Electronic letter

Fibrinogen genotypes (α and β) are associated with plasma fibrinogen levels in Chinese

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EDITOR—Several prospective epidemiological studies have consistently shown that raised plasma fibrinogen is an independent risk factor for coronary heart disease (CHD).[1–4] Clinical studies have also confirmed that plasma fibrinogen levels are related to the extent of coronary atherosclerosis in CHD patients.[5–7] Two other studies showed that raised fibrinogen level is an independent risk factor for mortality among male CHD patients.[8 9]

Plasma fibrinogen is an acute phase protein, levels of which rise rapidly in response to infection, inflammation, injury, or other trauma.[10] Many other environmental factors are also associated with increased plasma fibrinogen levels. They include age, obesity, blood cholesterol, lipoprotein,[11–13] hypertension,[1] diabetes mellitus,[14] and for women use of oral contraceptives and menopause.[11 12] Diet does not influence fibrinogen levels, whereas smoking can dramatically increase them.[11 12] This is dose dependent[1 15] and is reversible on cessation of smoking.[11] Moreover, it has been observed that a substantial part of the relationship between smoking and ischaemic heart disease is mediated through the effect of smoking on plasma fibrinogen levels.[3]

The genetic contribution to plasma fibrinogen levels has been widely investigated. The heritability of fibrinogen has been estimated to be 0.51 by path analysis.[16] Two twin studies estimated heritability to be 0.29-0.30.[17 18] Several restriction fragment length polymorphisms (RFLPs) of the fibrinogen gene are known to influence plasma fibrinogen level. A −455G/A RFLP at the promoter region (β gene) has been found to be associated with differences of fibrinogen levels in several white populations.[19–23] Another β gene −148C/T RFLP accounted for 4% of the total variance of fibrinogen levels in an American population.[24] In two British studies, the percentage of fibrinogen variance that could be explained by a *Bcl*I RFLP at the 3' end of the β gene was 2.4% in one[19] and 9% in another.[25] Although the *Taq*I RFLP at the α gene failed to show significant genotypic association with plasma fibrinogen levels in most of the studies, the combination of *Taq*I and *Bcl*I RFLPs explained 15% of fibrinogen variance,[25] while that of *Taq*I and −455G/A accounted for 8.9% of fibrinogen variance.[19] However, such a genotype association was not confirmed in other studies.[17 20 24 26] Many factors are responsible for the discrepancies in different studies, one of which is the variation in composition of their population samples particularly with regards to the ratio of males to females and smokers to non-smokers. In addition, these studies are also inconsistent in adjustment for a variety of well known confounding factors affecting plasma fibrinogen levels, such as age, sex, and body mass index (BMI).

In view of the controversy over the effect of genetic variations on plasma fibrinogen levels, we studied the genotype association of six fibrinogen polymorphisms with plasma fibrinogen levels in healthy Singaporean Chinese, a population with a relatively low prevalence of CHD. These included the −455G/A, −148C/T, +1689T/G, and *Bcl*I RFLPs on the β gene as well as the *Taq*I and T312A RFLPs on the α gene. We also investigated possible effect of interactions between environmental factors such as smoking and fibrinogen genotypes on plasma fibrinogen levels.

Subjects and methods

SUBJECTS

The participants for this study were recruited from volunteers who attended regular medical examinations as required for employment at the Singapore Anti-Tuberculosis Association (SATA) Chest and Heart Clinic. The response rate was about 70%. After excluding 36 subjects who suffered from hypertension, CHD, or diabetes mellitus, 403 healthy Chinese (191 males and 212 females) aged 20 to 74 were included in the present study. Since plasma fibrinogen levels are known to decline slowly on cessation of smoking, taking five or more years to return to the basal level,[27] we defined only two categories, non-smokers and smokers. The latter includes both current and ex-smokers who had stopped smoking for less than five years. BMI was calculated by the formula: BMI = Wt (Kg)/Ht^2(m^2).

LABORATORY INVESTIGATIONS

The details of blood sample collection were as previously described.[28] Plasma fibrinogen was estimated based on the method of Clauss[29] using an assay kit purchased from Biopool International, Sweden. The fibrinogen concentration was expressed as grams per litre (g/l).

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Table 1 Fragment length of PCR products for six RFLPs in the fibrinogen gene

RFLP	*Gene*	*Location*	*Amplicon size (bp)*	*Allele designation*	*Digested fragment length (bp)*
*Taq*I	α gene	3' end	900	T_2 T_1	*Taq*I(+): 100, 800 *Taq*I(−): 900
T312A	α gene	A/G (codon 312)	584	T312 A312	*Rsa*I(+): 78, 39, 170, 136, 56, 44, 39 *Rsa*I(−): 117, 170, 136, 56, 44, 39
−455G/A	β gene	G/A−455 (promoter)	1301	G^{-455} A^{-455}	*Hae*III(+): 272, 194, 835 *Hae*III(−): 466, 835
−148C/T	β gene	C/T−148 (promoter)	1301	C^{-148} T^{-148}	*Hin*dIII(+): 575, 383, 343 *Hin*dIII(−): 958, 343
+1689T/G	β gene	T/G+1689 (intron 1)	710	G^{+1689} T^{+1689}	*Ava*II(+): 250, 60, 400 *Ava*II(−): 310, 400
*Bcl*I	β gene	3' end	2500	B_2 B_1	*Bcl*I(+): 1400, 1100 *Bcl*I(−): 2500

Underlined fragments are constant bands in both alleles from each polymorphism as an internal control of enzyme activity.

The intra-assay coefficient of variation was 3% and inter-assay coefficient of variation was 4.8%.

Genomic DNA was extracted from white cells as described by Parzer and Mannhalter.[30] Six RFLPs were investigated using polymerase chain reaction (PCR) with the corresponding oligonucleotide primers.[31 32] Amplified PCR products were digested by appropriate restriction enzymes, except for the T312A RFLP, which needed simultaneous digestion by *Dde*I and *Rsa*I. Such double digestion eliminated comigrating digested products which may obscure the polymorphic fragments. Digested PCR fragments were separated by 1.5% agarose for *Bcl*I, 2% for *Taq*I, −455G/A, and −148C/T RFLPs, 3% for the +1689T/G RFLP, and 4% for the T312A RFLP. The fragment lengths of all PCR products and designations of the alleles are summarised in table 1.

STATISTICAL ANALYSIS

Allele frequencies were calculated by the gene counting method. Pairwise linkage disequilibrium coefficients (Δ) were estimated according to the equation given by Chakravarti *et al*.[33] The distribution of fibrinogen concentration was skewed as with previous reports.[25 34] As such, fibrinogen levels were $\log_{10}$ transformed for all the statistical analysis. For simplicity, geometric means (antilog value of the $\log_{10}$ means) of plasma fibrinogen are presented. Stepwise multiple regression analysis was performed on plasma fibrinogen so as to identify covariates that are independently associated with plasma fibrinogen levels. ANOVA was then performed to estimate the fibrinogen variance ($R^2 \times 100$) that would be explained by fibrinogen genotypes after adjustment for age, BMI, and any other significant variables identified by multiple regression in the various groups. All statistical analysis was performed using the SPSS statistical package. Significance level was set at 0.05.

Results

In the 403 healthy Chinese, the rare allele frequencies in −455G/A, −148C/T, +1689T/G, *Bcl*I, *Taq*I, and T312A RFLPs were 0.24 (95% confidence interval (CI) 0.21-0.27), 0.24 (95% CI 0.21-0.27), 0.24 (95% CI 0.21-0.27), 0.24 (95% CI 0.21-0.27), 0.43 (95% CI 0.39-0.46), and 0.43 (95% CI 0.40-0.47), respectively. Genotype distribution in all loci examined did not deviate significantly from a population in Hardy-Weinberg equilibrium, in the entire group, in separate gender groups, in smokers, or in non-smokers (p>0.05). All RFLP pairs within the α gene or β gene were in strong linkage disequilibrium with each other (Δ=0.95-0.97). The −455G/A-−148C/T pair was in complete linkage disequilibrium (Δ=1.000, p<0.0001). Weaker but significant disequilibrium of all intergene pairs was also observed (Δ=0.42-0.45, p<0.005).

ASSOCIATION OF POLYMORPHISMS WITH FIBRINOGEN LEVELS

β gene

Some earlier studies had shown that genotype association with plasma fibrinogen levels is gender specific.[21] In the present study, gender was found to be a significant variable contributing to plasma fibrinogen levels in multiple regression analysis (β=0.34, p<0.001). The one way ANOVA was therefore performed separately for men and women (table 2). Since the −455G/A and the −148C/T polymorphic loci were in complete linkage disequilibrium, only the data for the −455G/A RFLP is presented in this paper. Although significant genotype association was observed in women, there was a clear trend in both genders that rare homozygotes of the β gene (AA^{-455}, GG^{+1689}, and B_2B_2) had the highest mean fibrinogen levels while heterozygotes had levels intermediate between the two homozygous forms. This suggests the presence of a codominant effect. No evidence of genotype-genotype interaction within the β gene was found in men or women (p>0.05).

α gene

Significant genotype associations were observed in men, with rare allele homozygotes (T_2T_2 and A312A312) having the lowest plasma fibrinogen concentrations (p>0.005). The same trend was present in women although the difference did not attain statistical significance (p>0.05). When the 95% CIs of fibrinogen levels in the three genotypes of *Taq*I and T312A were examined, both the common homozygotes and the heterozygotes shared a similar level of fibrinogen while the rare homozygotes had significantly lower fibrinogen, indicating the recessive nature of the rare T_2 and A312 alleles. The genotypes of *Taq*I and T312A RFLPs explained 6.07% and 5.36% of fibrinogen variance, respectively (table 2). No evidence of genotype-genotype interaction

Table 2 Adjusted fibrinogen (FBG) levels (g/l) of different genotypes and $R^2 \times 100$ values in men and women

	Men			Women		
Genotype	No	FBG*	$R^2 \times 100$ (p)	No	FBG*	$R^2 \times 100$ (p)
−455G/A RFLP						
G/G^{-455}	107	2.51		118	2.75	
G/A^{-455}	72	2.63	1.27 (NS)	86	2.88	2.74 (0.027)
A/A^{-455}	11	2.69		8	3.02	
+1689T/G RFLP						
T/T^{+1689}	106	2.57		123	2.75	
T/G^{+1689}	72	2.69	1.89 (NS)	79	2.88	2.39 (0.042)
G/G^{+1689}	12	2.75		10	3.09	
BclI RFLP						
B_1B_1	110	2.57		123	2.75	
B_1B_2	70	2.69	1.59 (NS)	79	2.88	2.97 (0.021)
B_2B_2	11	2.75		10	3.09	
TaqI RFLP						
T_1T_1	55	2.63		80	2.88	
T_1T_2	94	2.75	6.07 (0.001)	94	2.82	0.80 (NS)
T_2T_2	40	2.40		37	2.69	
T312A RFLP						
T312T312	54	2.63		78	2.88	
T312A312	97	2.75	5.36 (0.003)	95	2.82	0.74 (NS)
A312A312	39	2.40		39	2.69	

*FBG = geometric means of fibrinogen levels adjusted for age, BMI, and other covariates. NS: non-significant.

Table 3 Adjusted plasma fibrinogen levels (g/l) of different genotypes in smokers and non-smokers (men)

	Non-smokers (n=131)			Smokers (n=60)		
Genotype	No	FBG*	$R^2 \times 100$ (p)	No	FBG*	$R^2 \times 100$ (p)
−455G/A RFLP						
G/G^{-455}	66	2.57	0.46 (NS)	41	2.51	3.38 (NS)
G/A^{-455} & A/A^{-455}	64	2.69		19	2.75	
+1689T/G RFLP						
T/T^{+1689}	66	2.57	0.37 (NS)	40	2.46	8.76 (0.022)
T/G^{+1689} & G/G^{+1689}	64	2.69		20	2.88	
BclI RFLP						
B_1B_1	68	2.57	0.44 (NS)	42	2.46	6.60 (0.049)
B_1B_2 & B_2B_2	63	2.69		18	2.82	
TaqI RFLP						
T_1T_1	39	2.63	2.79 (NS)	16	2.57	18.10 (0.004)
T_1T_2	65	2.69		29	2.82	
T_2T_2	26	2.46		14	2.14	
T312A RFLP						
T312T312	38	2.63	2.35 (NS)	16	2.57	16.28 (0.007)
T312A312	67	2.69		30	2.75	
A312A312	25	2.46		14	2.19	

*FBG = geometric means of plasma fibrinogen adjusted for age, BMI, and other significant covariates. NS: non-significant.

within the α gene was found in men or women (p>0.05).

Combination of α and β genes

The impact of combined genotypes of the α and β genes on plasma fibrinogen levels was subsequently examined. Considering that the linkage disequilibrium of all inter-gene pairs was very similar (Δ=0.42-0.45), the −455G/A RFLP is located in the promoter region of the β gene, and that the furthest T312A RFLP is located in the coding region of the α gene, haplotypes from these two RFLPs should provide the maximum information. However, since unequivocal haplotypes could not be obtained from subjects who were heterozygous at any locus of the two RFLPs, the available sample size became much smaller. Therefore, an alternative method using genotype combinations was adopted to evaluate the combined effect of the two RFLPs in this study.

Of the nine theoretically possible types of genotype combinations, only eight were present in this study. The A/A^{-455}/A312A312 combination was absent owing to low frequencies of these two rare alleles. As would be expected from observation of a single RFLP analysis, fibrinogen levels in the A/A^{-455}/T312A312 and G/G^{-455}/A312A312 groups were among the highest and lowest respectively (data not shown). The effect of genotype combinations was not significant in the women. The effect of genotype combinations was significant in the men ($R^2 \times 100$ is 6.74, p=0.044), but not to such an extent as to provide further contributions to the plasma fibrinogen variance in addition to the individual RFLPs alone. Therefore, this type of genotype combination in fibrinogen is of minimal practical use.

INTERACTION BETWEEN GENOTYPE AND SMOKING ON FIBRINOGEN LEVELS

In order to evaluate the effect of smoking on fibrinogen genotype association, we further stratified men into smokers and non-smokers. The number of female smokers was too small for a similar statistical analysis. Since the group size became smaller after stratification, the homozygotes and carriers of rare alleles of the β gene were pooled.

In men, no difference was detected in anthropometric parameters (age, BMI), blood lipids (total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, triglycerides) and plasma fibrinogen levels between smokers and non-smokers (p>0.05). However, significant genotype association was confined only to smokers (table 3). As before, the highest fibrinogen levels were associated with the rare homozygotes for the β gene, and the lowest fibrinogen levels with rare homozygotes for the α gene. The α gene RFLPs explained 16.3-18.1% of fibrinogen variance while those at the β gene explained 6.6-8.8% (p<0.01). When RFLPs at the β gene were included in the ANOVA model together with those at the α gene, the effect of the former was no longer significant. No genotype-genotype interaction was found between the α and β genes. The interaction between smoking and genotypes did not reach a level of statistical significance (p>0.05).

Discussion

In this study on the Chinese, β gene genotype associations with plasma fibrinogen levels remained significant in both men and women, despite adjustment for significant covariates. The rare alleles (A^{-455}, T^{-148}, B_2, and G^{+1689}) of the β gene were associated with higher fibrinogen levels. Such results are consistent with those observed in white populations.[19–25] One in vitro study showed that two alleles at −455G/A possess different binding characters for nuclear factors and further transfection assay showed that the A^{-455} allele significantly enhanced the transcription rate of the β gene.[35] Therefore, one of the possible mechanisms for genotype association at the β gene is the result of nucleotide substitution at a functional site such as −455G/A. The substitution affects β fibrinogen synthesis and, in turn, plasma fibrinogen level. The other three RFLPs in the β gene may be

markers of −455G/A RFLP owing to the results of complete (−455G/A *v* −148C/T) or strong (−455G/A *v* +1689T/G and −455G/A *v* *Bcl*I) linkage disequilibrium.

Further analysis in men showed that significant genotype association was confined to smokers (table 3). Since no difference in anthropometric and biochemical parameters, including plasma fibrinogen levels, was found between smokers and non-smokers, it appears that there is some kind of interaction between smoking and fibrinogen genotypes. As with our present findings, a European case-control study[23] on myocardial infarction found a trend towards higher fibrinogen levels in A^{-455} carriers in smokers but not in non-smokers. Green *et al*[20] reported that in male Swedes A^{-455} carriers had significantly higher fibrinogen levels than G^{-455} homozygotes, and that this genetic variation explained 12% of fibrinogen variance. Such a significant genotype association was confined to smokers only, and the interaction between smoking and fibrinogen genotypes was found to be significant (p=0.043). A more recent report on male Inuit smokers also confirmed significant genotype association at −455G/A and *Bcl*I loci.[21] However, a British study reported a significant genotype association between the −455G/A RFLP and fibrinogen levels with the same trend as ours, but in both smokers and non-smokers.[19] There was also no evidence of interaction between smoking and genotypes in a large Copenhagen City Heart study in the Danish.[36] Four possible factors may be responsible for such discrepancies. Firstly, the classification of smokers varied. In the Swedish, the Inuit, and the present study, ex-smokers were classified in the smoker group because plasma fibrinogen takes five years or more to return to basal levels.[27] However, in the British study, ex-smokers were classified as non-smokers. Secondly, the different amount of tobacco consumed per day by smokers would lead to different plasma fibrinogen levels and thus confound genotype association. Thirdly, the sample size was relatively small in the Swedish and Inuit studies. For example, sample size for non-smokers in the Swedish study[20] was only 25, while that for smokers in the Inuit study[21] was nearly 50 of which only two subjects carried the rare allele A^{-455}. Fourthly, mean age was different in these studies. In studies with significant genotype association observed in smokers, mean age was 30 for the Inuit and 40 for both the Swedish and Chinese populations. In contrast, subjects in studies that showed significant genotype association in both smokers and non-smokers were older. The British and Copenhagen cohorts had mean ages of 55 and 58, respectively. It is well established that plasma fibrinogen levels increase with age.

A plausible molecular mechanism for the smoker specific genotype association of the β gene with plasma fibrinogen levels could be proposed. It is known that the region from −150 to −82 bp upstream of the initiation site of transcription of the β fibrinogen gene is responsible for IL-6 induction.[37] Recently, three IL-6 response elements were described.[38] Smoking stimulates macrophages in the lungs to produce IL-6, which is then released into the circulation. When IL-6 reaches the liver, it stimulates the synthesis of fibrinogen protein by inducing the binding of nuclear transcriptional factors to IL-6 responsive elements of the β fibrinogen gene.[39 40] On the other hand, the four RFLPs of the β gene studied are in strong linkage disequilibrium with each other and, in particular, −148C/T RFLP at −150 bp upstream of the transcription start site is next to the IL-6 responsive elements. Recent studies have suggested that −148C/T influences the interaction between nuclear proteins and IL-6 response elements.[41 42] As mentioned earlier, one in vitro study found that base substitution at −455G/A affects the binding of nuclear proteins to the promoter region of the β gene.[35] Therefore, base substitutions at some functional RFLPs of the four linked loci may affect the binding of nuclear proteins to IL-6 responsive elements in the β gene, thereby influencing the transcription of β fibrinogen. Since the formation of β chains is the rate limiting step in the synthesis of the whole fibrinogen molecule,[43] base substitution in RFLPs of the β gene may influence the synthesis of fibrinogen protein.

It was also interesting to observe significant genotype association in the α gene in male smokers. The rare homozygotes for the *Taq*I or T312A RFLP had the lowest fibrinogen levels relative to the other two genotypes (tables 2 and 3). This trend is similar for the non-smoker males although the difference did not reach a statistically significant level. The sample size was too small meaningfully to stratify the females by smoking. In male smokers, the two α gene RFLPs appear to be associated strongly with fibrinogen levels relative to those in the β gene. When RFLPs at the β gene were included in the ANOVA model together with those of the α gene, the effect of the former was no longer significant. This suggests that the genotype association observed in the α gene may be of primary influence. The T312A RFLP is located in the coding region and could be functionally important, while the *Taq*I RFLP is located in the intron and may only be the marker of the former as a result of almost complete linkage disequilibrium. Based on the findings in the Chinese males, the effect of the two RFLPs at the α gene appears to be modulated by smoking.

In summary, this study in the Chinese confirms previous observations in white populations that −455G/A, −148C/T, +1689T/G, and *Bcl*I RFLPs in the β fibrinogen gene were associated with differences in plasma fibrinogen levels.[19 21 22 24] The A^{-455}, T^{-148}, G^{+1689}, and B_2 alleles were associated with higher fibrinogen levels. In males, such significant genotype association was confined to smokers only. In addition, strong genotype association in the α gene was also observed and confined to the Chinese male smokers. It explained more than 16% of fibrinogen variance in male smokers. This suggests a possible gene-environment interaction between smoking and fibrinogen genotypes.

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