An increased frequency of the 5A allele in the promoter region of the MMP3 gene is associated with abdominal aortic aneurysms

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Matrix metalloproteinase 3 (MMP3), is over expressed in the wall of abdominal aortic aneurysms (AAA), while inactivation of the gene expressing this enzyme is associated with reduced aneurysm formation in an experimental model. The 5A allele of the 5A/6A polymorphism in the promoter region of the MMP3 gene is associated with enhanced MMP3 expression. This study aimed to determine whether the presence of the 5A allele in the MMP3 promoter is a risk factor for AAA, and if this allele is associated with an increased expression of MMP3 in the aneurysm wall. We compared the frequencies of the 5A and 6A alleles in AAA (n = 405), aortic occlusive disease (AOD) (n = 123) and controls (n = 405). The 5A allele frequency was higher in AAA compared with controls (odds ratio – OR 1.32, P = 0.005) and AOD (OR 1.684, P = 0.0004), but was similar in AOD compared to controls (OR 0.78, P = 0.1). The ORs of the 5A/6A and the 5A/5A genotypes were 1.35 and 1.79, compared with 6A homozygotes. Although wall from 5A homozygotes contained 17% more MMP3 mRNA than homozygotes (P = 0.049) the significance of this was lost when adjusted for age and sex (P = 0.069), and size (P = 0.30). Wall from 5A homozygotes did however contain over 45% more MMP3 protein than heterozygotes (P = 0.009 when corrected for age and sex and P = 0.043 when corrected for aneurysm size). It appears that an abnormality in the MMP3 gene is part of the genetic profile that predisposes to aneurysmal disease.

INTRODUCTION

Abdominal aortic aneurysm (AAA) is a common condition with an estimated prevalence of 20–40 cases per 100 000 population, accounting for 1–2% of all deaths in the UK. This rises to 2.2% of deaths of men aged 70–75 and is responsible for 5% of all unexpected deaths of men over the age of 55. The incidence of AAA has been steadily increasing for the last three decades (1).

Aneurysm expansion is characterized by atherosclerosis, inflammation, loss of elastin and increased turnover of collagen in the aortic wall (2–4), but the pathogenesis of the disease is still not fully understood. Monogenic diseases that have been associated with aortic aneurysm and dissections (TAAD) include Marfan’s syndrome, commonly caused by mutations in the fibrillin 1 gene (5,6). The abnormal fibrillin may weaken the structure of the aortic wall or affect the signalling of transforming growth factor beta (TGFβ, 7), which is an important mediator of tissue remodelling. Mutations in the TGFβ receptors type 1 or 2 can cause familial thoracic aortic aneurysm with dissection (8), although other genes for this condition remain to be identified. Pseudoxanthoma elasticum (9,10), autosomal dominant cutis laxa (caused by mutations in the elastin gene, 11) and autosomal recessive cutis laxa (caused by fibrillin-4 gene mutations, 12), have also been associated with aortic aneurysm. These conditions are, however, most often associated with aneurysmal dilata-

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tion of the ascending aorta. Ehlers Danlos syndrome type IV in which there are mutations in the COL3A1 gene, has been associated with a variety of disorders of the large blood vessels, but usually causes vascular rupture rather than aneurysm development.

There is an approximately seven-fold increase in the prevalence of aortic aneurysms in the male siblings of affected individuals compared with controls and an 18-fold risk of AAA in male siblings of affected individuals aged over 60 years (14,15). A monogenic disorder resulting in AAA has not been found, but it is plausible that an inherited predisposition to ‘sporadic’ AAA may exist.

Variant genes encoding the proteins involved in extracellular matrix turnover, including matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) have been considered strong candidates for a genetic susceptibility to AAA. MMPs are a family of endopeptidases that are produced by a wide range of cell types found in the aneurysm wall including inflammatory cells, fibroblasts and smooth muscle cells. MMPs degrade the components of extracellular matrix, including elastin and collagen, and have been implicated in the proteolysis that accompanies aneurysm expansion (16–20). The amount of MMP activity in tissue can be regulated at the transcriptional level, by pro-enzyme activation and by naturally occurring TIMPs of metalloproteinases (21,22). We have previously shown that the levels of stromelysin-1 (MMP3) mRNA are over two orders of magnitude higher in the aneurysm wall compared with the wall from aortic occlusive disease (AOD, 23) which is more than any of 14 other MMPs measured in the same samples.

The gene coding for MMP3 is located on the long arm of chromosome-11 (24) and consists of 10 exons, although there may be other shorter splice forms in addition to its full length transcript. A large number of polymorphisms have been reported in this gene, but the 5A/6A polymorphism in the promoter region (1612 base pairs upstream from exon 1) has been most extensively investigated as this polymorphism increases the activity of the MMP3 promoter (25). A higher frequency of the 5A allele was found in patients with AAA in one small Finnish study although the comparison with controls failed to achieve significance after correction for multiple testing (26). The homozygous 5A genotype of MMP3 and the 5A allele were more frequent in patients with aneurysmal coronary artery disease than in those with coronary occlusive disease (27).

The aim of this study was to determine whether the presence of the 5A allele in the MMP3 promoter was a risk factor for AAA, and to establish whether this promoter polymorphism affects gene expression in the aneurysm wall. The frequency of the 5A and 6A allele was also compared in patients with AAA and AOD to investigate whether the altered expression of MMP3 may be a determinant of whether an aneurysm develops in the presence of atheroma.

RESULTS

Patient demographics

The demographics for patients with AAA and AOD are presented in Table 1. The male to female ratio of ~5:1 was presented in Table 1. The male to female ratio of ~5:1 was significantly different between the AAA and AOD groups.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total</th>
<th>AAA</th>
<th>AOD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A/5A</td>
<td>405</td>
<td>21</td>
<td>21</td>
<td>94</td>
</tr>
<tr>
<td>5A/6A</td>
<td>123</td>
<td>21</td>
<td>17.1%</td>
<td></td>
</tr>
<tr>
<td>5A/5A</td>
<td>208</td>
<td>64</td>
<td>52.0%</td>
<td></td>
</tr>
<tr>
<td>6A/6A</td>
<td>74</td>
<td>38</td>
<td>30.9%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>405</td>
<td>123</td>
<td>101</td>
<td></td>
</tr>
</tbody>
</table>

Association of the presence of the 5A allele with AAA

The frequency of the 5A allele was significantly higher in patients with AAA compared with both the control population [RR 1.15 (95% CI 1.04–1.27), OR 1.32 (95% CI 1.09–1.61), P = 0.0053, with a power of 81%], and patients with AOD [RR 1.13 (95% CI 1.06–1.21), OR 1.68 (95% CI 1.26–2.25), P = 0.0004, Table 2]. Conversely, the frequency of the 6A allele was highest in patients with AOD than in patients with AAA. The frequency of the 5A allele was not significantly higher [OR 0.78 (95% CI 0.59–1.05), P = 0.097] between the AOD and the control population.

The ORs of the 5A/6A and the 5A/5A genotypes compared with the 6A homozygotes were 1.35 and 1.79, respectively.

Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Age category</th>
<th>AAA</th>
<th>AOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-54</td>
<td>123</td>
<td>101</td>
</tr>
<tr>
<td>55-64</td>
<td>21</td>
<td>21</td>
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<tr>
<td>65-74</td>
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<td>21</td>
</tr>
<tr>
<td>75 and over</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 2. Genotype and allelic frequencies, in patients with the AAA, AOD and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Total</th>
<th>AAA</th>
<th>AOD</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>5A</td>
<td>454</td>
<td>106</td>
<td>43.1%</td>
<td></td>
</tr>
<tr>
<td>6A</td>
<td>356</td>
<td>140</td>
<td>36.6%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>810</td>
<td>246</td>
<td>30.4%</td>
<td></td>
</tr>
</tbody>
</table>

**P < 0.01.**

***P < 0.001.***
Comparison of MMP3 gene expression in 5A and 6A homozygotes and heterozygotes

There was no difference in aneurysm diameter between the 5A/5A (6.4 ± 0.24 cm) and 5A/6A (6.2 ± 0.14 cm, \( P = 0.38 \)) groups. The two 6A/6A aneurysms had diameters of 6.0 and 5.9 cm. There was also no correlation between AAA diameter and MMP3 protein expression (\( r = 0.14, P = 0.43 \)). The mean ages of the 5A/5A genotypes (73.9 ± 2.3 years) and the 5A/6A genotypes (70.6 ± 1.4 years) were not different (\( P = 0.2 \)). The ages of the two 6A/6A genotypes were 62 and 78 years. The proportion of males to females in each group (5A/5A – 0F/17M; 5A/6A – 5F/32M) was also not significantly different (\( P > 0.05 \), Fisher’s exact test).

### Protein

Significantly greater amounts of MMP3 protein were present in the aneurysm wall of homozygous 5A patients (344 ± 36 ng/mg soluble protein) than in the aneurysm wall of 5A/6A heterozygotes (235 ± 22 ng/mg soluble protein, \( P = 0.015 \), Fig. 1). MMP3 protein concentration in the two patients with the 6A/6A genotype was 190 and 170 ng/mg soluble protein. The association between MMP3 protein concentration levels and genotype were robust to correction in a linear model with age and sex (\( P = 0.009 \)), but the significance was reduced when aneurysm diameter was included in the model (\( P = 0.043 \)). This is likely to be a consequence of the missing data on size (10/52 individuals), as aneurysm diameter made no significant contribution to the MMP3 levels (\( P = 0.63 \)).

### RNA

Agilent® analysis (not shown) showed that good quality mRNA was extracted from all the samples. More MMP3-cDNA copies were detected in aneurysmal tissue from 5A homozygous patients (3.23 ± 0.14) than in heterozygotes (2.75 ± 0.19, \( P = 0.049 \), Fig. 2). The significance of this was however lost when correction was made for age and sex (\( P = 0.069 \)) and AAA size (\( P = 0.30 \)) using the linear model. Tissue was obtained from only two patients with the 6A/6A genotype. The ratios of MMP3 cDNA to β-2-microglobulin cDNA in these two patients were 2.85 and 2.49.

**DISCUSSION**

This study shows that the 5A allele in the promoter region of the MMP3 gene occurs more frequently in AAA patients compared with both the control population and with patients who have AOD. This increased frequency of the transcriptionally more active 5A-promoter supports the non-significant trend shown by the much smaller Finnish study (26). The difference in the 5A/6A allele frequencies between the Finnish control (0.47) and the Southeast English populations (0.49) may represent a racial difference, but may also have occurred because of the relatively small sample size of the Finnish control cohort studied. The frequencies of the 5A and 6A alleles in our control population are consistent with published MMP3 control population data for South England (28). Comparison of 5A-allele frequencies in mixed Canadian and Belgian cohorts failed to show a significant difference between AAA and controls (29). The 5A allele frequency in controls from this study (0.49) also differed from Canadian and Belgian control populations (0.53 and 0.48, respectively).

The strengths of the present study are that all the patient and control groups were of similar ethnicity (white) and from one geographical region of the UK. Also the power calculation was based on the actual genetic data from a pilot study for this same region. Controls were not, however, specifically matched for environmental risk factors for aortic aneurysm, as we were comparing allele frequency in cases, to allele frequencies in the population from which they came. Selection of controls by specific phenotypes (such as smoker/non-smoker) would in itself risk introducing bias, and would be irrelevant, as we were not screening controls to exclude aneurysms. The expected incidence of aortic aneurysms in the control population is ~4%. These were not excluded from our analysis as we felt that their exclusion would bias towards a significant result. The patients recruited in our AAA group had larger aneurysms compared with other studies who recruited patients with an aortic diameter of ≥3.0 cm. The patients with AOD all had evidence of occlusive aortic disease rather than occlusive peripheral vascular disease. All control AAA and AOD patients...
came from the same population (whites from Southeast England). In the absence of genomic controls, it is not possible to be absolutely sure that there is no population stratification that could lead to false positives, although stratification is always difficult to control.

Epidemiological studies have shown that a family history is a recognized risk factor for aneurysm development (15,30). Sixteen percent of the AAA patients in this study had at least one member of the family affected, usually a brother or a father. Other studies have also shown an inverse association between diabetes and AAA (31). Significantly fewer patients with diabetes were present in the aneurysm group.

This study also shows that there is a link between the 5A/5A genotype and MMP3 gene expression. The aortic aneurysmal wall of patients, who were homozygous for the 5A-allele, contained greater quantities (>45%) of MMP3 protein than the wall from 5A/6A heterozygotes; suggesting that the 5A-allele causes greater MMP3 production in these patients. Although we also found that levels of MMP3 mRNA were higher in the aneurysm wall, the significance of this finding was lost when the data were corrected for age, sex and aneurysm size. This may have been the result of missing data (~20% of our data set in this analysis). Discrepancies between mRNA and protein are, however, not an unusual occurrence. Transcription is only one level of regulation in the cell; genes are therefore frequently not expressed in direct proportion to their level of mRNA (32–34). Our protein data do however, confirm the in vitro experiments, in which transient transfection of cells using an MMP3 promoter/reporter gene construct (containing the 5A-allele at the polymorphic site) led to greater expression of the reporter gene (as measured by increased reporter activity) than a similar construct containing the 6A allele (25). The increasing ORs of the 5A/6A and the 5A/5A genotypes, compared with the 6A homozygotes, also suggest that the risk of AAA increases with each copy of the 5A allele carried.

The relationship between genotype (or allelic frequency) and MMP3 gene expression was only investigated in samples from the aneurysm wall as it was felt that as AAA is a localized condition, the relatively high concentrations in the wall would be more relevant to disease progression. The number of patients in which aortic aneurysmal tissue could be obtained for mRNA and protein analysis was also considerably less than the AAA patients recruited for genotyping. This was because tissue was only available from patients having open surgery and not from those recruited at post-operative follow-up visit, or from patients having an endovascular repair of their aneurysm (UKSAT, 30).

Studies in ApoE deficient mice have shown that inactivation of the MMP3 gene reduces aneurysm formation, but not atherosclerosis (35). Even in an experimental setting, however, it would be difficult to assess the effect of the modest over-expression of MMP3, found in this study, on matrix turnover in the aortic wall, as a controlled, defined chronic over-expression of MMP3, such as that observed in the aneurysm wall in man, would be hard to achieve.

MMP3 is expressed by the mononuclear infiltrate (probably macrophages) present in the aneurysm wall (36) and digests collagen and other extracellular matrix proteins, which are important in maintaining the structural integrity of the aortic wall. It also plays a central role in the pericellular activation of other MMP proenzymes, including interstitial collagenase (MMP1, 37), matrilysin (MMP7, 38) and gelatinases A (MMP9, 39), which have previously been implicated in aneurysm expansion (16–20,40). It seems plausible, that increased production of MMP3 might weaken the aortic wall and cause further proteolysis by activating latent, constitutively produced metalloproteinases. The cause of the increased MMP3 expression in certain individuals prone to aneurysm formation is not known, but a combination of genetic and environmental factors may be responsible.

The data presented show that there is an association between AAA and the 5A allele of the MMP3 gene. The 5A/6A polymorphism may, however, be in linkage disequilibrium with other polymorphisms in the gene, but the functional studies suggest a difference in promoter activity can be caused directly by this polymorphism. Taken together, these data provide strong evidence for the contribution of the 5A allele in the promoter region of the MMP3 gene to an inherited predisposition to AAA rather than AOD. Our data is confined to an analysis of a single gene, while aortic aneurysm predisposition is almost certainly polygenetic. It would appear that an abnormality in the MMP3 gene is part of this genetic profile.

The genotypic difference between AAA and AOD might explain why these vascular diseases share common risk factors, but result in different pathologies. The MMP3 gene might provide a useful target for future pharmaco-modulation aimed at preventing aneurysm expansion.

MATERIALS AND METHODS

Patients

Patients with AAA (n = 405) and AOD (n = 123) were identified from the vascular outpatient clinics at St Thomas’ Hospital, London, UK, a tertiary referral centre for vascular disorders. The anatomy and the dimensions of aneurysms were analysed by ultrasonography or by contrast enhanced computed tomography scans. The mean maximum diameter of the aneurysms was 6.4 cm (range 4.5–11.2 cm). Symptoms of occlusive disease were investigated by Doppler ultrasonography or angiography. Only patients with radiographic evidence of an aortic occlusion were included in the AOD cohort of this study. St Thomas’ Hospital research and ethics committee approved a pilot genotype study on patients with AAA compared with healthy subjects from Southeast England. DNA patients with AAA and AOD were compared with DNA from a control sample population (n = 405, white from Southeast England) representing all subjects over 50 in the general population (obtained from the Department of Medical and Molecular Genetics, Guy’s Hospital, King’s College London). Four percent (approximately) of this control population would be expected to have AAA. These were not excluded from the final analysis.

Genotyping

Blood was taken from consented patients and stored at –20 °C in EDTA tubes (Vacutainer, Becton-Dickinson). DNA was
extracted from the white-cell pellet obtained from blood, using a modified salt-extraction method (41). The promoter region of interest was amplified by a polymerase chain reaction (PCR) in a 96-well plate (Thermosprint, Bilatec AG) using a thermocycler (Genius, Techne, UK). Genomic DNA (200–800 ng) was amplified in a 20 μl reaction containing: 2 μl of 10X Buffer with 15 mM MgCl₂, (Taq DNA Polymerase buffer Promega); 0.4 μl dNTPs (10 mM, Promega); 1 unit of Taq DNA Polymerase (Promega); and 40 ng of forward (5’T(5 ′FAM)-GATTACAGACATGGGTCACG-3’) and reverse primers (5′-GAATTCACTACCTGGCACC-3’, Sigma-Genosys Ltd). The following PCR cycling conditions were used: denaturation at 95°C for 2 min followed by 25 cycles of 95°C for 40 s; 55°C for 40 s; 72°C for 1.5 min; and a final extension step of 72°C for 5 min. The fluorescent PCR product (1 μl) was added to a 15 μl master mixture consisting of HiDi formamide (250 μl, Applied Biosystems) Genescan-350 Rox Standard (6 μl, Applied Biosystems) and blue-dextran loading buffer (10 μl, Applied Biosystems). This mixture was denatured at 98°C for 2 min and the plates cooled on ice to stop the denaturing process. The samples were analysed on an ABI3100 Prism analyser (Applied Biosystems) and electrophoresed using a POP-6 polymer at 15 kV at 60°C. The pattern of fluorescent peaks was analysed using Genotyper 3.7 software to determine the genotype (5A/5A, 5A/6A and 6A/6A, Fig. 3). Single peaks at 180 and 181 bp represents the 5A and 6A homozygote respectively.

Aortic tissue
Aortic tissue was obtained intraoperatively, from consented patients undergoing open repair of their aneurysm or bypass of their occluded aorta. The aortic specimen was immediately cut into 3 mm cubes, snap-frozen in liquid nitrogen and stored at −80°C for extraction of mRNA and protein. The snap-frozen aortic tissue (0.1–0.2 g) was pulverized using a reciprocating ball and cup Microdismembrator (Braun, Germany) before RNA or protein were extracted.

MMP3 messenger RNA
Total RNA was extracted from the pulverized tissue using 1 ml of Trizol, 0.2 ml Chloroform, 0.5 ml Isopropanol and high-speed centrifugation. The RNA was washed in 75% ethanol and stored in nuclease-free water at −20°C. The integrity and concentration of the RNA was analysed using the RNA 600 Nano Assay protocol on an Agilent 2100 Bioanalyzer. The cDNA was synthesized in a reverse transcription reaction using Superscript™ First—Strand Synthesis System for RT—PCR (Invitrogen Life Technologies) according to the supplied protocol. The resulting cDNA reaction was then amplified by real-time PCR using a TaqMan® MMP3 Genomic Assay (HS00233962_m1, Applied Biosystems).

Figure 3. Chromatogram identifying PCR products for 5A (red) and 6A (blue) allele genotypes in the MMP3 promoter.

MMP3 protein
MMP3 was extracted from pulverized tissue using a protein extraction buffer containing 0.1% Triton X-100 and 0.2 ml protease inhibitor cocktail (Set III, Calbiochem) in PBS pH 7.4. The resultant homogenates were centrifuged for 15 min at 15 000 g and 4°C, and the supernatants aliquoted and stored at −80°C. Tissue MMP3 levels were measured by ELISA (human total MMP3, Quantikine, R&D Systems) according to the manufacturer’s instructions. The concentration of soluble protein in each sample was measured using the Coomassie® Plus Protein Assay Reagent Kit (Pierce Chemical Company, IL, USA). MMP3 concentrations were standardized by expressing them as a ratio of the soluble protein concentration in the extract (μg MMP3/mg soluble protein), and the ratios expressed as mean ± standard error of the mean (sem).

Statistical analysis
Allelic frequencies and patient demographics were analysed using the Chi-square test. The ratios of MMP3 cDNA to β2-microglobulin, the standardized MMP3 protein concentrations in patients homozygous and heterozygous for the 5A and 6A alleles and the differences were compared using a two-tailed unpaired t-test with Welch’s correction. A linear model was fitted to correct MMP3 protein concentration and mRNA levels for potential confounders of age, sex and aneurysm diameter using R v2.2.2 for Windows (http://www.r-project.org).

Conflict of Interest statement. None declared.

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REFERENCES


