Investigation of the C242T polymorphism of NAD(P)H oxidase p22 \textit{phox} gene and ischaemic heart disease using family-based association methods

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ABSTRACT

Ischaemic heart disease is a complex phenotype arising from the interaction of genetic and environmental factors. Excessive production of reactive oxygen species leading to endothelial dysfunction is believed to be important in the pathogenesis of ischaemic heart disease. The NAD(P)H oxidase system generates superoxide anions in vascular cells; however, the role of the C242T polymorphism of the NAD(P)H oxidase p22 \textit{phox} gene in ischaemic heart disease is unclear due to contradictory results from case-control studies. Consequently, we applied family-based association tests to investigate the role of this polymorphism in ischaemic heart disease in a well-defined Irish population. A total of 1023 individuals from 388 families (discordant sibships and parent/child trios) were recruited. Linkage disequilibrium between the polymorphism and ischaemic heart disease was tested using the combined transmission disequilibrium test (TDT)/sib-TDT (cTDT) and pedigree disequilibrium test (PDT). Both cTDT and PDT analyses found no statistically significant excess transmission of either allele to affected individuals (\(P = 0.30\) and \(P = 0.28\), respectively). Using robust family-based association tests specifically designed for the study of complex diseases, we found no evidence that the C242T polymorphism of the p22 \textit{phox} gene has a significant role in the development of ischaemic heart disease in our population.

INTRODUCTION

Ischaemic heart disease (IHD) is the leading cause of death worldwide [1]. As with most common chronic diseases, it arises due to the complex interplay of genetic and environmental factors. Evidence supporting a genetic component to IHD includes the observation that the risk of developing IHD is substantially increased in first-degree relatives of individuals with early-onset IHD [2]. Twin study data also clearly demonstrate that, at younger ages, death from IHD is more likely to be influenced by genetic factors in both men and women than at older ages, although the effect did persist into the seventh and eighth decades [3]. Certain aspects of the pathogenesis of IHD are understood, but the interplay of environmental and genetic factors is poorly defined; thus the specific genes involved, their number and relative importance remain speculative.

Excessive production of reactive oxygen species, including superoxide (\(O_2^•\)), \(H_2O_2\) and nitric oxide (NO*), is believed to be important in the pathogenesis of IHD by causing endothelial dysfunction [4]. NAD(P)H oxidases

Key words: ischaemic heart disease, NAD(P)H oxidase, polymorphism, reactive oxygen species.

Abbreviations: IHD, ischaemic heart disease; PDT, pedigree disequilibrium test; TDT, transmission disequilibrium test; cTDT, combined TDT/sib-TDT.

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are membrane-associated enzymes which catalyse the 1-electron reduction of oxygen using either NADH or NADPH as the electron donor, and they are the most important source of superoxide anion, the precursor of a variety of potent oxidants in vascular cells and myocytes [5]. It has been hypothesized that polymorphisms which disrupt the function of NAD(P)H oxidase might alter the risk of developing IHD in a given individual.

NAD(P)H oxidase is comprised of several distinct subunits, including p22 phox, which acts as the final electron transporter from NADPH to molecular oxygen [6]. The CYBA gene, which codes for p22 phox, is located on chromosome 16q24 and has several allelic variants, some of which cause chronic granulomatous disease [7–11]. It has been proposed that the C242T nucleotide transition, which results in replacement of histidine by tyrosine at amino acid position 72, may alter superoxide production, and it has therefore been implicated as a potential genetic risk factor for IHD [12].

Nevertheless, there remains considerable controversy as to whether the C242T polymorphism of the p22 phox gene is associated with IHD, as case-control studies to date have produced conflicting results [12–14]. Case-control studies have a well-recognized vulnerability to spurious results for several reasons, including the risk that bias may be introduced by unrecognized differences in the sampling of cases and controls [15].

In order to overcome these problems, we investigated the presence of linkage disequilibrium between the C242T polymorphism of the p22 phox gene and IHD in a well-defined Irish population using two family-based association methods [16,17]. The methods we used included the combined transmission disequilibrium test (TDT)/sib-TDT (cTDT) [16] and the pedigree disequilibrium test (PDT) [17]. Both are powerful tests designed specifically for the study of complex diseases such as IHD and are resistant to the problems of population admixture inherent in traditional case-control studies.

METHODS

Study population
Between August 1999 and April 2002, we recruited 1023 individuals from 388 families. The inclusion criteria are described in detail in a previous publication [18]. Briefly, all subjects were Caucasian whose parents and grandparents were born in Ireland. Each family was required to have at least one member affected with proven premature IHD (disease onset ≤ 55 years for males and ≤ 60 years for females) and at least one unaffected sibling and/or both parents surviving.

The affected siblings had proven IHD. This was defined as the presence of one or more of the following features: (i) a history of acute myocardial infarction (as defined by World Health Organization criteria) [19]; (ii) a history of unstable angina (typical chest pain with dynamic ECG changes or minor elevations in cardiac markers); and (iii) obstructive coronary artery disease angiographically (> 70 % luminal stenosis). The affected siblings were recruited from patients referred to the cardiology centres in the Royal Victoria Hospital and Belfast City Hospital, Belfast, U.K.

For inclusion, the unaffected siblings were required to: (i) be older than the affected sibling was at the onset of IHD; (ii) have no symptoms of angina or possible myocardial infarction using the ‘Rose chest pain on effort and possible infarction questionnaire’ [20]; (iii) have no history of IHD diagnosed by a doctor; and (iv) have a resting 12-lead ECG showing no evidence of ischaemia or previous myocardial infarction (independently coded using the ‘Minnesota code’) [21]. All subjects were also assessed to allow determination of IHD risk factors.

The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and was approved by the Research Ethics Committee of Queen’s University Belfast. Fully informed consent was obtained from all subjects.

DNA procedures
DNA was extracted from peripheral whole blood using a salting-out method [22]. The p22 phox genotypes were determined by PCR amplification of the region containing the mutation, followed by RsaI digestion and agarose-gel electrophoresis, as described by Schächinger et al. [23]. Genotyping was repeated in 10 % of the samples randomly selected as a quality control measure. Each gel was read by two observers unaware of the subject’s disease status.

Statistical analysis
The presence of linkage disequilibrium between the C242T polymorphism of the p22 phox gene and IHD was determined using cTDT and PDT. These family-based association tests establish the presence of linkage disequilibrium by testing for unequal transmission of either allele from parents to affected offspring and/or unequal sharing of either allele between discordant sibships.

cTDT [16] combines TDT with sib-TDT. cTDT was performed using publicly available software (http://genomics.med.upenn.edu/spielman/TDT.htm) and the results were also verified using a spreadsheet.

TDT uses data from families in which marker genotypes are known for the parents and the affected offspring (a trio), but only parents who are heterozygous for the marker alleles are informative for the test. TDT tests for unequal transmission of a genetic marker from a heterozygous parent to an affected offspring. For sib-TDT disease-discordant sibships are informative if there is at least one affected and one unaffected sibling with different marker genotypes. sib-TDT compares the marker frequency in affected individuals versus their unaffected
Table 1  Composition of families

<table>
<thead>
<tr>
<th>Family Structure</th>
<th>Families (n)</th>
<th>Subjects (n)</th>
<th>Affected subjects (n)</th>
<th>Unaffected sibs (n)</th>
<th>Disease discordant sib pairs (n)</th>
<th>Parent pairs (n)</th>
<th>Trios (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1AS and 1US</td>
<td>206</td>
<td>412</td>
<td>206</td>
<td>206</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1AS and 2US</td>
<td>81</td>
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<td>81</td>
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<td>1AS and 3US</td>
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<td>68</td>
<td>17</td>
<td>51</td>
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<td>0</td>
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<tr>
<td>1AS and 4US</td>
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<td>2</td>
<td>8</td>
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<td>0</td>
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<tr>
<td>1AS and 5US</td>
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<td>6</td>
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<td>5</td>
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<tr>
<td>2AS and 1US</td>
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<td>6</td>
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<td>2AS and 2P</td>
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<td>2</td>
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<tr>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3AS and 1US</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>388</td>
<td>1023</td>
<td>418</td>
<td>495</td>
<td>542</td>
<td>55</td>
<td>58</td>
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</table>

Table 2  Sibling characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Affected siblings (n = 418)</th>
<th>Unaffected siblings (n = 495)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age when IHD diagnosed (years)</td>
<td>46.3 ± 6.4</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Age at study entry (years)</td>
<td>51.3 ± 7.5</td>
<td>55.5 ± 8.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sex (males; n)</td>
<td>335 (80.1)</td>
<td>233 (47.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Non-smoker (n)</td>
<td>76 (18.2)</td>
<td>211 (42.6)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>141 (33.7)</td>
<td>257 (51.9)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
<td>44 (10.5)</td>
<td>21 (4.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hypercholesterolaemia (n)</td>
<td>388 (92.8)</td>
<td>410 (82.8)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>28.6 ± 4.3</td>
<td>28.3 ± 4.6</td>
<td>0.31</td>
</tr>
</tbody>
</table>

siblings. To ensure the analyses gave valid tests of association only, one trio or discordant sibship was included from each family. In families with multiple disease discordant sibships, the sibship with the maximally discordant genotype was selected as described by Curtis [24].

PDT [17] is another family-based association test. This test was performed using publicly available software (http://wwwchh.mc.duke.edu/software/pdt.html) and verified using a spreadsheet. PDT can use data from related nuclear families (trios and discordant sibships) from extended pedigrees and remain a valid test of linkage disequilibrium. Informative extended pedigrees contain a minimum of one informative trio and/or discordant sibship as described for cTDT. The rationale for using PDT is that it allows increased used of data compared with cTDT.

Prospective power calculations for family-based association studies of complex diseases are problematic, as they require a model of inheritance to be specified and the number of informative families can be difficult to predict. We therefore chose to assess power retrospectively.

The independent samples t test was used to compare means for quantitative variables and the χ² test was used for qualitative variables. All statistical tests were performed at the 5 % significance level (two-tailed).

RESULTS

We studied 1023 individuals from 388 families, including 418 affected individuals, 495 unaffected siblings and 110 parents. This yielded 542 disease-discordant sibling pairs and 58 trios (both parents and affected offspring). Table 1 shows the composition of the families studied.

The characteristics of the affected and unaffected siblings are shown in Table 2. The affected siblings were statistically significantly more likely to be male, smokers, suffer from diabetes mellitus and have increased
cholesterol compared with the unaffected siblings. In contrast, the affected siblings were less likely to have hypertension. Both groups were overweight with no significant difference in terms of body mass index.

Despite repeated attempts, the DNA from one individual could not be genotyped. This resulted in the exclusion of this family (a disease-discordant sibling pair) from further analysis. The allele frequencies were determined using the successfully genotyped probands (i.e., one individual per family). Of the 387 probands genotyped, 174 (45.0 %), 172 (44.4 %) and 41 (10.6 %) had CC, CT and TT genotypes respectively, giving a T allele frequency of 32.8 % among our cases: Hardy–Weinberg equilibrium was apparent.

**cTDT**

After genotyping and after selection of a single discordant sibship or trio per family, 147 discordant sibships and 54 transmissions from 43 trios (423 individuals) were informative for analysis. There was no statistically significant excess transmission of either allele to affected individuals \( P = 0.30 \), as shown in Table 3.

**PDT**

After genotyping, 191 pedigrees (514 individuals) were informative for analysis. There was an excess of 13 C alleles among the 155 contributing discordant sibships and an excess of 10 C alleles among the 43 informative trios. However, this excess did not reach statistical significance and produced a PDT statistic \( Z = 1.09 \) \( P = 0.28 \).

**DISCUSSION**

In the present study, we have found no evidence of linkage disequilibrium between the C242T polymorphism of the p22 \textit{phox} gene and IHD using two family-based association tests [16,17] in a well-defined Irish population.

We undertook our study because previous case-control studies of this polymorphism in IHD have produced conflicting results. Inoue and co-workers [12], in a study of Japanese patients, found that individuals carrying the T allele were at reduced risk of IHD. Subsequently, other groups [13,14] found no evidence of association between this polymorphism and IHD. In contrast, an Australian group [25] found that the T allele was associated with increased risk of IHD in younger males, but not in the overall population, and an American group [5] found more progression of angiographic atherosclerosis in individuals with the T allele. High rates of spurious associations are observed in case-control studies and several factors probably contribute to the disparate results for the C242T p22 \textit{phox} polymorphism, including differences in the ethnic backgrounds of the populations, definitions of phenotype and statistical methods. One of the major strengths of our present study is its use of family-based association tests, which have important advantages over the case-control method used in these studies. Family-based tests have been specifically designed for the investigation of the role of candidate genes in complex diseases and avoid the effects of confounding due to population stratification intrinsic to case-control studies [15]. These tests are therefore ideally suited for investigating further and helping resolve the controversial role of this polymorphism in IHD. Furthermore, our study subjects were carefully phenotyped and drawn from a relatively homogeneous Caucasian population. We found a T allele frequency of 32.8 %, consistent with the frequency observed previously in European [13], Australian [25] and American [5] populations, but markedly higher than that seen in a Japanese [12] one.

The C242T polymorphism of the p22 \textit{phox} gene has been proposed as a genetic risk factor for IHD based on the hypothesis that the polymorphism results in variants of NAD(P)H oxidase that produce different amounts of reactive oxygen species and thereby result in altered risk of developing IHD. The C242T polymorphism of the p22 \textit{phox} gene results in a substitution of His72 with a tyrosine residue and it has been speculated that this may modulate NAD(P)H oxidase enzyme activity by affecting its haem-binding site. Azumi and co-workers [26], in a study of human coronary artery sections taken at autopsy, found that as atherosclerosis progressed the expression of p22 phox increased throughout the vessel wall. A recent study by Guzik et al. [27] found that the 242T allele was associated with significantly lower basal and NADH-stimulated vascular superoxide production in human saphenous vein and internal mammary artery samples from patients undergoing routine coronary artery bypass surgery. These findings support the hypothesis that the T variant of the polymorphism results in reduced production of reactive oxygen species by NAD(P)H oxidases and so may be protective against IHD. Why did we find no evidence of such an effect in our study?

First, the functional data reported by Guzik et al. [27] have limitations [28]. The assay method used can generate oxygen free radicals itself and may be imprecise. In addition, extrapolation of \textit{ex vivo} results, particularly data from saphenous veins, to the \textit{in vivo} process of

**Table 3**  

<table>
<thead>
<tr>
<th>Transmission of C allele to affected individuals</th>
<th>Observed</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDT</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>Sib-TDT</td>
<td>181</td>
<td>178.5</td>
</tr>
<tr>
<td>cTDT</td>
<td>214</td>
<td>205.5</td>
</tr>
</tbody>
</table>

\( Z = 1.04; \ P = 0.30\) (two-tailed with continuity correction).
coronary atherosclerosis may be unwarranted. More in vivo functional data have been published recently [23]. Schächinger and co-workers [23] studied 93 patients undergoing coronary angiography and found carriers of the CC genotype to have a significantly blunted endothelium-dependent dilator response. They suggested that the T allele may therefore be protective against IHD. These data support those of Guzik et al. [27]. Nevertheless, doubt still exists as to whether the C242T p22 phox polymorphism is biologically significant.

Secondly, we investigated only one polymorphism of the p22 phox gene. The p22 phox protein is a subunit of NAD(P)H oxidase. The biology and systems governing the function of this important enzyme, its interaction with antioxidant defence systems, other genes and environmental factors are exceedingly complex and poorly understood. It may be that, in certain subgroups or ethnic groups, this polymorphism does play a role in IHD risk; however, such a study would require much larger numbers and is likely to also require a design capable of testing for the impact of multiple other interacting genes simultaneously.

Thirdly, we cannot exclude the possibility that this polymorphism has only a minor role in susceptibility to IHD, which was not demonstrable in the present study of 1023 individuals and that a type 2 error occurred. However, using the method described by Spielman and Ewens [16] for cTDI, we retrospectively estimate that a sample of 190 minimally informative families will have over 95% power to detect as significant (P < 0.05; two-tailed) a deviation from 50 to 65% in the rate of transmission of an allele to affected individuals in our study population. We also had 80% power to detect a deviation from 50 to 60% in the rate of allele transmission to affected individuals.

In conclusion, using robust family-based association tests specifically designed for the study of complex diseases, we found no evidence that the C242T polymorphism of the p22 phox gene plays a significant role in the development of IHD.

ACKNOWLEDGMENTS

This research was supported by a Royal Victoria Hospital Research Fellowship (to M.S.), the Northern Ireland Chest, Heart and Stroke Association, the Research and Development Office, Northern Ireland and the Heart Trust Fund (Royal Victoria Hospital). We acknowledge the technical assistance of Christine Belton.

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