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Restriction fragment length polymorphisms in the Apo B gene in relation to coronary heart disease in a Southern Asian population

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Summary

We have examined DNA polymorphisms associated with the apolipoprotein B gene in 95 Sri Lankan males with ischaemic heart disease and 95 matched controls. For polymorphisms detected using the XbaI or MspI enzymes the allele frequency in Sri Lankans contrasted markedly from that in Caucasians. Overall, there was no significant association of any allele studied with coronary disease cases in this sample. There was, however, a significant difference observed between the XbaI allele frequency in normotriglyceridaemic or normocholesterolaemic CHD cases compared with the allele frequency in the controls.

Introduction

The rate of coronary heart disease (CHD) has been reported to be higher in Southern Asians than in people of other ethnic descent [1,2]. In a recent study of myocardial infarction in immigrant Asians and white men in Britain, a higher relative rate of infarction and more extensive atheroma was reported in Asians [3]. The reason for the greater propensity of Asians to develop atheroma is not clear. The identification of the basis of premature and accelerated atherogenesis in Asians would make a valuable contribution to the understanding of coronary atherosclerosis.

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Studies on twins and families have shown that there is a substantial genetic component in the aetiology of CHD [4]. In recent years many studies focussed on candidate genes (genes whose products are suspected to be involved in the pathogenesis of atherosclerosis) have been carried out on white populations. However, as far as we are aware, no such investigations have been done on any Southern Asian population.

Elevated plasma Apo B and LDL levels are associated with an increased prevalence of atherosclerosis [5]. Hence genetic variation in Apo B is one likely cause of abnormal lipid metabolism causing atherosclerosis. We investigated the possible aetiological role of genetic variation at the Apo B gene locus for the development of CHD in individuals from Sri Lanka.

Materials and methods

Subjects

The patients were 95 Sri Lankan males aged 37–65 yr who were shown by history and electrocardiographic examination to have ischaemic heart disease when they presented to the Teaching Hospital, Peradeniya between 1986 and 1988. They were matched with an equal number of controls who were healthy Sri Lankan males of the same age and ethnic group recruited from a population in which a prevalence study of ischaemic heart disease had been conducted in 1988. The controls had no history of angina or myocardial infarction and had normal electrocardiograms.

Materials

Chemicals were obtained from Sigma Chemical Co. Ltd. (Poole) or BDH Biochemical Reagents UK (Poole). Restriction enzymes (XbaI and EcoRI) were from Gibco BRL (Paisley) and MspI was from Boehringer Mannheim (Lewes, East Sussex). The probes, pABC and pBH2 were gifts from Dr. S. Humphries of the Charing Cross Sunley Research Centre, Hammersmith, London. pABC contains a 3.5 kb EcoRI fragment of the Apo B gene [11] and pBH2 contains cDNA from the 3′ end of the gene (see Fig. 1b). The DNA markers used for the gels were lambda HindIII fragments bought from Gibco-BRL. Radiochemicals were obtained from Du Pont (NEN), (Stevenage, Hertfordshire) and Hybond-N was supplied by Amersham International plc (Amersham).

Lipid analysis

Venous blood was drawn from all individuals after a 14 h fast, mixed with K_2 EDTA at a final concentration of 2.5 mmol/l and separated into plasma and cells by centrifugation at $1411 \times g$ (3000 rpm) for 5 min at room temperature. The cells and plasma fractions were frozen separately at $-20\,^{\circ}C$ prior to analysis.

Total plasma cholesterol was measured using an enzymatic kit (Cat. No. 816302, Boehringer Mannheim GmbH). The same procedure was used to measure HDL

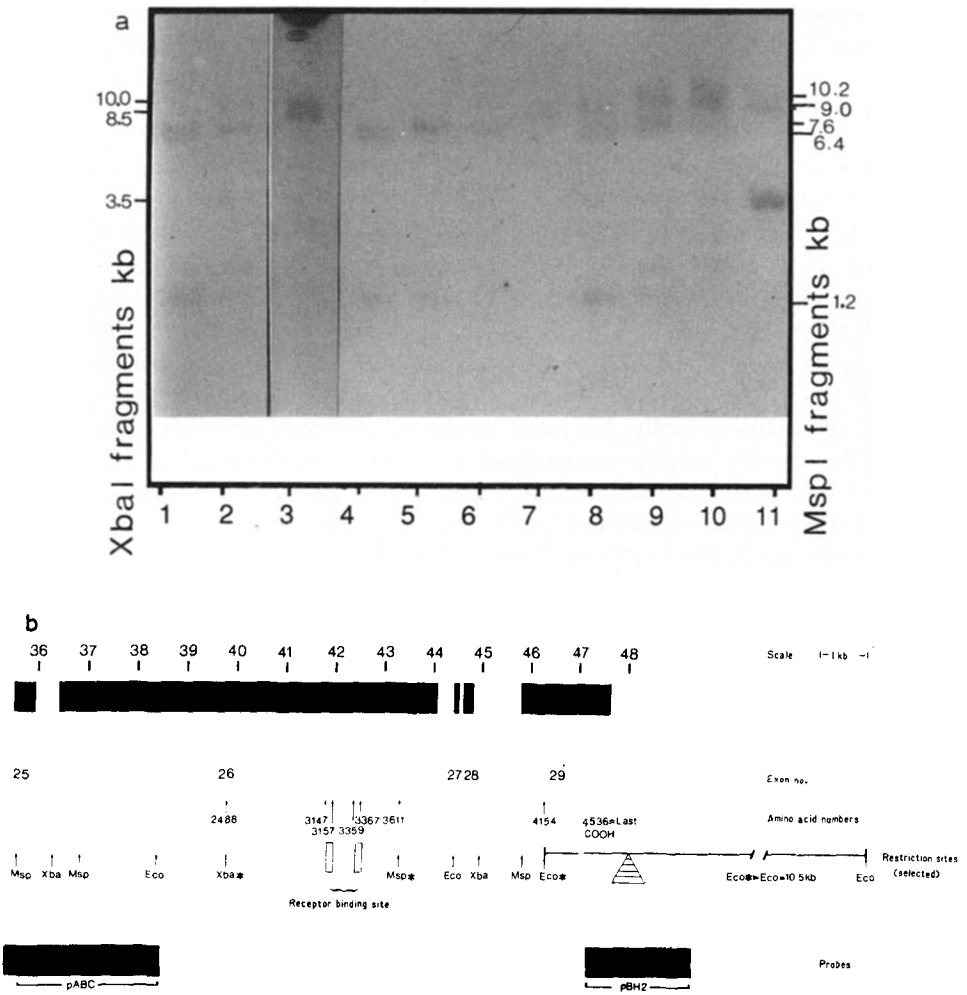

Fig. 1. a. Southern blot analysis of MspI and XbaI polymorphisms. The hybridisation pattern obtained using increasing MspI concentrations in 3 individuals (Individual A; Lanes 1–3, Individual B; Lanes 4–7 and Individual C; Lanes 8–11). Lanes 1, 4 and 8, 75 U MspI/5 μg DNA; Lanes 5 and 9, 50 U MspI/5 μg DNA; Lanes 2, 6 and 10, 25 U MspI/5 μg DNA; Lanes 3, 7 and 11, 20 U XbaI/5 μg DNA. Probe used was pABC labelled as in 'Methods'. Genotypes: Individual A, M1M1, X1X1; Individual B, M1M1, X1X1; Individual C, M1M2, X2X2. b. Map of the 3′ end of the Apo B gene. The scale begins at the 5′ end of the mRNA and all the exons preceding exon 25 are small, < 0.4 kilobase pairs (kb) in size. On the top line the filled in black regions are the exons with the blank introns in between. Exon 26 is a very large exon and encodes the receptor binding site [22]. The relevant restriction sites are shown and the triangle at the 3′ end of the gene represents a small insertion-deletion mutation. The DNA corresponding to the probes used is shown on the bottom line.

cholesterol after precipitation of plasma LDL and very low density lipoprotein (VLDL) using heparin/manganese [6]. Triglycerides were measured using an Enzymatic test kit (Cat. No. 816370, Boehringer Mannheim GmbH).

The upper limits of the reference intervals for plasma total cholesterol and triglyceride concentration were taken as 6.7 mmol/l and 2.0 mmol/l [7].

RFLP detection

DNA was prepared from frozen whole blood by a Triton X-100 lysis method [8]. Five μg DNA was digested with the restriction enzymes XbaI, MspI or EcoRI in a total of 50 μl volume. The incubation buffer for EcoRI was 50 mmol/l Tris–HCl (pH 8.0), 10 mmol/l $MgCl_2$ 100 mmol/l NaCl and 4 mmol/l spermidine. For XbaI, the incubation buffer was 50 mmol/l Tris–HCl (pH 8.0), 10 mmol/l $MgCl_2$, 50 mmol/l NaCl and 4 mmol/l spermidine. The incubation buffer for MspI was Tris–HCl 10 mmol/l (pH 7.2), $MgCl_2$ 10 mmol/l 2-mercaptoethanol 1 mmol/l, Triton X-100 0.02% (v/v) and 4 mmol/l spermidine. All digests were initiated with 20 units of enzyme (usually 2 μl) and incubated overnight at 37°C. A further 10 units of enzyme were added next morning and incubation continued during the day. For the XbaI digests, this second addition was later shown to be unnecessary. This protocol was varied as described below for MspI digests to resolve a particular problem with methylated cytosine residues (^{m}CCGG).

The digested DNA was electrophoresed through 0.8% agarose gels in TAE buffer (0.04 mol/l Tris–acetate, 1 mmol/l K_2EDTA, pH 8.0) alongside markers from lambda DNA. After electrophoresis gels were stained with ethidium bromide and photographed. DNA was denatured in situ and transferred to 'Hybond-N' by Southern blotting [9]. The Hybond-N was irradiated on a UV transilluminator for 3 min to bind DNA. Probes were prepared by digestion of the appropriate plasmid, cutting the cloned fragment out of a low melting point agarose gel and labelling to a specific activity of $4–9 \times 10^5$ cpm/ng using the random priming method of [10]. The probe for detecting the XbaI and MspI polymorphisms was pABC and to detect the EcoRI polymorphism pBH2 was used. These were added to minimal volume of hybridisation mixture at a radioactivity concentration of 2.5×10^6 cpm per blot. Hybridisation and washing techniques were carried out as described [9]. The hybridised filters were autoradiographed for three to seven days.

Conventionally, the most common allele of an RFLP is known as N1 and the less common alleles as N2, N3 etc where N is the first letter of the enzyme used to determine the RFLP. Clearly, this system sometimes results in anomalies as the definition depends on the frequencies in the first population studied. Occasionally too, different research groups may have opposite meanings of N1 and N2. The XbaI RFLP arises from an alteration in the third base of the threonine codon 2488 ACC → ACT and does not change the amino acid sequence of the resulting apolipoprotein B [11]. In this paper the XbaI RFLP X1 allele (no cutting site) resulted in a band of 8.6 kb while the X2 allele resulted in a band of 3.5 kb (site was present) with a common band of 10 kb (see Fig. 1).

The MspI RFLP changes codon 3611 from CGG (arginine) to CAG (glutamine) [12] and the more common allele of this, M1, was the smaller 5.6 kb allele with M2 as the larger 8.8 kb allele without the cutting site. There were technical problems using this enzyme which are discussed in the next paragraph. The EcoRI RFLP

(fragment sizes 12.45 kb and 10.5 kb) changes codon 4154 from GAA (glucamic acid) to AAA (lysine) [11] and the more common allele R1, was that which had the cutting site and resulted in a band of 10.5 kb.

MspI digestion problems

The restriction enzyme MspI recognises and cuts at the DNA sequence 5′-CCGG. MspI is sensitive to methylation at the first cytosine residue [13] and human DNA is methylated at some cytosine residues. Figure 1b shows that the autoradiography result for individuals with the polymorphic MspI site on both chromosomes (M1M1 type) should have been a band at 6.4 kb and another at 1.2 kb. However, this pattern was complicated by poor cutting at the flanking sites. If the upstream MspI site was poorly recognised by the enzyme then a 7.6 kb band was detected at the expense of the 1.2 kb and the 6.4 kb. This problem was also observed with the polymorphic site itself. Great care was taken not to assign an individual as M1M2 where the DNA was poorly digested so for any samples which appeared to be M1M2, the results of increasing MspI concentrations during digestion was examined.

Figure 1a shows the Southern blot analysis of three samples originally considered M1M2 on further analysis. Three different concentrations of MspI were used, 75 U/5 μg DNA, 50 U/5 μg DNA and 25 U/5 μg DNA. An XbaI (20 U/5 μg DNA) digest of the same DNA was also run on the same agarose gel. After probing with pABC as described the concentration effect of the MspI is clearly seen and only one of these samples (individual C) was truly M1M2.

Statistical analysis

Differences between allele numbers in control and CHD patients were tested for significance by the χ^2 test. Differences between mean serum lipid levels in individuals with different genotypes were compared by a one-way analysis of variance to test the null hypothesis that there was no difference between the mean values for the three genotypes. Significance was estimated by calculating the variance ratio (F) and the number of degrees of freedom. Values for $P < 0.05$ were taken to indicate significance.

Results

Table I shows the allele frequencies at the XbaI locus in controls and patients with CHD. The overall X2 allele frequency was 0.16. The frequencies of the X1 allele did not differ significantly in the men with CHD compared with controls. In both the patients with CHD and in controls some individuals were hyperlipidaemic with plasma cholesterol above 6.7 mmol/l and plasma triglyceride above 2.0 mmol/l. Hypercholesterolaemic individuals had total plasma cholesterol above 6.7 mmol/l but total plasma triglyceride was below 2.0 mmol/l. Hypertriglyceridaemic individuals had total plasma triglyceride above 2.0 mmol/l but total plasma

TABLE I

Frequencies of XbaI alleles in control subjects and coronary heart disease (CHD) patients

	n	X1	*P*
Controls	190	0.800	n.s.
CHD	190	0.874	
Normocholesterolaemic Controls	172	0.808	< 0.05
Normocholesterolaemic CHD	166	0.885	
Normotriglyceridaemic Controls	166	0.795	< 0.025
Normotriglyceridaemic CHD	152	0.895	
Normolipidaemic Controls	156	0.801	n.s.
Normolipidaemic CHD	146	0.890	
Hyperlipidaemic Controls	34	0.794	n.s.
Hyperlipidaemic CHD	44	0.818	

n, number of alleles.
X1 = The ratio of the total number of alleles which were X1 type, ie restriction site absent.
P = The probability that the two populations in each set belong to a single population. The number of X1 and X2 alleles were counted for each group and the chi-squared test applied to determine whether the distribution of the alleles differed in each of the sets. n.s. indicates that $P > 0.05$.

cholesterol was below 6.7 mmol/l. The frequencies of the X1 alleles were increased in both normo-cholesterolaemic and normotriglyceridaemic patients with CHD compared with the frequencies of these alleles in controls with normal plasma cholesterol and triglyceride levels respectively.

The distribution of the serum lipid concentrations in individuals with the three genotypes at the XbaI locus are shown in Table II. Mean values for serum total cholesterol and LDL-cholesterol levels in individuals with different genotypes did

TABLE II

Serum lipid and lipoprotein concentrations in control subjects and CHD patients in each of the three genotypes at the XbaI locus

		X1X1	X1X2	X2X2
Controls (*n*)		62	28	5
CHD (*n*)		72	22	1
Total cholesterol	Controls	5.28 ± 1.16	5.01 ± 1.41	4.92 ± 0.62
(mmol/l)	CHD	5.50 ± 1.12	5.70 ± 1.20	6.0
Triglyceride	Controls	1.41 ± 0.71	1.48 ± 0.67	1.31 ± 0.46
(mmol/l)	CHD	1.57 ± 0.66	1.94 ± 1.14 ***	1.63
HDL-cholesterol	Controls	1.08 ± 0.24 *	1.1 ± 0.36 **	1.43 ± 0.20
(mmol/l)	CHD	0.96 ± 0.30	0.87 ± 0.39	0.65
LDL-cholesterol	Controls	3.42 ± 1.05	3.42 ± 1.27	3.03 ± 0.30
(mmol/l)	CHD	3.88 ± 0.99	3.87 ± 1.00	4.61

CHD, Coronary heart disease; all values are mean ± SD.
* X1X1 vs. X2X2 $P < 0.05$,
** X1X2 vs. X2X2 $P < 0.05$,
*** X1X1 vs. X1X2 $P < 0.05$.

TABLE III

Frequencies of MspI and EcoRI alleles in Apo B coding sequence in control subjects and coronary heart disease (CHD) patients

RFLP	Sample	*n*	Rare allele frequency	*P*
MspI	Controls	138	0.043	n.s.
	CHD	140	0.021	
EcoRI	Controls	32	0.031	n.s.
	Patients	32	0.063	

not differ significantly in the controls or patients with CHD. Plasma triglyceride levels in individuals with different genotypes in the control group and the plasma HDL level in individuals with different genotypes in the CHD group also showed no significant differences. However, there was a significant difference between the mean plasma HDL-cholesterol levels in controls with different genotypes, the lowest levels occurring in individuals with the X1X1 genotype and the highest in X2X2. The difference between the X1X1 and X2X2 values or between the X1X2 and X2X2 values were significant and the X1X2 mean value was intermediate between the two homozygotes. The X1X1, X1X2 difference was of too small a magnitude to be important. Plasma triglyceride levels in patients with CHD heterozygous for the restriction site, were significantly higher than in X1X1 patients and as only one individual with CHD had a genotype X2X2, his triglyceride level was not taken into account.

There was no significant difference in the frequencies of EcoRI and MspI RFLPs in the coding sequence of Apo B gene in controls and in patients (Table III). There was apparent linkage disequilibrium between XbaI and MspI RFLPs (Table IV)

As shown in Fig. 1 incomplete digestion of DNA with MspI restriction enzyme could result in incorrect assignment of MspI genotypes. To avoid this all samples which have been assigned M1M2 genotype have had MspI RFLP repeated with DNA digests prepared with higher concentration of MspI.

TABLE IV

Observed number of individuals with different paired genotypes for XbaI and MspI RFLPs of the Apo B gene

		XbaI polymorphism		
		X1X1	X1X2	X2X2
MspI	M1M1	95 (93.5)	32 (36.9)	3 (3.6)
Polymorphism	M1M2	0 (3.4)	2 (1.4)	3 (0.1)
	M2M2	0 (0.00)	0 (0.00)	0 (0.00)

Number expected if genotypes are in linkage equilibrium are given in parentheses.

Discussion

The frequency of the X2 allele in normal Sri Lankan subjects is 0.2. This contrasts both with the reported frequency of 0.5 in Caucasians [14,15–17] and that of 0.04 in Japanese [18]. The frequency of the rare allele of the MspI polymorphism in the Sri Lankan population was only 0.03 which is considerably less than the frequency in Caucasians reported as 0.12 [12] or 0.11 [19]. It will be interesting to see the frequencies for Indian and other populations. Does the Sri Lankan population reflect the Indian founders? Sri Lanka was colonised by Indo-Arians from the Ganges region starting in the sixth century, BC [20] and these people form the Sinhalese majority found in Sri Lanka today.

Several groups of workers have reported a positive association between the X1 allele and CHD [15,16], although this has not been found in all studies [17,18]. Hegele et al. [16] found a significant association between X1 allele and myocardial infarction in whites in the Boston area. Myant et al. [15] also reported a higher frequency of X1 allele in CHD patients than in control subjects though the difference was not significant. In the Seattle population [17] and in a Japanese population [18] such an association could not be demonstrated. In the present study the X1 allele was 7.4% more frequent in CHD patients than in controls but this difference was not significant. The selection of patients for this study has been random and not based on any particular phenotypic feature such as age of onset or major associated risk factor. In view of the heterogeneity of coronary heart disease and the random selection of cases, it is not surprising that a larger difference between cases and controls were not found. The widely different frequency of the *X*2 allele in different populations may be one of the reasons for the discrepancy of results in different studies on the association between XbaI polymorphism of the Apo B gene and CHD. The inconsistencies between the reports so far published may also arise from variation of X2 allele frequency within genetic subgroups of a population. In the present study patients and controls come from the same gene pool, i.e. same ethnic subgroup and same geographical locality. Our findings imply that some individuals who inherit the X1 allele coinherit a predisposing mutation to CHD linked to the Apo B gene.

Associations between the M2 allele and coronary heart disease [16] and the R2 allele and coronary heart disease [15–17], have been reported. There was no association between MspI RFLP or EcoRI RFLP and coronary heart disease in our study population. The Apo B MspI RFLP has been reported to be in linkage equilibrium with the XbaI RFLP [12]. We did not observe the X1M2 haplotype within our subjects and neither did Rajput-Williams et al. [19] in 290 Caucasian subjects.

Several groups have employed different strategies to overcome the problem of a methylated cytosine-sensitive enzyme such as MspI. Apparent lack of cutting at the polymorphic site can of course result in misdiagnosis of genotype. Huang et al. [12] used 80 U MspI per 10 μg DNA on their original digests but we preferred to check all our M2 possibilities again in case of individual sensitivity to digestion due to different degrees of methylation of different DNA samples to digestion. Rajput-Wil-

liams et al. [19] do not comment on the difficulties of elucidating the RFLP but Soria et al. [21] evade the methylated cytosine problem quite neatly by amplifying the region surrounding the MspI site using the polymerase chain reaction and then testing the PCR product (which will not be methylated and hence MspI will cut easily) for presence or absence of the MspI site. In the figure presented here, it will be noticed that the differential sensitivity of MspI sites to digestion is not limited to the polymorphic site at amino acid number 3611. The flanking upstream site at amino acid number 1485 [22] is not polymorphic but is poorly cut in some samples unless there is a very high concentration of MspI present so that a larger 'shadow' band is frequently seen on autoradiograms which consists of the M1 band of 6.4 kb still attached to an upstream fragment of 1.2 kb making up a total size of 7.6 kb. The concentration of this 7.6 kb band can be seen to decrease as the MspI concentration in the digest increased. However, because of our increasing MspI concentrations we are confident of our genotype designations in each of the cases presented in this paper.

Several workers have reported a significant association between X1 allele and lower levels of cholesterol and Apo B lipoprotein levels [11,14,23]. However this has not been found in other studies [16,17,24]. There was no significant association between X1 allele and serum cholesterol and low density lipoprotein levels in Sri Lankan subjects. The lack of association of X1 with serum cholesterol, low density lipoprotein cholesterol and its association with susceptibility to coronary heart disease suggest that regions of apolipoprotein B other than the LDL receptor-binding region are important in determining an individual's susceptibility to atherosclerosis. There is some evidence for this hypothesis from animal experiments. Swine with an allotypic variant of apolipoprotein B, LpB7, have normal or decreased low density lipoprotein with enhanced accumulation of cholesterol esters in macrophages and increased susceptibility to atherosclerosis [25,26].

The significantly low plasma HDL-cholesterol in those with the X1X1 genotype in the control group may have occurred by chance. However the same trend towards low HDL levels in those with X1X1 genotype has been observed [15,16]. Triglyceride-rich lipoproteins contribute significantly to the synthesis of HDL precursors. Therefore HDL synthesis and plasma triglyceride transport are intimately connected. This suggests the possibility that variation in the amino acid sequence of the Apo B-100 affects the transfer of lipids and apoproteins from triglyceride-rich lipoproteins to HDL.

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