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Association of an allele on chromosome 9 and abdominal aortic aneurysm

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Abstract

Objective—Abdominal aortic aneurysm (AAA) has been recognized as a multi-factorial disease with both genetic and environmental risk factors. A locus residing within non-coding DNA on chromosome 9p21.3 has recently been associated with AAA. To further investigate the significance of this site for AAA, we performed an association study on a large group of 3,371 men aged 65–83 years of whom 513 had an AAA.

Methods—All men were assessed for other risk factors in a uniform way and an ultrasound of the abdominal aorta was performed.

Results—Our findings validated the strong association of the chromosome 9p21.3 SNPs rs10757278 and rs1333049 with AAA and demonstrated the upregulation of LINE-1 elements at the site of AAA.

Conclusion—This study confirms a reproducible association between risk alleles on chromosome 9p21.3 and AAA. We also provide preliminary evidence for an association of LINE-1 elements with AAA which will require further investigation.

Keywords

Abdominal aortic aneurysm; risk locus; retrotransposons

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INTRODUCTION

The association of chromosome 9p21.3 locus, identified by the single nucleotide polymorphisms (SNPs), rs10757278 or rs1333049, with abdominal aortic aneurysm (AAA), was originally recognized in a multicentre case-control study performed on individuals that originated from seven different populations [1]. The most significant association with AAA was found in the Icelandic group (odds ratio (OR) ~ 1.4, $p=2.6 \times 10^{-5}$). Significant associations were found in 5 of the 7 cohorts. A further analysis in which subjects with known coronary heart disease were excluded led to marked reduction in the reported significance of the association for most groups (e.g. New Zealand $p=0.097$, United Kingdom $p=0.038$, Iceland $p=0.013$). This raises the possibility that this association may simply reflect confounding with atherosclerosis and/or its risk factors. A more recent study reported an association between rs1333049 and AAA in a UK population with OR of ~2 [2]. The investigators reported that this association was maintained after adjusting for other AAA risk factors, including smoking, dyslipidemia, coronary heart disease (CHD), age and family history, although only half of the subjects had risk factor data for this analysis. A further case-control study included individuals from UK and Western Australia and neither population had any significant association between rs10757278 and AAA alone but findings were compatible with the ORs found in earlier studies [3].

The chromosome 9p21.3 risk locus is believed to reside within non-coding DNA. A large part of the human genome is repetitive DNA and the majority of these repetitive sequences represents retrotransposons; a class of mobile genetic elements that transpose repeatedly within their host genomes [4]. Among them, long interspersed nuclear elements 1 (LINE-1s) are known to mediate wide variation in human genome [5], and their invasiveness may lead to pleiotropic effects influencing multiple phenotypic traits. Based on our *in silico* analyses we postulated that the risk alleles on 9p21.3 could be associated with LINE-1 elements and sought evidence to support this theory within aortic biopsies.

MATERIALS AND METHODS

Based on power calculations using Quanto v.1.2.4 (<http://hydra.usc.edu/GxE/>) we included 600 men with AAA and 3,000 without AAA from the Health In Men Study (HIMS) [6] in an attempt to replicate previous associations of 9p21.3 locus with AAA at an OR of ≥ 1.3 (power = 80%, minor allele frequency MAF = 0.40, two-sided $P < 0.05$). All subjects included (Supplementary Data 1) had undergone abdominal ultrasound. AAA was defined as maximum infrarenal aortic diameter ≥ 30 mm. The definitions of clinical risk factors such as hypertension, dyslipidaemia, and CHD were as previously described [7].

Aortic wall biopsies were collected in RNeasy[®] solution (Ambion) from a group of 24 patients undergoing open AAA repair in Queensland, Australia (Supplementary Data 2). Samples were obtained from the body of the aneurysm (site of maximum dilatation) and the non-aneurysmal proximal aortic neck just below the renal arteries. Total RNA was extracted using RNeasy[®] Mini Kit (Qiagen) according to manufacturer's instructions. Ethical approval was granted from the relevant committees and all subjects gave written informed consent to their involvement in the study.

Four SNPs (rs564398, rs10757278, rs1333049 and rs10811661) were genotyped using the Illumina Golden Gate[®] assay at University of Western Australia. Patients in which genotyping failed for any of the four SNPs were excluded from the statistical analysis. In total, we included 513 out of 600 AAA cases and 2,858 out of 3,000 men without AAA (Supplementary Data 1).

Gene structures were predicted within the segment of 500-kb in both directions from rs1333049 (chromosome 9 position 22,115,503; human genomic coordinates version hg18) using a

combination of Fgenesh v2.6 gene prediction tool, BLAST search, and the InterProScan protein sequences databases. We predicted a “prototype gene” with reverse transcriptase or long interspersed nuclear element 1 (LINE-1) reverse transcriptase related activity containing rs1333049 and rs10757278 SNPs in its intron 4 (Supplementary Data 3). The same segment was subsequently screened for all LINE-1 elements using the web-based tools L1Xplorer and the L1Base database (<http://line1.bioapps.biozentrum.uni-wuerzburg.de/l1xplorer.php>).

A subset of 100 (20 AAA cases and 80 controls) men was selected from a total of 3,371 (513 and 2,858 AAA cases and controls, respectively) using a stratified random sampling procedure to assess the LINE-1 insertions at chromosome 9p21.3 (Supplementary Data 1). We adopted the method called L1 display [8] to verify the LINE-1 insertions identified by *in silico* analysis.

Quantitative real-time PCR (qPCR) was performed for both open reading frame 1 and 2 (ORF1 and ORF2) of the LINE-1 mRNA as well as for phorbolin-1 and 2 (APOBEC3A and APOBEC3B) genes. APOBEC3A and APOBEC3B were assessed, in addition to LINE-1, since they are the primary intracellular inhibitors of LINE-1 mediated retrotransposition. The relative expression of the gene of interest in each sample was calculated by using the concentration-Ct-standard curve method and normalized using the average expression of GAPDH for each sample. GAPDH was chosen as the “housekeeping” gene since analyses showed its expression to be unaffected by AAA biopsy site or chromosome 9p21.3 genotype. The QuantiTect SYBR Green one-step RT-PCR Kit (Qiagen) was used according to the manufacturer's instructions with 100ng of total RNA as template. SYBR[®] Green PCR primers were designed using the PREMIER Biosoft's AlleleID 7 (ORF1: 5'-GAAAGTGATAGGGAGAATGG-3' and 5'-TTATGTGTCCTTGGAGTTGC-3', ORF2: 5'-TCAATGGAACAGAACAGAGTC-3' and 5'-GTGCCATATGTCCTGAATGG-3', APOBEC3A: 5'-CTACCTGTGCTACGAAGTG-3' and 5'-GGAGATGAACCAAGTGACC-3', and APOBEC3B: 5'-CGCCAGACCTACTTGTGC-3' and 5'-GAAGATTCTTAGCCTCGTTGC-3').

The associations of individual genotypes and haplotypes with AAA and LINE-1 expression level respectively were tested under additive, dominant and recessive models. All computations were undertaken using SimHap 1.0.0 [9]. Wilcoxon's signed ranks test and Spearman's Rho using the SPSS statistical package v.17.0.2 was performed to identify differences between ORF1, ORF2, APOBEC3A and APOBEC3B expression levels. Statistical significance was defined at the conventional 5% level.

RESULTS

The strongest association between the chromosome 9p21.3 risk locus and AAA was found under a dominant model of inheritance with OR=1.81 (95% CI 1.42-2.32, $p=2.5 \times 10^{-6}$) and OR=1.70 (95% CI 1.33-2.17, $p=1.9 \times 10^{-5}$) for rs10757278 and rs1333049, respectively (Table 1). Results were pooled with previous data using meta-analysis and showed combined OR ~1.3 for both rs10757278 and rs1333049 (Supplementary Data 4).

Nine full length LINE-1s were predicted to be inserted in the 9p21.3 region according to the L1Xplorer (Supplementary Data 5). Five insertions were phylogenetically older L1PA5s, two younger L1PA2s, and two non-canonical elements. Primers were designed for one older element (L1PA5 family) that corresponds to our candidate gene (ID5), and two younger L1PA2s residing in both downstream and upstream direction from rs1333049 or rs10757278 SNPs (ID1 and ID9). All three full-length LINE-1s were confirmed as authentic insertions with no insertional polymorphism in 100 subjects assessed (data not shown).

The median relative expression of ORF1, ORF2, and APOBEC3B in the aneurismal body was more than 1.5 fold higher than in the non-aneurismal neck (ORF1: 2.22 vs. 1.36, $p=0.033$; ORF2: 2.06 vs. 1.36, $p=0.044$; and APOBEC3B: 0.66 vs. 0.40, $p=0.019$). Importantly the

expression of ORF1 and ORF2 was highly correlated ($r=0.96$, 95% CI 0.90-0.98, $p<0.001$). The expression of the inhibitor APOBEC3B gene within the AAA body biopsies was correlated to both ORF1 ($r=0.66$, 95% CI 0.34-0.84, $p<0.001$) and ORF2 ($r=0.72$, 95% CI 0.44-0.87, $p<0.001$).

We found no association between the genotypes and allele frequencies of rs10757278 and rs1333049 polymorphisms with ORF1 and ORF2 expression levels within AAA biopsies (data not shown). However the GG haplotype formed by rs10757278 and rs1333049 polymorphisms was significantly associated with increased expression of both ORF1 and ORF2 in biopsies from the body of human AAAs (Table 2). No association was found between rs10757278 and rs1333049 haplotypes and ORF1 or ORF2 expression in biopsies from the macroscopically normal AAA neck.

DISCUSSION

This study confirms an important association of a risk locus on chromosome 9p21.3 with AAA. From a clinical perspective, the small effect size and the fact that this risk locus resides within the segment of non-coding DNA limits the diagnostic, prognostic, and therapeutic application of this polymorphism. The effect size is however in keeping with our current understanding of AAA as a multi-factorial disease with genetic and environmental components [10]. This study also provides preliminary evidence for an association of LINE-1 elements with AAA. Our *in silico* analysis suggested that LINE-1 insertion adjacent to rs10757278 or rs1333049 could explain the association of these SNPs with AAA. However while confirming the insertion of these candidate LINE-1s at the predicted site we found there was no insertional polymorphism in subjects assessed. We did however demonstrate that mRNA for LINE-1 was upregulated within AAA biopsies. These findings suggest that LINE-1 retrotransposition activity that requires the protein products of both ORF1 and ORF 2 [11] could be present within human AAA which has previously been thought to be restricted to human embryonic development [12]. The upregulation of LINE-1 inhibitor gene (APOBEC3B) also indirectly confirms these findings suggesting ongoing cellular defence against LINE-1 activity. A haplotype involving the two genetic polymorphisms within 9p21.3 was associated with upregulation of LINE-1 mRNA within AAA biopsies suggesting that this locus may effect the expression of an active LINE-1 element at a currently unidentified site on chromosome 9p21.3. Larger studies will be required to confirm this finding, although access to aortic biopsies is now limited due to the increase in endoluminal AAA repair. The association between 9p21.3 haplotype and LINE-1 expression is, however, consistent with the recently proven gene regulatory function of this site. Within mice the deletion of the 9p21.3 equivalent non-coding DNA segment was found to be associated with severe reduction in the expression of Cdkn2a and Cdkn2b which are situated at distant sites on 9p21.3 in humans [13]. It is possible that the effect of this risk locus is not limited to Cdkn2a and Cdkn2b but also influences loci controlling LINE-1 expression. Further studies, such as chip-sequencing assays, will be required to assess this in more detail in humans. The mechanism responsible for the association of LINE-1 with AAA is not evident in the current study. Studies of active transposable elements suggest that they can alter gene expression in either destructive, or less often, beneficial directions especially when active genes are targeted [14,15]. Such insertions into genes that are essential for normal vascular maintenance could promote the pathogenesis of cardiovascular diseases including AAA. Further studies will be required to assess the mechanisms in more detail.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1
Multivariate association of SNPs with AAA in 3,371 HIMS subjects (adjusted for other risk factors).

SNP	Genotype	Additive Model				Dominant Model				Recessive Model			
		OR	95% CI	P	AIC	OR	95% CI	P	AIC	OR	95% CI	P	AIC
rs564398	AA	-	-	-	2552.5 NS	-	-	-	2556.3 NS	-	-	-	2550.6 NS
	AG	1.03	0.82-1.28	0.807		0.94	0.76-1.16	0.557		0.71	0.54-0.94	0.016	
	GG	0.72	0.53-0.98	0.037		-	-	-		-	-	-	
rs10757278	AA	-	-	-	2534.6 6.0×10^{-6}	-	-	-	2532.8 10.0×10^{-7}	-	-	-	2555.1 NS
	AG	1.84	1.42-2.38	4.1×10^{-6}		1.81	1.42-2.32	2.5×10^{-6}		1.16	0.92-1.46	0.210	
	GG	1.76	1.31-2.37	<0.001		-	-	-		-	-	-	
	GG	-	-	-		-	-	-		-	-	-	
rs1333049	GC	1.71	1.33-2.21	3.5×10^{-5}	2539.1 5.6×10^{-5}	1.70	1.33-2.17	1.9×10^{-5}	2537.2 9.9×10^{-6}	-	-	-	2555.1 NS
	CC	1.67	1.25-2.25	<0.001		-	-	-		1.16	0.92-1.47	0.203	
	TT	-	-	-		-	-	-		-	-	-	
rs10811661	TC	1.16	0.93-1.44	0.193	2557.0 NS	1.15	0.93-1.42	0.201	2555.1 NS	-	-	-	2556.7 NS
	CC	1.07	0.60-1.92	0.820		-	-	-		1.03	0.57-1.83	0.932	
	CC	-	-	-		-	-	-		-	-	-	

AIC, Akaike's information criterion; NS, Non-significant model; Adjusted for covariates (Supplementary Data 1).

Table 2

Univariate associations of the rs10757278-rs1333049 GG haplotype with relative expression of LINE-1's ORF1 and ORF2.

Site	ORF	Copy	Additive Model				Dominant Model				Recessive Model			
			Coefficient	P	Mean	AIC	Coefficient	P	Mean	AIC	Coefficient	P	Mean	AIC
Body	1	0	-	-	2.61	70.40 0.0294	-	-	2.61	75.26 NS	-	-	2.51	69.73 0.0132
		1	-0.60	0.4507	2.01		-	-	2.95		-			
		2	3.17	0.0252	5.78		0.34	0.5780	2.95		3.28	0.0204	5.79	
	2	0	-	-	2.43	65.54 NS	-	-	2.43	68.75 NS	-	-	2.33	64.98 0.0387
		1	-0.55	0.4455	1.88		0.14	0.6030	2.57		-	-		
		2	2.27	0.0636	4.70		-	-	-		2.37	0.0530	4.70	
Neck	1	0	-	-	1.54	61.73 NS	-	-	1.54	60.56 NS	-	-	1.63	62.03 NS
		1	0.55	0.4750	2.09		0.76	0.2780	2.30		-	-		
		2	1.39	0.1720	2.93		-	-	-		1.30	0.2040	2.93	
	2	0	-	-	1.56	71.09 NS	-	-	1.56	69.35 NS	-	-	1.61	70.65 NS
		1	0.22	0.5430	1.78		0.29	0.6300	1.85		-	-		
		2	0.43	0.7220	1.99		-	-	-		0.38	0.7550	1.99	