Interactive effect of the p53 gene and cigarette smoking on coronary artery disease

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Abstract

Objective: p53 is a tumour suppressor protein involved in the control of cell growth and has an established role in carcinogenesis, particularly in relation to smoking. It may also be related to arteriosclerosis by affecting smooth muscle cell proliferation, a feature of atherogenesis. Methods: We explored a role for p53 in atherogenesis by assessing the association between two DNA polymorphisms of the p53 gene (MspI at intron 6 and HaeIII at intron 1) and angiographically documented coronary artery disease (CAD) in 654 Australian Caucasian patients. Results: There was a significant interactive effect of the two polymorphisms and cigarette smoking on CAD in a logistic regression analysis (P = 0.0039) but no association between CAD and either individual p53 polymorphic marker. CAD occurrence was more frequent in non-smoking patients with rare alleles at both sites (85.0%) compared to those homozygous for common alleles at both sites (70.4%). However, this was not seen in smokers (85.7 vs 82.8%). In all 654 patients cigarette smoking remained a significant predictor of CAD irrespective of p53 genotypes (P = 0.0065). Conclusions: Our findings identify an interactive effect of both p53 polymorphisms and cigarette smoking on the occurrence of coronary artery disease in that non-smoking patients with rare alleles at both sites had increased incidence of CAD. They illustrate the relevance of genotype-specific and environment-dependent enhanced cardiovascular risk and foreshadow a need for further studies to establish functional changes. © 1997 Elsevier Science B.V.

Keywords: p53 gene; DNA polymorphism; Cigarette smoking; Coronary artery disease; Gene–environment interaction

1. Introduction

A large body of evidence has established that both circulating and local arterial wall factors participate in the initiation and progression of atherosclerotic lesions [1,2]. Among the vascular wall changes are endothelial dysfunction, uncontrollable proliferation of vascular smooth muscle cells (VSMC) and abnormal accumulation of extracellular matrix. Each of these may be influenced by both genetic and environmental factors [1–5] and so too may the relevant circulating variables [4,5].

Among the many pathological processes occurring in the vascular wall during atherogenesis, endothelial dysfunction has been thought a marker for the initial lesion and VSMC proliferation important for its progression [1–3]. Many factors may be involved in enhancing abnormal proliferation. p53 is a tumour suppressor protein which is expressed in many types of human cells and is involved in the control of cell proliferation [6–9]. It also plays an important role in regulating the growth of VSMC [8–10]. Loss of p53 activity causes unrestrained growth while increased levels of p53 arrest cells in the G1 phase of the cell cycle. Mutant alleles of p53 are frequently found and are also often expressed at elevated levels in tumour cells [6–8,11]. They tend to bind to the wild-type subunit making it unable to function and in this way may alter the regulation of cell growth. Mutations have been reported in every codon of the five highly conserved domains from exon 5 through to 9 in various cancers [6]. Cigarette smoking appears to inactivate p53 by inducing mutations [12,13] and therefore we reasoned that p53 may influence the contribution of smoking to atherogenesis. Indeed, sev-
eral polymorphisms in the non-coding region of the p53 have also been shown to predispose an individual who smokes, or who is a passive smoker, to the development of tumours [14,15], although this was not found in a recent Japanese study [16].

There are similarities between atherogenesis and benign tumour formation although in atherogenesis the process of proliferation is less aggressive and more chronic [17,18]. It is possible, therefore, that while major p53 mutations at exons may lead to the development of malignant changes, some DNA variations at the p53 gene associated with quantitative changes in p53 production or minor alterations in p53 function may also be associated with the comparatively mild growth enhancement found in atherogenesis. Data about such a relationship are sparse (only two studies) and controversial [9,19]. Recently, Speir et al. showed a potential interactive effect of p53 and human cytomegalovirus on coronary re-stenosis after angioplasty [9]. A role for p53 in atherogenesis is also supported indirectly by the findings of Isner et al. [20]. They reported that apoptosis, a process to which p53 contributes [7,10], occurs in human atherosclerosis. However, D’Agostini et al. failed to show any relationship between a p53 DNA polymorphism and coronary artery disease (CAD) [19].

Atherosclerosis is a multifactorial process and many factors, particularly cigarette smoking [21,22], could confound a potential relationship between p53 and CAD. With this in mind, we explored a possible relation between a p53 DNA polymorphism and coronary artery disease (CAD) [19].

2. Methods

2.1. The patients

We studied Caucasian patients aged 65 years or less, both men and women, consecutively referred to the East-ern Heart Clinic at Prince Henry Hospital for coronary angiography over an 18-month period in 1994 and 1995. We excluded only patients shown to have significant left main disease (> 50% luminal obstruction) because it was difficult to categorise this small proportion of the total (5.0%) within the classification system we used (see below). A written consent was obtained from every patient after a full explanation of the study which was approved by the Ethics Committee of the University of New South Wales.

A 4 ml venous blood sample was drawn into an EDTA sample tube from patients before the angiogram after a 6–14 h fast. The blood sample was centrifuged within 2 h and plasma and cellular components stored separately at −70°C in aliquot until analysis. DNA was extracted from the frozen cellular blood component by a salting-out method [25]. The extracted DNA was stored at 4°C until analysis.

2.2. Determination of the polymorphisms in the p53 gene

The HaeIII polymorphic marker is located at intron 1 of the p53 gene as described by Ito et al. [23]. The relevant DNA fragment was amplified by the 5’-TTCCGCTGT- TCTTCCCATG-3’ for the upstream and 5’-TGTTGTAATGCCACCTCG-3’ for the downstream primers in the PCR. The amplification was performed for 35 cycles with annealing temperature of 60°C in a Hybaid Thermal cycler in a total reaction of 50 μl containing 180 μM dNTPs, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 50 pmol of each primer and 2 units Taq polymerase. The HaeIII digest of the amplified fragment was subjected to electrophoresis through 8% acrylamide gel and identifies two alleles H1 58bp and 37bp, and H2 95bp in which H1 is a common allele.

The MspI polymorphism is at intron 6, a G → A substitution 61 bp downstream from exon 6 of the p53 gene (17p13) as described by McDaniel et al. [24]. This base change abolishes a MspI restriction site and can be detected after digestion of the relevant PCR fragment. The primers used for the PCR were 5’-AGGTCTGTGTT- TGCAACTGGG-3’ for the upstream and 5’-GAG-GTCAAAATAGCACGAGG-3’ for the downstream primers. The amplification was conducted for 35 cycles with the annealing temperature of 59°C in a total reaction volume of 50 μl with contents the same as the one above except for the specific pair of primers. The amplified 107 bp fragment was digested by MspI (M1 allele, 63 bp, 44 bp; M2 allele, 107 bp) in which M1 is a common allele.

2.3. Coronary angiographic documentation of CAD

The presence and severity of coronary stenosis was determined by coronary angiograms which were assessed by two cardiologists who were unaware that the patients...
were to be included in the study. Each angiogram was classified as revealing either no coronary lesion or lesions with less than or more than 50% luminal stenosis. For CAD incidence, patients were classified as having or not having angiographically demonstrable CAD; and for CAD severity we grouped the patients into those having one, two, or three major epicardial coronary arteries with more than 50% luminal obstruction. We also used the Green Lane coronary scoring system [25] which provides a numerical value for lesion severity and takes account of the amount of myocardium supplied by an affected vessel; the maximal score is 15.

2.4. Documentation of cigarette smoking

We documented smoking status by grouping patients into those who were non-smokers and had never smoked and into those who were or had been smokers. Smokers were further grouped as current smokers who had regularly smoked at least 5 cigarettes per day for at least the previous 3 months and ex-smokers who had stopped smoking for more than 1 year. The life-time smoking dose in pack-years (1 pack-year = smoking one pack of 20 cigarettes each day for 1 year) was recorded as described previously [25].

2.5. Statistical analysis

The frequencies of the alleles and genotypes among different subgroups were compared by $\chi^2$-test. ANOVA was used for comparison of quantitative variables among 3 groups or more and Student’s t-test for two group comparisons. A logistic regression analysis was used to assess the independent contributions to CAD of various risk factors while controlling for other variables using the SPSS-X statistical package. In the logistic analysis, predictive variables are entered into the model either independently (described as main effect) or jointly (described as interactive effect).

3. Results

3.1. Genetic characteristics of the patients

The demographic information for the 654 patients (male 441, female 161) with or without angiographically demonstrable CAD is shown in Table 1. Genotype distributions of the p53 MspI and HaeIII polymorphisms between the two groups are not different and are in Hardy-Weinberg equilibrium as shown in Table 1. Although these two polymorphic markers are located more than 11 200 bp apart, they are in linkage disequilibrium ($\chi^2 = 9.89, df = 4, P = 0.042$).

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of patients in the study (mean ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without CAD</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Smokers (%) *</td>
</tr>
<tr>
<td>Life-time smoking dose (pack-years)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
</tr>
<tr>
<td>TC/HDL-C</td>
</tr>
<tr>
<td>Lp(a) (mg/l)</td>
</tr>
<tr>
<td>Male/Female</td>
</tr>
</tbody>
</table>

**Smokers include both ex- and current smokers. * P < 0.01, ** P < 0.001 by Student’s t-test or $\chi^2$ comparison. Values are presented as mean ± s.e.m. * H2 is the rare allele of the p53 HaeIII polymorphism and M2 is the rare allele of the p53 MspI polymorphism.

3.2. p53 polymorphisms and occurrence of angiographically documented CAD

Among the 654 patients there were 68 men and 60 women who had angiographically normal coronary arteries (Table 1). Using a simple $\chi^2$-analysis, there were no relationships between the MspI or HaeIII polymorphisms and the absence of CAD ($\chi^2 = 0.025, P = 0.98$, and $\chi^2 = 1.64, P = 0.44$, respectively).

We assessed the relationship between the p53 polymorphisms and CAD occurrence in a logistic regression analysis whilst controlling for the other potentially confounding variables age, sex, presence of hypertension or diabetes, body mass index, total cholesterol (TC), HDL cholesterol (HDL-C), lipoprotein(a), TC/HDL-C ratio and cigarette smoking. We entered both MspI, HaeIII genotypes, smoking status [in terms of smokers (current and ex-smokers) and non-smokers], sex, presence of hypertension or diabetes, BMI and other lipoprotein variables as individual terms, and MspI* smoking, HaeIII* smoking, MspI* HaeIII* smoking, MspI* HaeIII* sex, MspI* HaeIII* age as interactive terms in a stepwise likelihood ratio model. For current and ex-smokers in this patient population relationships with both CAD occurrence and severity were the same and depended upon the life-time smoking dose (in pack-years) as we have reported previously [25]. Therefore we grouped current and ex-smokers...
The observed number of cases and the column percentage (in brackets) are presented. The association between genotypes and the number of significantly diseased vessels was assessed by χ²-test as followings: (a) χ² = 5.08, P = 0.16; (b) χ² = 0.85, P = 0.83; (c) χ² = 10.48, P = 0.01.
a small number of patients with rare alleles in this subgroup.

3.3. p53 polymorphisms and CAD severity

To explore a possible association between p53 and CAD severity, as opposed to occurrence, we first assessed severity from the number of significantly diseased vessels (Table 3). The frequency distribution of the rare alleles was not different among those with or without significantly diseased vessels (> 50% luminal obstruction). In a $\chi^2$ comparison, neither of the p53 polymorphic markers was associated with the number of significantly diseased vessels ($\chi^2 = 5.08$, $P = 0.16$ for $MspI$ polymorphism and $\chi^2 = 0.85$, $P = 0.83$ for $HaeIII$ polymorphism and $\chi^2 = 10.48$, $P = 0.105$ for both polymorphisms). In a log-linear analysis, we also found no three-way interactions among p53 polymorphisms, cigarette smoking and the number of significantly diseased vessels ($\chi^2 = 4.39$, $P = 0.623$) whilst smoking remained a significant predictor for CAD severity ($\chi^2 = 19.5$, $P = 0.0002$).

Although non-smoking patients with the rare alleles at both sites tended to have higher coronary scores as shown in Table 4, the differences were not statistically significant. Using ANOVA the p53 polymorphisms had no main effect on the scores ($P = 0.54$). The p53 polymorphisms had no interactive effects with sex ($P = 0.194$), smoking ($P = 0.061$), or age ($P = 0.226$) on coronary scores whilst sex ($P = 0.0001$), age ($P = 0.00001$) and cigarette smoking ($P = 0.007$) all exerted major main effects on the scores.

4. Discussion

The study identifies a significant interactive effect between both p53 polymorphisms and cigarette smoking on CAD occurrence ($P = 0.0039$) whilst showing that the p53 $HaeIII$ and $MspI$ polymorphisms are not individually associated with CAD occurrence and severity (Tables 1 and 3). The presence of the p53 rare alleles at both sites was associated with more frequent CAD in non-smokers, but not in smoking patients. Although the incidence of CAD among smokers was increased independent of genetic profiles, the p53 polymorphism did appear to modify the association quantitatively. The frequency of CAD occurrence in patients homozygous for the wild-type at both or either site was much higher in smokers than that in non-smokers (Table 2), an observation further supported by the differences in life-time smoking dose among smokers. However, this difference was not seen in patients with rare alleles at both sites. The findings of our study are consistent with the notion that certain genetically related CAD risk factors are environmentally modifiable, and that some environmental CAD risk factors could also be affected by specific genotypes.

It is well established that cigarette smoking is a major risk factor for CAD. Smoking may enhance atherogenesis by many mechanisms as reviewed previously [21,22,26,27], but our present findings are consistent with the conclusion that p53 is one of the target molecules smoking affects in relation to atherogenesis. Denissenko et al. have also recently reported the involvement of p53 gene mutations in smokers who develop lung cancer [13]. Although the mechanism(s) remains unknown, we could speculate that smoking may quantitatively decrease the expression of functional p53, which in turn leads to abnormal proliferation of many cells including vascular smooth muscle cells and therefore promotes atherogenesis. Vascular smooth muscle cell proliferation contributes to atherogenesis. Furthermore, p53-related atherogenesis could also be mediated through p53-dependent apoptosis. Wild-type p53 induces apoptosis of vascular smooth muscle cells, particularly cells infected with viruses [10]. Thus expression of mutant p53 could block this wild-type p53 function and suppress apoptosis [10,20]. The occurrence of apoptosis in atheromatous lesions has been described although the mechanisms are not clear [20]. Specifically, our study shows that to determine the true association between p53 and CAD it is essential to control for smoking. Since both the $MspI$ and $HaeIII$ polymorphic markers are on introns, we postulate that they are in linkage with some functional effects which induce either quantitative or qualitative changes in p53 gene expression. Our results indicate that this relates to the presence of the rare alleles at both sites since altered CAD risk was only observed when both rare alleles were present. It has been shown that intron 4 of p53 influences gene expression [28,29], and Peller et al. have identified an association between the $MspI$ polymorphism at intron 6 and cancer predisposition [30]. There is therefore a likelihood of a functional role for the polymorphisms we have studied. In vitro experiments are required to explore functional effects and to assess environment-dependent changes in p53 gene expression.

Although the size of the patient population we studied is appropriate for evaluation of the main effect of the polymorphisms, for interactive effects the power is reduced because of the degrees of freedom. This is particularly true for CAD severity and we found no association between the p53 polymorphisms and CAD severity. In conclusion, our current study is consistent with an associa-

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Table 4

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Non-smokers</th>
<th>Smokers</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>$H1$ or $M1$</td>
<td>4.39 ± 0.32 (118)</td>
<td>5.39 ± 0.24 (264)</td>
<td>5.08 ± 0.20 (382)</td>
</tr>
<tr>
<td>$H2$ or $M2$</td>
<td>4.01 ± 0.44 (78)</td>
<td>5.76 ± 0.32 (144)</td>
<td>5.17 ± 0.27 (222)</td>
</tr>
<tr>
<td>$H2$ and $M2$</td>
<td>5.44 ± 0.14 (20)</td>
<td>5.34 ± 0.71 (230)</td>
<td>5.39 ± 0.54 (50)</td>
</tr>
</tbody>
</table>

$F$- and $P$-values were obtained by ANOVA.
tation between p53 polymorphisms and CAD occurrence which is influenced by cigarette smoking. Cigarette smoking remains a powerful CAD risk factor regardless of the p53 genotype and may mask the relationships with the p53 genotypes we assessed whereas in non-smoking patients with rare alleles at both sites the increased incidence of CAD is evident.

Acknowledgements

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References


