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A single nucleotide polymorphism in exon 3 of the kallikrein 1 gene is associated with large but not small abdominal aortic aneurysm

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ABSTRACT

Objective: Abdominal aortic aneurysm (AAA) is a late onset degenerative condition with an inherited component thought to be due to multiple risk alleles. A locus on chromosomes 19q13 has been previously associated with AAA. The gene encoding kallikrein 1 (*KLK1*) is located on chromosome 19q13 and the single nucleotide polymorphism (SNP) rs5516 has been previously shown to lead to structural changes in the *KLK1* transcription regulatory region. The aim of this study was to investigate whether rs5516 was associated with AAA and aortic diameter.

Methods: We performed a case-control study on two independent subject groups from Western Australia (n=1304) and Queensland (n=325) of which 609 and 225 had an AAA, respectively. In addition we analysed RNA extracted from abdominal aortic biopsies from 12 patients undergoing AAA surgery and 6 organ donors.

Results: After adjusting for other risk factors the G allele of the rs5516 polymorphism was associated with large but not small AAA using a dominant model in the Western Australian men and a recessive model in Queensland subjects. In subjects with large AAA the G allele was associated with aortic diameter. The short splice variant of *KLK1* was upregulated within AAA compared to control biopsies.

Conclusion: This study suggests that a genetic polymorphism in *KLK1* may contribute to the risk of developing later stage AAA.

Keywords

Abdominal aortic aneurysm; kallikrein 1, splice variants

INTRODUCTION

Screening studies indicate that ~ 5% of men aged > 60 years have an abdominal aortic aneurysm (AAA) [1]. Important risk factors for AAA include age, smoking, coronary heart disease, hypertension and family history [2]. Subjects with a first degree relative with a history of AAA have a approximately 2-fold increased risk of developing the condition [3]. Genome-wide screening of patients with a family history of AAA using affected relative pairs suggested two linkage regions on chromosomes 19q13 and 4q31 with a logarithm of odds (LOD) scores of approximately 5 and 4, respectively [4]. These two genomic regions have been termed AAA1 and AAA2 loci in the Online Mendelian Inheritance in Man (OMIM) database [5]. Both AAA susceptibility loci were further confirmed using DNA linkage analyses approaches in different populations [6, 7]. One of the genes located within the AAA1 locus encodes kallikrein 1 (KLK1) [8]. Kallikrein 1 is a serine protease which converts low molecular weight kininogen to Lys-bradykinin, a peptide which has a range of biological actions relevant to AAA such as promotion of inflammation [9, 10, 11]. There are two chief receptors stimulated by kinins, namely B1 and B2. Deficiency of the B1 kinin receptor has been associated with increased risk of AAA development in a mouse model in which aneurysm was induced by angiotensin II infusion [12]. Single nucleotide polymorphisms (SNPs) in *KLK1* have previously been associated with intracranial aneurysm [13] but not been assessed for association with AAA.

We selected rs5516 (g.23591691C>G) located within exon 3 of the *KLK1* gene for study on the basis of its involvement in exonic splicing enhancer (ESE) activity [14]. During this process a mature mRNA is formed and sequence variations within ESE sites may alter the relative expression of mRNA isoforms i.e. the relative types of splice variants [15]. The *KLK1* gene has been shown to express two alternatively spliced variants: intron 3 spliced (short) and intron 3 retained (long) isoforms [16]. The aim of the present study was to assess

the association of rs5516 SNP with AAA within two separate case-control series imaged for AAA. We also postulated that alternative splicing variants of *KLK1* could be associated with AAA and sought evidence to support this theory within aortic biopsies.

MATERIAL AND METHODS

Patients

In order to assess the association of the rs5516 SNP with AAA we examined two case-control groups: a) 609 men with AAA and 695 without AAA from the Western Australian Health In Men Study (HIMS) [17, 18]; b) 225 patients with AAA and 100 subjects who had symptoms of lower limb athero-thrombosis but no AAA from Australia's north east state of Queensland [19, 20]. HIMS subjects had undergone abdominal ultrasound, while Queensland subjects were imaged by computed tomography angiography (CTA). The reproducibility of ultrasound was assessed during subject recruitment and 95% confidence intervals were < 3mm [17]. Maximum axial infrarenal aortic diameter in Queensland subjects was assessed using the CTA viewer function on the Philips workstation (MxView Visualization Workstation Software, Philips Electronics Australia). Maximum diameter was recorded in millimeters to the nearest 0.1 mm [19, 20]. Small AAAs were defined as a maximum infrarenal aortic diameter measuring 30 to 49mm and large AAAs as those ≥ 50mm. Controls had maximum infrarenal aortic diameter ≤ 25mm. The definitions of risk factors such as hypertension, dyslipidemia, diabetes, coronary heart disease (CHD) and smoking were as previously described [21].

Aortic wall biopsies were collected in RNAlater® solution (Ambion) from a group of 12 men undergoing open AAA repair and 6 organ donors in Queensland, Australia. AAA samples were obtained from the body of the aneurysm (site of maximum dilatation). Donor abdominal aortic tissues were used as controls. Total RNA was extracted using RNeasy®

Mini Kit (Qiagen) according to manufacturer's instructions. Ethical approval was granted from the relevant committees.

Genotyping

The human kallikrein 1 is a highly polymorphic gene with more than 120 single nucleotide polymorphisms (SNPs) including seven missense variations [22]. Among these SNPs rs5516 was selected for genotyping because of its high level of heterozygosity (0.464) in Caucasians [22] allowing detection of all three possible genotypes in relatively small numbers of individuals. This SNP has been reported to modify the gene expression regulatory sequences in exon 3 of the *KLK1* gene. Genotyping of the HIMS and Queensland subjects was carried out at the Australian Genome Research Facility, University of Queensland, using the Sequenom's MassARRAY system that utilizes a homogenous MassExtend (hME – single base extension) reaction termed iPLEX GOLD. Genotype calls were made using SpectroTYPER™ RT software (Sequenom Inc., San Diego, CA, USA). Genotyping efficiency of rs5516 was 97% and 98% for HIMS and Queensland groups, respectively.

Tissue expression

Quantitative real-time PCR (qPCR) was performed for both short and long splice variants of the *KLK1* mRNA. The relative expression of these isoforms 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 similar in aortic biopsies from AAA patients and organ donors. 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 RASE (Real-time PCR Annotation of Splicing Events) tool [23] for both *KLK1* short variant (5'-ATGCTGTGAAGGTCGTGGAGTT-3' and 5'- CACTGGAGATCATCTGGAAATGAGAAA-3', reference sequence NM_002257)

and *KLK1* long variant (5'-GGTTCGTAGTCTCATTTCC-3' and 5'-AAGTCTGTCACCTTCTGG-3', reference sequence AY429508). Qiagen's QT01192646 QuantiTect® Primer Assay was used to assess GAPDH expression.

Statistical analyses

Univariate association of continuous and nominal covariates with AAA was examined by Mann–Whitney U test and Fisher exact test, respectively. Our primary binary outcome was the presence of small or large AAAs. The secondary quantitative outcome was maximum infrarenal aortic diameter. Logistic regression was used to model the effects of multiple covariates on binary and quantitative outcomes under dominant and recessive models. Hypertension, coronary heart disease, dyslipidemia, smoking and gender (Queensland group only), in addition to rs5516 genotype, were examined for inclusion in the model. Forward stepwise variable selection procedures were performed where only significant covariates were retained in the models. A dominant model measured differences between rs5516 CC homozygotes and G allele carriers, while a recessive model compared GG homozygotes with C allele carriers. All computations were undertaken using SimHap v1.0.2 [24]. Hardy-Weinberg equilibrium was tested using HPlus v3.1 [25]. Results from the HIMS and Queensland groups were meta-analyzed by Comprehensive Meta Analysis v2 (Biostat, Inc.). Combined odds ratio (OR) and standardized mean difference (SMD) in maximum aortic diameter were determined using fixed effects models. SMD represents the difference in means between two groups divided by the pooled standard deviation of the measurements [26]. Mann–Whitney U test using the PASW Statistics 18 Release 18.0.0 package was performed to identify differences between *KLK1* short and long splice variants expression levels. Statistical significance was defined at the conventional 5% level.

Power calculation

The available sample sizes had an 80% power to detect an effect size (OR) of approximately 1.5 and 2.3 for the association of the risk allele with small AAA in the HIMS and Queensland subjects, respectively. The available sample sizes had an 80% power to detect an OR of approximately 4.2 and 3.0 for the association of the risk allele with large AAA in the HIMS and Queensland subjects, respectively. Alpha was assumed to be 0.05. Calculations were performed by using the PS: Power and Sample Size Calculation v3.0 software [27].

RESULTS

HIMS subjects

Genotyping was carried out in 1304 HIMS subjects of whom 19 (2%) had large and 590 (45%) had small AAAs (Table 1). Genotype distribution of rs5516 passed testing for Hardy-Weinberg equilibrium (P=0.1178) in controls and therefore was assessed for association with AAA and aortic diameter. Multivariate analysis showed that the rs5516 minor (G) allele was significantly associated with large but not small AAA with OR=2.97, P=0.044, under a dominant model (Table 2). Rs5516 was also significantly associated with aortic diameter in HIMS men with large AAA (Table 3). The effect was seen under a recessive model where minor allele GG homozygotes had significantly larger mean aortic diameter (about 19mm greater, P=0.002) compared with C allele carriers.

Queensland subjects

Genotyping was performed on 325 Queensland subject of whom 60 (18%) had large AAAs, 165 (51%) had small AAAs, and 100 (31%) no AAAs (Table 1). The genotype frequencies of rs5516 were consistent with Hardy-Weinberg equilibrium (P=0.5128) in controls and therefore we assessed the association of rs5516 with AAA and aortic diameter.

There was a significant association between GG genotype and large AAA (OR=4.20, P=0.024, Table 2). Rs5516 was not associated with small AAAs. Rs5516 was also significantly associated with aortic diameter in Queensland subjects with large AAA (Table 3). Individuals with GG genotype had significantly larger mean aortic diameter (about 11mm greater, P=0.009) compared with C allele carriers.

Meta-analysis

Data was combined from both case-control studies under dominant and recessive models (Table 4). Under a recessive model the G allele was associated with large AAA at borderline significance (OR 2.4, P=0.056). Under a similar recessive model the G allele was strongly associated with bigger diameter large AAAs (standardized mean difference 0.59, P<0.001) (Table 4).

Aortic expression of KLK1 splice variants

The relative expression of both short and long variants of *KLK1* mRNA was markedly increased in biopsies from AAAs compared to those from normal abdominal aortas (Table 5). The ratio of short and long mRNAs in the aortic biopsies from the two subject groups was also markedly different. In subjects with AAA the short variant of *KLK1* mRNA was expressed 5-fold more commonly than the long variant. In contrast in the biopsies from organ donors the long variant of *KLK1* mRNA was expressed 10-fold more commonly than the short variant (Table 5).

DISCUSSION

The main findings of this study were that the G allele of *KLK1* rs5516 polymorphism was associated with large but not small AAAs after adjusting for other risk factors in both

groups examined. In the patients with large AAAs the G allele was also associated with the size of the AAA. These findings suggest that changes in KLK1 activity could be relevant to later stage AAA progression.

Previous studies have suggested that aortic diameter is an important predictor of the natural history for rupture in AAAs. For small AAAs (40-55mm) it is estimated that the rupture rate is approximately 1% per year, while for AAAs of 70mm or more the rupture rate is estimated to be approximately 30% per year [28, 29]. It has been suggested that the evolution of AAA occurs in a number of stages including initiation, enlargement and rupture. Progressive aortic wall dilatation is associated with cycles of extracellular matrix degradation [30] and inflammation, involving proteolytic enzymes produced by invading inflammatory cells [31, 2]. Our findings suggest a role of the *KLK1* gene in large but not small AAA. Larger AAAs have some distinct features, such as a greater volume of intraluminal thrombus which contains cytokines, neutrophils, proteolytic enzymes and platelets [32, 33]. Kininogen has been demonstrated to bind to the surface of the cells contained within AAA thrombus, such as platelets, which bring substrate for tissue kallikrein expressed by infiltrating inflammatory cells, such as neutrophils. Kininogen has also been demonstrated within endothelial cells prevalent in areas of neovascularisation typically found in the wall of AAA [34]. Kinins released by kallikrein can then promote further inflammation which is believed critical in AAA progression [11]. Inhibition of platelet aggregation limits AAA progression and neutrophil accumulation within a guinea pig to rat aortic transplant AAA model and could potentially act via changes in kinins [35].

Previous genome-wide screening studies suggested that a locus on chromosome 19q13 was linked with AAA [4-7]. *KLK1* is one of more than a hundred genes located in this region and has been examined in this study. A number of other genes encoded within this region could be associated with AAA. In a recent study, 615 tagging SNPs within

chromosome 19q13 that also included the *KLK1* locus were assessed in 1024 Dutch subjects [36]. In this series, however, Baas and colleagues reported that none of these SNPs showed any evidence of association with AAA. A number of differences exist between the subjects examined in the study of Baas and colleagues and the current investigation. In the current study in the independent populations examined cases and controls were recruited in the same way, i.e. from out-patient vascular clinics in Queensland and by population screening in Western Australia. All subjects were imaged and similar risk factor data recorded. In contrast in the Dutch study the control group was mainly recruited separately from blood donors who did not undergo imaging and risk factor assessment and were generally much younger than the AAA cases. Some of these differences may explain the disparate results reported.

The current study also provides preliminary evidence for an association of the short *KLK1* splice variants with AAA. Splicing requires both a recognition site and regulatory sequences such as exonic splicing silencers (ESSs) and enhancers (ESEs) that regulate accurate splicing of the pre-mRNA into messenger RNA (mRNA). The *KLK1* gene has two alternative transcripts due to the retention of its third intron [16]. These include a classical short variant and the long isoform in which intron 3 is retained. Both the short and long isoforms were upregulated in AAA biopsies however the short variant was expressed at 5-fold greater levels. In contrast, in biopsies of organ donors, the predominant *KLK1* isoform was the long form. It is possible that rs5516 influences the balance between splicing and retention of the *KLK1* third intron, as this SNP is located within its ESE site.

The current study has a number of limitations. We included well phenotyped individuals in which all subjects were imaged for AAA presence, however detailed imaging of coronary or cerebrovascular arteries was not performed. Risk factor information was collected by standardized questionnaires and adjusted for in the multivariate analyses. The sample sizes available were however relatively small, particularly with respect to the number

of subjects with large AAAs. The high odds ratios identified in this study are not typical of those expected for genetic risk alleles for multigenic complex diseases [4, 6, 7]. Previous studies for example have reported odds ratios for the association of a locus on chromosome 9p21.3 with AAA of approximately 1.4 [37, 38]. Also our findings were not completely consistent in both groups assessed. In the HIMS subjects the association between rs5516 and large AAA was demonstrated in a dominant model, while in the Queensland subjects this association was only found using a recessive model. Further studies in other populations are required to confirm our findings.

In conclusion, to our knowledge this is the first published report examining the association of a functional SNP in *KLK1* gene with AAA. Our findings suggest that rs5516 is associated with large but not small AAA. The findings highlight the possibility that distinct genetic mechanisms may contribute to the risks of AAA initiation and progression. Further large studies are required to provide more definitive evidence.

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Table 1: Characteristics of subjects undergoing *KLK1* genotyping

Subjects	**HIMS**			**Queensland**		
Characteristic	**Large AAA**	**Small AAA**	**No AAA**	**Large AAA**	**Small AAA**	**No AAA**
Number	19	590	695	60	165	100
Aortic diameter (mm)	58.7±9.9	35.4±4.83	21.2±1.9	60.5±11.8	38.5±5.5	19.9±3.2
Women	0	0	0	7 (12%)	39 (24%)	36 (36%)
Age (years)	73.2±4.4	73.3±4.4	73.1±4.4	72.7±6.2*	71.7±8.0*	65.4±10.3
Hypertension	12 (63%)*	315 (53%)*	263 (38%)	40 (68%)	121 (73%)	68 (68%)
Diabetes mellitus	1 (5%)	62 (11%)*	49 (7%)	10 (17%)*	31 (19%)*	35 (35%)
Dyslipidemia	5 (26%)	265 (45%)*	231 (33%)	29 (49%)	114 (70%)*	53 (53%)
Coronary heart disease	11 (58%)*	233 (40%)*	123 (18%)	35 (58%)*	93 (56%)*	37 (37%)
Ever smoker	17 (89%)*	504 (85%)*	431 (62%)	45 (76%)	137 (83%)	74 (74%)

Nominal variables are presented as numbers; continuous variables are presented as mean ± standard deviation. Continuous variables were compared between subjects with large or small AAA and controls using Mann Whitney U test (*$p<0.05$); Nominal variables were compared between subjects with large or small AAA and controls using Fisher exact test (*$p<0.05$).

Table 2: Multivariate association of rs5516 with AAA

Group	AAA	Genotype	N		Dominant Model				Recessive Model			
			AAA	No AAA	OR	95% CI	P	AIC	OR	95% CI	P	AIC
HIMS	Small AAA	CC	291	341	-	-	-	1440	-	-	-	1439
		CG	242	279	1.03	0.80-1.32	0.814	NS				NS
		GG	57	75					0.86	0.57-1.29	0.460	
	Large AAA	CC	5	341	-	-	-	158	-	-	-	162
		CG	11	279	2.97	1.03-8.58	0.044	0.032				NS
		GG	3	75					1.31	0.36-4.77	0.687	
Queensland	Small AAA	CC	63	52	-	-	-	339	-	-	-	342
		CG	89	42	1.70	1.01-2.87	0.047	NS				NS
		GG	13	6					1.65	0.59-4.64	0.340	
	Large AAA	CC	31	52	-	-	-	202	-	-	-	197
		CG	20	42	1.12	0.56-2.22	0.747	NS				0.019
		GG	9	6					4.20	1.21-14.54	0.024	

N, number of individuals; CI, confidence intervals; AIC, Akaike's information criterion; NS, Non-significant model.

The association between rs5516 with AAA was adjusted for hypertension, coronary heart disease, dyslipidemia, and smoking in the HIMS group and for gender, hypertension, coronary heart disease, dyslipidemia and smoking in the Queensland group.

Table 3: Multivariate association of rs5516 with aortic diameter

Group		Genotype	Frequency	Dominant Model					Recessive Model				
				Coefficient	SE	P	Mean	AIC	Coefficient	SE	P	Mean	AIC
HIMS	Control	CC	0.491	-	-	-	21.2	2845	-	-	-	21.2	2845
		CG	0.401	0.0524	0.142	0.712	21.3	NS					NS
		GG	0.108						0.0306	0.228	0.894	21.2	
	Small AAA	CC	0.493	-	-	-	35.3	3542	-	-	-	35.3	3542
		CG	0.410	0.0393	0.399	0.921	35.3	NS					NS
		GG	0.097						0.1470	0.678	0.828	35.4	
	Large AAA	CC	0.263	-	-	-	61.3	150	-	-	-	57.6	136
		CG	0.579	-0.9267	5.967	0.879	60.4	NS					0.0002
		GG	0.158						19.2290	5.056	0.002	76.8	
Queensland	Control	CC	0.520	-	-	-	20.0	491	-	-	-	19.9	491
		CG	0.420	-0.2468	0.577	0.670	19.8	NS					NS
		GG	0.060						0.1069	1.230	0.931	20.0	
	Small	CC	0.382	-	-	-	38.7	1042	-	-	-	38.5	1042

	AAA	CG	0.539	-0.4273	0.900	0.636	38.3	NS					NS
		GG	0.079						-1.0990	1.624	0.499	37.4	
	Large AAA	CC	0.517	-	-	-	58.3	467 NS	-	-	-	58.7	462 0.0058
		CG	0.333	4.4692	3.159	0.163	62.8						
		GG	0.150						11.3066	4.184	0.009	70.0	

SE, standard error; Mean, mean aortic diameter in mm; AIC, Akaike's information criterion; NS, non-significant model.

The association between rs5516 with aortic diameter was adjusted for hypertension, coronary heart disease, dyslipidemia and smoking in the HIMS group and for gender, hypertension, coronary heart disease, dyslipidemia and smoking in the Queensland group.

Table 4: Meta-analysis assessing the association of the G allele of rs5516 with large AAA and aortic diameter

Group	Dominant model							Recessive model						
	Large AAA			Aortic diameter of large AAAs				Large AAA			Aortic diameter of large AAAs			
	OR	95% CI	P	SMD	SE	95% CI	P	OR	95% CI	P	SMD	SE	95% CI	P
HIMS	2.97	1.03-8.58	0.044	-0.04	0.23	-0.49-0.42	0.877	1.31	0.36-4.77	0.687	0.88	0.23	0.43-1.34	<0.001
Queensland	1.12	0.56-2.22	0.747	0.23	0.16	-0.09-0.55	0.158	4.20	1.21-14.54	0.024	0.44	0.17	0.12-0.77	0.008
Combined	1.50	0.84-2.67	0.172	0.14	0.13	-0.12-0.41	0.288	2.40	0.98-5.88	0.056	0.59	0.14	0.33-0.85	<0.001

OR, odds ratio; CI, 95% confidence intervals; SMD, standardized mean difference; SE, standard error.

Table 5: Relative expressions of *KLK1* splice variants in 12 AAA and 6 control biopsies.

KLK1 variant	Sample	N	Median	IQR	P
Short	AAA	12	1.928	1.194-2.262	0.001
	No AAA	6	0.007	0.006-0.010	
Long	AAA	12	0.238	0.132-0.793	0.021
	No AAA	6	0.062	0.046-0.098	
Short to Long ratio	AAA	12	5.110	3.224-16.140	0.002
	No AAA	6	0.133	0.054-0.149	

N, number; IQR, interquartile range; P, two-sided P-value