assuming a dominant model. We initially performed multiple logistic regression analyses to determine the associations of individual SNVs with the risk of AAA, with adjustments for gender, age, and/or smoking habits. Next, each association was adjusted for individual risk factors, such as hypertension, diabetes, hyperlipidemia, and atherosclerosis obliterans, and for complications, such as AAI, PAI, and CSI. We performed all analyses with the statistical package for Social Science for Windows (SPSS), version 19 (IBM, Chicago, IL USA).

2.5 Immunohistochemical analysis

We performed immunohistochemistry to evaluate the expression of the protein, WAS/WASL interacting protein family 3 (WIPF3), in the archived atheromatous plaques and aortic media tissues from three subjects with AAA. Briefly,
formalin-fixed and paraffin-embedded sections were deparaffinized, hydrated, immersed in 0.01 mol/L citrate buffer (pH 6.0), and heated for 40 min in a warm bath (95 °C). Staining was performed with an EnVision+ Rabbit/HRP kit (Dako, Glostrup, Denmark). The primary antibody was a rabbit polyclonal antibody to WIPF3 (HPA041211 Sigma-Aldrich, Missouri, USA), applied at a dilution of 1:50.

2.6 Ethics statement

This study protocol was approved by the Ethics Committees of the Tokyo Medical and Dental University (2009-19-8) and the Tokyo Metropolitan Geriatric Hospital (2009-482). Written informed consent was obtained from family members of all participants before autopsy.

3 Results

3.1 Characteristics of the study population

The characteristics of the study population are shown in Table 1. Out of 2263 patients, we found AAA in 118 subjects. Overall, the mean age at death was 80.65 ± 8.86 years. The average age at death was significantly higher in subjects with AAA than in subjects without AAA (84.08 ± 7.90 vs. 80.46 ± 8.88 years, P < 0.001).

In this study, the prevalence of AAA was not significantly different between males and females (5.4% and 5.0%, respectively). The smoking rate was higher among subjects with AAA (60.0%) compared to those without AAA (51.9%), although this difference was not significant (P = 0.109). With regard to the clinical findings, hypertension was more prevalent in subjects with AAA than in those without AAA (P = 0.001). Hyperlipidemia was more prevalent in subject with AAA than in those without AAA, however it did not reach statistical significance. Diabetes was more prevalent in subjects without AAA than in those with AAA, but this difference also did not reach statistical significance. Arteriosclerosis obliterance was more prevalent in subjects with AAA (P < 0.001).

Pathological arteriosclerosis measured with the AAI, PAI, and CSI, were significantly more severe in subjects with AAA than in subjects without AAA (P < 0.001).

Table 1. Characteristics of the study subjects with (+) and without (−) AAA.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Available data N(%)</th>
<th>All subjects</th>
<th>AAA (+)</th>
<th>AAA (-)</th>
<th>χ² test P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>2263</td>
<td>118</td>
<td>2145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at death, yrs</td>
<td>80.65 ± 8.86</td>
<td>84.08 ± 7.90</td>
<td>80.46 ± 8.88</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>2263 (100%)</td>
<td>1255</td>
<td>68 (5.4%)</td>
<td>1187 (94.6%)</td>
<td>0.636</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>1008</td>
<td>50 (5.0%)</td>
<td>958 (95.0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>2076 (91.7%)</td>
<td>1085/2076 (52.3%)</td>
<td>63/105 (60.0%)</td>
<td>1022/1971 (51.9%)</td>
<td>0.109</td>
</tr>
<tr>
<td>Clinical findings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>734/2223 (33.0%)</td>
<td>56/118 (47.5%)</td>
<td>678/2105 (32.2%)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>370/2223 (16.6%)</td>
<td>14/118 (11.9%)</td>
<td>356/2105 (16.9%)</td>
<td>0.164</td>
<td></td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>2223 (98.2%)</td>
<td>82/2223 (3.7%)</td>
<td>74/2105 (3.5%)</td>
<td>0.076</td>
<td></td>
</tr>
<tr>
<td>Arteriosclerosis obliterans, n (%)</td>
<td>80/2223 (3.6%)</td>
<td>14/118 (11.9%)</td>
<td>66/2105 (3.1%)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Pathological findings</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAI, n (%)</td>
<td>2225 (98.3%)</td>
<td>103/2225 (49.6%)</td>
<td>1000/2111 (47.4%)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>PAI, n (%)</td>
<td>1969 (87.0%)</td>
<td>496/1969 (25.2%)</td>
<td>448/1878 (23.9%)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>CSI, n (%)</td>
<td>1363 (60.2%)</td>
<td>354/1363 (26.0%)</td>
<td>319/1289 (24.7%)</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

N(%): number of subjects with available data (a percentage out of 2263 subjects); n(%): number of subjects in a group (percentage of all subjects with available data, relative to each characteristic). Pathological findings were defined as a score within the upper 25th percentile of each group. AAA: abdominal aortic aneurysm; AAI: aortic atherosclerosis index; CSI: Coronary stenosis index; PAI: Pathological arteriosclerotic index.

Maeda Y, et al. AAA susceptible genes
Based on the association study, assuming a dominant model, the minor (G) allele of rs3750092 in WIPF3 was protective against AAA (p.E321G, OR: 0.36, 95% CI: 0.24–0.56). For SNVs in the LIPA gene, the minor (C) allele of rs1051338 (p.T16P, OR: 2.50, 95% CI: 1.70–3.66) and the minor (G) allele of rs2246942 (intronic, OR: 2.32, 95% CI: 1.58–3.41) were risk factors for AAA. When the analyses were adjusted for age/gender or for age/gender-smoking habits, no essential difference was observed. This finding indicated that those factors did not affect the genetic association (Table 2). Moreover, the results were not essentially different, when we applied further adjustments for the clinical findings of hypertension, diabetes, hyperlipidemia, and arteriosclerosis obliterans, or when we applied adjustments for the pathological findings of AAI, PAI, and CSI (Supplementary Table).

### 3.3 Immunohistochemical analysis of WIPF3 protein expression

We used immunohistochemistry to determine the presence of WIPF3 protein in aortic aneurysm lesions, including atheromatous plaques, and in the aortic wall (Figure 2). Immunostaining showed no signal for WIPF3 in the aortic media, but the signal was positive in atheromatous plaques.
Figure 2. Immunohistochemical analysis of WIPF3 protein in aortic lesions from three patients with AAA [(A) & (B) × 400, (C–F) × 200]. (A–C): Representative images of atheromatous plaque sections that were positively stained for WIPF3. WIPF3 immunoreactivity was detected in the foamy cells of atheromatous plaques (arrows). In case 3 (C), positive staining was observed in the athromatous matrix (*). (D–F): Representative images of aortic media sections immunohistochemically stained for WIPF3 in the same three patients with AAA. No positive reaction was detected. AAA: abdominal aortic aneurysm; WIPF3: WAS/WASL interacting protein family 3.

These results suggested that WIPF3 was expressed in only atheromatous lesions. High magnification microscopy revealed that WIPF3 was expressed in foamy macrophages within the atheromatous plaques.

4 Discussion

In this study, we conducted an analysis of 24,423 SNVs in exon regions to determine their associations with AAA. Although no SNVs/genes fulfilled the Bonferroni criteria of significance, we detected non-synonymous SNVs in WIPF3 and LIPA that may affect AAA susceptibility.

In our study population, the AAA prevalence was similar between males and females. This finding contrasted with the general observation that the prevalence of AAA is approximately five times higher in males than in females.[4] However, some studies showed that the prevalence of AAA gradually increased with age in females and males. Current reports from Japan revealed that, among older individuals, the AAA prevalence was similar between sexes. AAA was
detected in 5.7% of females aged over 80 years and 5.7% of men aged 70–79 years. Thus, our data appeared to be consistent with observations that the prevalence was not gender-dependent in the older Japanese population. We found no significant difference in smoking prevalence between subjects with and without AAA in our study population. Although there is strong evidence that smoking is a risk factor of AAA, the effect of smoking was not apparent in our population. One reason for the lack of a significant effect of smoking might be that the smokers in our study population included both current and ex-smokers. Our information was limited in detail; thus, we had no data on when ex-smokers had stopped smoking. The clinical findings of hypertension, hyperlipidemia, and arteriosclerosis obliterans were more prevalent in subjects with AAA than in those without AAA. The higher prevalence of diabetes in subjects without AAA than those with AAA was an expected observation, because diabetes decreases aortic wall expansion. The high severity we observed in the AAI, PAI, and CSI in subjects with AAA confirmed that arteriosclerosis was a significant risk factor of AAA. In our previous report, where we investigated part of the current population, we found evidence to support the predictive abilities of PAI and CSI as markers for the risk of atherosclerosis and also coronary artery disease (CAD). Among all the SNVs investigated in this study, the SNV with the lowest p-value was rs3750092 in the WIPF3 gene; this SNV was followed by rs1051338 and rs2246942 in LIPA gene, which showed a high LD (r² = 0.907, D' = 0.968, from 1000 genomes (phase_1 JPT data)). Previous GWAS and replication studies have shown that rs2246942 and the two SNVs with a high LD were associated with CAD. In an expression quantitative trait locus (eQTL) analysis, an increased level of LIPA mRNA was associated with the risk for CAD. Variants in LIPA were also associated with lipid traits, indicating the importance of lipids in CAD. However, target genes identified by GWAS should be analyzed in functional studies to improve our understanding of the underlying mechanism. LIPA encodes lysosomal acid lipase, which plays an important role in macrophage cholesterol ester homeostasis. Because atherosclerosis is an underlying condition for both CAD and AAA, variations in LIPA may be a common risk factor in both diseases. However, the association of LIPA variations with AAA was not affected by adjustment with atherosclerotic changes may suggest that it is an independent risk factor (Supplementary Table).

Our results indicated that the rs3750092 variant of the WIPF3 gene was protective against AAA. The WIPF3 protein binds to the neural Wiskott-Aldrich syndrome protein (N-WASP), and together, they play a key role in regulating the actin cytoskeleton and cell movement. The WIPF3 protein is abundant in the brain and testis, but its expression in other tissues was not well known. We demonstrated that WIPF3 protein was expressed in macrophages of atheromatous plaques. Interestingly, other studies have shown that N-WASP was required for podosome formation in macrophages. Macrophages are known to play a critical role in AAA formation. They infiltrate the wall of AAA vessels and produce cytotoxic mediators, such as cytokines, perforin, and matrix metalloproteinase proteinases (MMPs). These mediators contribute to the elimination and degeneration of smooth muscle cells during the pathogenesis of AAA. Both candidate genes identified in the present study, WIPF3 and LIPA, function in macrophages; thus, the pathogenesis of AAA might be affected by macrophage characteristics.

Further studies are needed to confirm these associations in larger sample sizes and to investigate the molecular mechanisms associated with these SNVs. For example, the mechanism underlying the role of NWASP/WIPF3 in AAA progression is currently unknown. In addition, larger sample sizes are needed to confirm our findings that the genetic associations remained positive after adjusting for AAI, PAI, and CSI, and that they were independent from the risk of atherosclerosis. Future functional and physiological analyses in animal models are required to determine the causal relationship between WIPF3/LIPA and AAA development. This study had some limitations. First, we could not avoid a selection bias and a survival bias. Second, we had insufficient clinical data and a small sample size, due to the post-mortem study design. According to a summary report of the vital statistics in Japan, 2014, more than 80% of older individuals (65 years old and above) die in a hospital. The autopsy rate in this hospital was 40%, and the autopsies were conducted without focusing on a particular disease. The characteristics of the subjects in our study did not differ greatly from those reported for the general population of older residents in Tokyo, Japan. Due to the small sample size, we had low statistical power for detecting the associations between AAA and the genes reported in this study. In addition, due to the retrospective nature of this study, it was difficult to collect information on WIPF3 and LIPA protein expression levels; thus, we could not analyze associations between protein expression levels and AAA lesion diameters. Consequently, our results should be regarded as hypothesis-generating, and the WIPF3 and LIPA genes should be considered candidate risk factors for AAA. More research is required to confirm our findings.
Acknowledgements

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References


Longo GM, Xiong W, Greiner TC, et al. Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms.


Supplemental Figure. Quantile-quantile (Q-Q) plot of the data shown in the Manhattan plot in Figure 1.
### Supplementary Table. Effects of various adjustment factors on the strengths of genetic associations with AAA.

<table>
<thead>
<tr>
<th>Adjustment factor</th>
<th>(WIPF3) (rs3750092)</th>
<th>(LIPA) (rs1051338)</th>
<th>(LIPA) (rs2246942)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(P)-value</td>
<td>(P)-value</td>
<td>(P)-value</td>
</tr>
<tr>
<td>Age</td>
<td>(6.09 \times 10^{-6})</td>
<td>(3.49 \times 10^{-5})</td>
<td>(2.79 \times 10^{-6})</td>
</tr>
<tr>
<td>Gender</td>
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<td>(6.29 \times 10^{-6})</td>
<td>(2.79 \times 10^{-6})</td>
</tr>
<tr>
<td>Smoking</td>
<td>(1.63 \times 10^{-3})</td>
<td>(1.59 \times 10^{-3})</td>
<td>(2.51 \times 10^{-3})</td>
</tr>
<tr>
<td>Hypertension</td>
<td>(7.24 \times 10^{-6})</td>
<td>(1.11 \times 10^{-6})</td>
<td>(2.56 \times 10^{-6})</td>
</tr>
<tr>
<td>Diabetes</td>
<td>(7.24 \times 10^{-6})</td>
<td>(6.64 \times 10^{-6})</td>
<td>(2.56 \times 10^{-6})</td>
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<tr>
<td>Hyperlipidemia</td>
<td>(7.24 \times 10^{-6})</td>
<td>(6.93 \times 10^{-6})</td>
<td>(2.56 \times 10^{-6})</td>
</tr>
<tr>
<td>Arteriosclerosis obliterans</td>
<td>(7.24 \times 10^{-6})</td>
<td>(9.83 \times 10^{-6})</td>
<td>(2.56 \times 10^{-6})</td>
</tr>
<tr>
<td>AAI</td>
<td>(7.45 \times 10^{-6})</td>
<td>(1.72 \times 10^{-6})</td>
<td>(3.06 \times 10^{-6})</td>
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<td>PAI</td>
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<td>(8.25 \times 10^{-5})</td>
<td>(5.31 \times 10^{-6})</td>
</tr>
<tr>
<td>CSI</td>
<td>(4.83 \times 10^{-5})</td>
<td>(5.94 \times 10^{-5})</td>
<td>(4.01 \times 10^{-5})</td>
</tr>
</tbody>
</table>

\(P\)-values were calculated, assuming a dominant model. AAA: abdominal aortic aneurysm; AAI: aortic atherosclerosis index; CSI: Coronary stenosis index; PAI: Pathological arteriosclerotic index.