RESEARCH NOTE

Genome association study of human chromosome 13 and susceptibility to coronary artery disease in a Chinese population

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Introduction

Coronary artery disease (CAD) is a complex disease and many genetic factors underlie pathogenesis of CAD. Several loci that are associated with susceptibility to CAD have been found. To find the genetic loci associated with CAD in Chinese population we performed a genome scan on chromosome 13 in CAD patients and healthy people from Shandong peninsula. Fourteen microsatellite markers on chromosome 13, spaced at approximately 10 cM, were used to screen DNA pool samples of 156 CAD patients and 1000 normal controls. Statistical analysis was performed using CLUMP software to compare the differences in allele frequency at each locus between the two pooled samples. We found significant statistical differences at *D13S263* and *D13S156* loci between allele frequencies in patients and those in controls (both, $P < 0.05$). The data provide support for the existence of two regions on chromosome 13 associated with CAD in this population.

CAD is one of the most important health problems worldwide causing a high rate of mortality and morbidity in both developed and developing countries. Classic epidemiological studies have revealed many risk factors for CAD, including increasing age, gender, hypertension, dyslipidaemia, obesity, diabetes and smoking (McEvoy *et al*. 2011). However, some populations are more susceptible than others to CAD even when exposed to very similar environments (Lanktree and Hegele 2009). Previous epidemiological surveys, pedigree investigations and twin studies have all suggested that there is a strong genetic factor underlying this complex disease (Marenberg *et al*. 1994; Silberberg *et al*. 1998). Genomewide association studies (GWAS), have been widely used to identify chromosomal regions associated with diseases. Many genes associated with susceptibility to CAD have been discovered. Some of these genes have been found in most populations studied, but reports on chromosome 13 and CAD are rare (Roberts *et al*. 2011). Susceptibility genes for CAD at 13q12, 13q13.1, 13q14.11 and 13q34 were found in some populations, but unlike other chromosomal studies (e.g. chromosomes 1, 3 and 9), studies on chromosome 13 have not been replicated in Chinese subjects (Knoblauch *et al*. 2000; Arking *et al*. 2003; Helgadottir *et al*. 2004; Koch *et al*. 2007; Assimes *et al*. 2008). We therefore performed a chromosome-wide scan in samples from CAD patients and controls in Shandong peninsula of China. To the best of our knowledge, the present study is the first to describe the relationship between chromosome 13 and CAD in a Chinese population. The present results may help to characterize susceptibility genes that affect cardiovascular disease in this Chinese population.

Materials and methods

Subjects

The patient group consisted of 156 CAD patients, 121 males and 35 females, aged between 17 and 61. CAD had been confirmed by coronary angiography (CAG) in Qilu Hospital

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of Shandong University in the period from May 2007 to October 2007. Criteria for inclusion were as follows: (i) clinical presentation; (ii) electrocardiogram (ECG) changes in line with 1979 WHO diagnostic criteria (Beanlands *et al*. 2007) for development of coronary heart disease; (iii) CAG that showed that at least one coronary artery stenosis ≥ 50% diameter; (iv) exclusion of familial hypercholesterolaemia, severe liver and kidney diseases. The control group consisted of 1000 apparently healthy blood donors selected from the Blood Center of Shandong province (702 males and 298 females), aged between 17 and 55. However, blood lipid level, hypertension, diabetes and CAD history were not screened because of the large sample size. The cases and controls were all from Shandong peninsula, with no blood relationship between individuals. This study obtained ethical approval from Medical Ethics Committee of Qilu Hospital of Shadong University and written informed consent of all subjects.

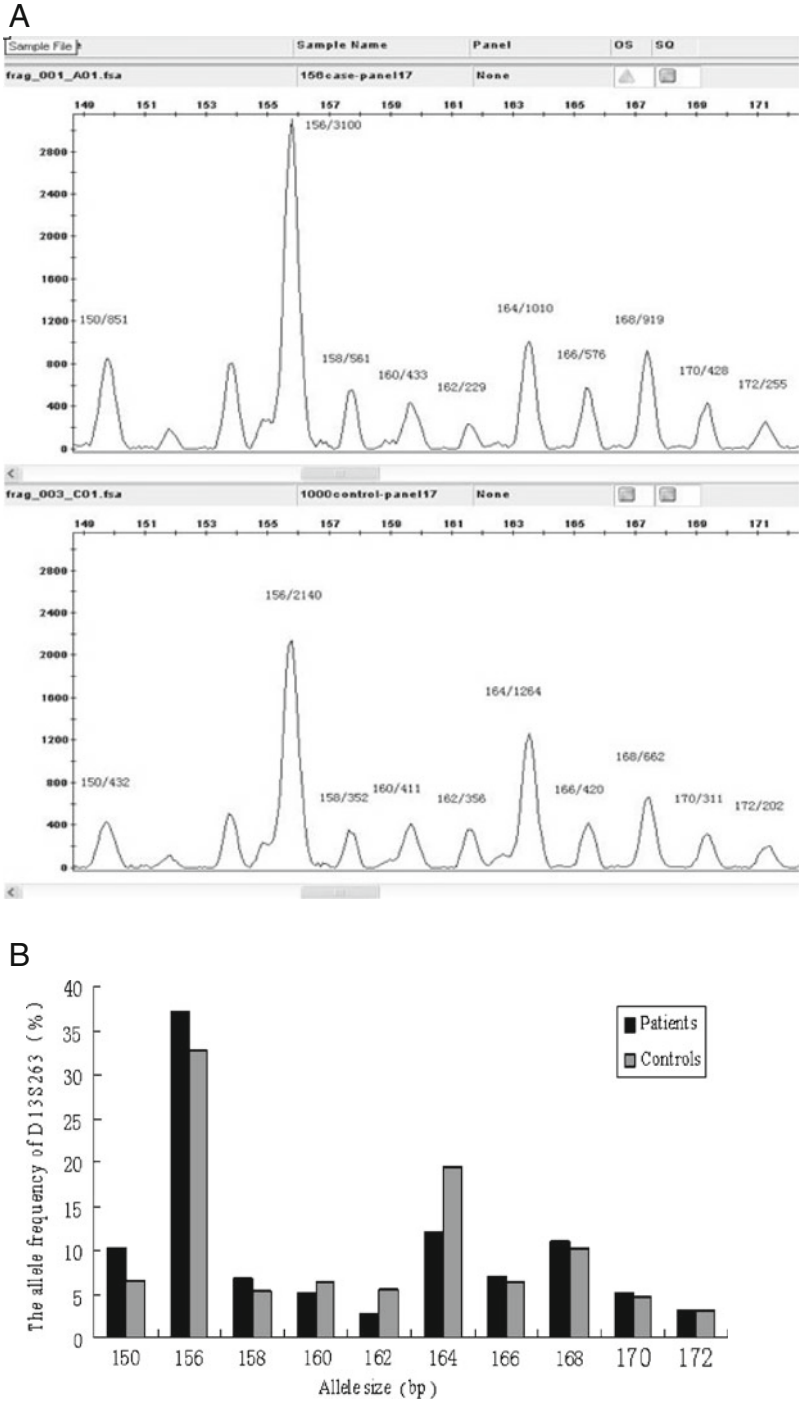

Figure 1. **A** Allele image pattern (AIP) for *D13S263* in patients (top) and controls (bottom). **B** Allele frequencies for *D13S263* in patients and controls.

Preparation of DNA pools

Genomic DNA was extracted from peripheral arterial blood (5 mL, taken into EDTA) by a modified phenol–chloroform method (Iacovacci *et al*. 2003). DNA concentration was measured in duplicate using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), samples showing high variation (> 10% difference) were measured again and the mean of the three measurements was used (Liguori *et al*. 2003). DNA samples were diluted to 20 ng/μL as working concentrations. Two DNA pools were made, one from CAD patients and one from controls.

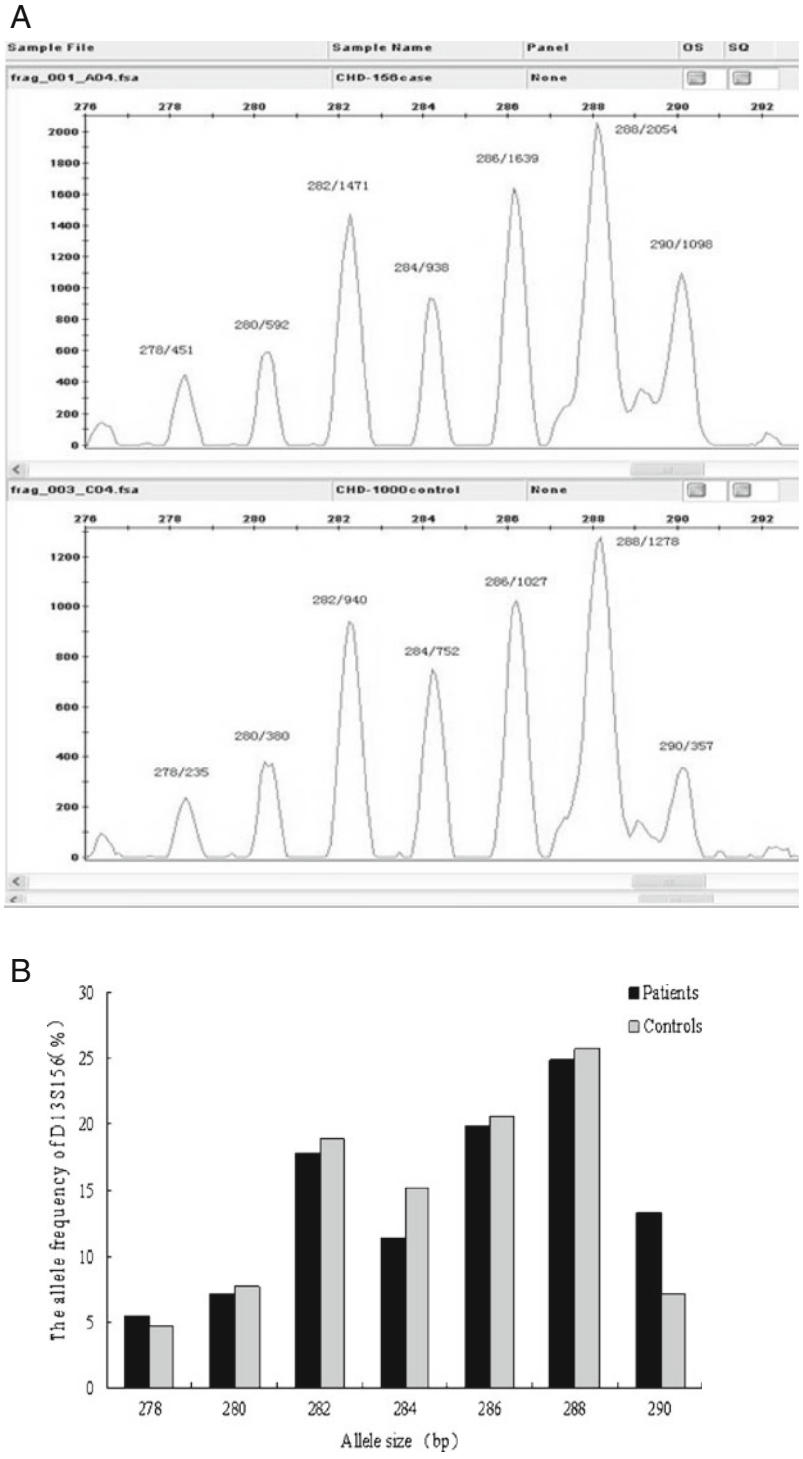

Figure 2. **A** Allele image pattern (AIP) of *D13S156* in patients (top) and controls (bottom). **B** Allele frequencies for *D13S156* in patients and controls.

Figure 3. Location of *D13S263* and *D13S156* on chromosome 13.

Genotyping

We used microsatellite markers from the ABI PRISM Linkage MappingSet MD-10 (Applied Biosystems, Foster City, USA) comprising 14 markers with an average spacing of 10 cm on chromosome 13. Multiplex polymerase chain reaction (multiplex PCR) in a 15 μL volume was performed with 60 ng of genomic DNA, 5 μM of primer, 9 μL of Takala multiplex PCR reaction mix, 0.6 U of Takala DNA polymerase. Amplification was performed on a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Foster City, USA) with the following parameters: 95°C for 12 min initial denaturation, followed by 10 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, and 20 cycles of 89°C for 15 s, 55°C for 15 s, 72°C for 30 s, and then terminated at 72°C for 10 min. Electrophoresis was carried out on ABI PRISM 3130-Avant Genetic Analyzer, by mixing up of nine PCR products simultaneously which had no overlap of products of the same size range labelled with same dye. Genotyping was performed with GeneMapper 3.5 software (Liu *et al*. 2009) (Applied Biosystems, Foster City, USA).

Statistical analysis

The PCR amplified results of pooled DNA samples was constituted by a series of allele image pattern (AIP) maps. The number of peaks reflected the alleles in the DNA pools and the height of the each peak reflected the allele frequency of that allele. The predicted allele frequencies of microsatellites in the two DNA pools were first estimated by dividing each allele peak height obtained by the GeneMapper 3.5 software with the sum of all peak heights of that marker (Collins *et al*. 2000). Peak heights on the AIP maps were calculated using CLUMP software for chi-square test and $P < 0.05$ was taken as significant difference. Analysis of individual genotyping at loci *D13S263* and *D13S156* were also performed using CLUMP (Sham and Curtis 1995).

Results

The genotypes of all 14 microsatellite markers on chromosome 13 were verified at the end of the analysis. Figures 1A and 2A show the AIP maps. All the genetic loci were polymorphic in cases and controls, and the numbers of alleles on these loci ranged from 4 to 15. We used CLUMP software for comparing the difference in allele frequency of each locus between the two groups and contrasted the distributions of the alleles. Significant statistical differences were found between allele frequencies in cases and controls at markers *D13S263* ($\chi^2 = 15.412620$, df $= 0.002319$, $P = 0.014985$) and *D13S156* ($\chi^2 = 14.152888$, df $= 0.000170$, $P = 0.003996$). Figure 1B shows that there were 10 alleles at *D13S263* (150, 156, 158, 160, 162, 164, 166, 168, 170 and 172 bp), and the frequency of the 156 bp allele in cases was significantly higher than that in controls, while the frequency of the 164 bp allele in cases was significantly lower than that in controls. Figure 2B shows that there were seven alleles at *D13S156* (278, 280, 282, 284, 286, 288 and 290 bp), and the frequency of the 290 bp allele in cases was significantly higher than that in controls, while the frequency of the 284 bp allele in cases was significantly lower than that in controls.

The locations of these two loci on chromosome 13 are shown in figure 3 and all the statistical results are shown in table 1. Allele frequencies at the remaining 12 loci showed

Table 1. Association between CAD and microsatellite markers on chromosome 13.

Marker	Location	Position (UCSC)	No. of alleles	χ^2	P
D13S175	13q12.11	19746380- 19746734	6	4.377862	0.310689
D13S217	13q12.3	28269791-28270155	9	11.159211	0.063936
D13S171	13q13.1	32151912- 32152220	4	0.345870	0.935065
D13S218	13q13.3	37930231-37930566	6	5.746546	0.151848
*D13S263**	13q14.11	40978920-40979230	10	15.412620	0.014985
D13S153	13q14.2	47788735- 47789009	12	6.972342	0.513487
*D13S156**	13q22.1	73555426- 73555776	7	14.152888	0.003996
D13S170	13q31.1	80007095- 80007379	15	0.040886	1.000000
D13S265	13q31.3	89170921-89171101	9	4.034729	0.613387
D13S159	13q32.2	97851595- 97851757	15	5.329424	0.854146
D13S158	13q33.1	102774398- 102774526	6	7.306865	0.104895
D13S173	13q33.3	106604890- 106605187	11	5.489471	0.0.489510
D13S1265	13q33.3	108126458- 108126828	10	7.468988	0.290709
D13S285	13q34	111843383- 111843564	14	4.766732	0.856144

*$P < 0.05$ versus controls.

no significant statistical difference between patients and controls, and we conclude that they have no association with CAD.

Discussion

With completion of the Human Genome Project and other developments in molecular genetics, molecular biology techniques play an important role in research on CAD (Hsu and Smith 2012). Barcellos *et al*. (1997) proposed the DNA pooling approach, in which many individual DNA samples could be mixed in the PCR reaction. Compared to ordinary PCR, the advantages of pooling method are that it is economical and efficient, so it was applied in the Genetic Analysis of Multiple Sclerosis in Europeans (GAMES) project and achieved satisfactory results (Johnson and Griffiths 2005). In China, Chen *et al*. (2007) used this method for schizophrenia, and Xie *et al*. (2009) found two loci associated with CAD; these two studies further proved the effectiveness of this method. As a complex genetic disease, CAD may have genetic heterogeneity. From the AIP maps in our study, most genetic loci in patients and controls were similar, indicating that the samples selected had quite high homogeneity.

After scanning microsatellite markers on chromosome 13, we found that *D13S263* and *D13S156* had association with CAD. Susceptibility genes could be located in and around these regions. The location of marker *D13S263* is 13q14.11. Vanhoof *et al*. (1996) found that gene that encodes plasma carboxypeptidase B2 (*CPB2*) in humans is located here. CPB2, also known as thrombin-activatable fibrinolysis inhibitor (TAFI), is synthesized by the liver and circulates in plasma as a plasminogen-bound zymogen. As bradykinin is one of the substrates of TAFI, the *CPB2* gene that encodes TAFI is a candidate gene for blood pressure regulation. Boffa *et al*. (1999) identified a single-nucleotide polymorphism (SNP) in the *CPB2* coding region, 1057C > T, predicting an Ile325-to-Thr substitution in the mature TAFI polypeptide. Koschinsky *et al*. (2001) found that homozygotes for *CPB2* 1057T had significantly lower diastolic blood pressure than subjects with other *CPB2* genotypes in aboriginal Canadians. *CPB2* mutation can also lead to increase of fibrin, platelets and blood viscosity, which are critical mediators in pathogenesis of CAD (Santamaría *et al*. 2004). Tregouet *et al*. (2009) studied 1668 individuals with angiographically proven CAD and the results demonstrated that plasma-activated TAFI levels, measured by a TAFIa/TAFIai ELISA are strongly predictive of future cardiovascular death (CVD) in CAD patients, independently of any conventional cardiovascular risk factors. The contribution of *CPB2* polymorphisms to the variability of the total amount of TAFI (t-TAFI) levels was mainly due to two SNPs, the 2599 C > G and 1583T > A polymorphisms. In addition, the Ile isoform at the Ile325Thr site was associated with slightly decreased TAFIa/TAFIai levels. Even though the Ile325Thr and 1583T > A polymorphisms were associated with TAFIa/TAFIai levels, they did not significantly affect risk of CVD, suggesting that the increment of plasma TAFIa/TAFIai levels observed in patients with CVD in the future probably has another origin. However, these results did not assess whether elevated TAFIa/TAFIai levels played a causal role or were just secondary to the disease process. Our present results show significant statistical differences in allele frequencies in CAD patients and controls at 13q14.11. It is consistent with the results of several studies carried out in other populations (Vanhoof *et al*. 1996; Boffa *et al*. 1999; Koschinsky *et al*. 2001; Tregouet *et al*. 2009), and further supports the view that similar susceptibility genes might exist in Chinese population.

The most important finding in our study is that *D13S156* is associated with CAD in a Chinese Han population in Shandong province. Knoblauch *et al*. (2000) studied such a family in Israel and found a cholesterol-lowering gene located at 13q, defined by markers *D13S156* (13q22.1) and *D13S158* (13q33.1). They further confirmed the two genetic variances with regard to lipid concentrations. In a Syrian family with autosomal recessive hypercholesterolaemia, Al-Kateb *et al*. (2002) found linkage of the disorder to both 1p36.1-p35 and 13q22-q32. In affected family members, they identified homozygosity for an autosomal recessive mutation in the *ARH* gene on chromosome 1 and found evidence for an interaction between *ARH* and the chromosome 13 locus. They determined the exon–intron structure of *ARH*. All splice sites conform to the GT/AG rule, except intron 8, where the donor splice site GT was converted to GG. Classic experiments indicate that increased low-density lipoprotein cholesterol (LDL-C) is essential for development of atherosclerosis. On the other hand, low LDL-C levels are not only well tolerated, but appear to be necessary to avoid coronary atherosclerotic lesions (Ferdinand 2011). In our study, we found that *D13S156* (13q22.1) was associated with CAD, suggesting that there might be susceptibility genes for CAD located at this region or the actual gene might lie in some other chromosome but with a translocation at chromosome 13. However, levels of LDL of the CAD patients in our study were almost in the normal range (Sjouke *et al*. 2011); and only further study can show whether the mutation of this gene is similar to the findings of Knoblauch *et al.* (2000) and Al-Kateb *et al*. (2002). Sabater-Lleal *et al*. (2008) conducted a genomewide linkage scan for genes affecting variation in plasma TAFI levels in 398 subjects from 21 extended Spanish families and found a strong linkage on chromosome 13q, around the marker *D13S156*, where the structural gene that encodes TAFI is located. The gene location for TAFI is inconsistent with the result of Vanhoof *et al*. (1996), but they both are related to CAD and also supported by our results that *D13S263* or *D13S156* have relationship with CAD.

Our study also gave some negative results that are inconsistent with some previous reports. Helgadottir *et al*. (2004) mapped a gene that causes predisposition to myocardial infarction to location 13q12-13, A four-marker SNP haplotype spanning the gene *ALOX5AP*, which encodes

5-lipoxygenase activating protein (FLAP), is associated with a two-fold greater risk of myocardial infarction in Iceland, but Koch *et al*. (2007) failed to replicate the association of polymorphisms in the *ALOX5AP* gene and myocardial infarction. Knoblauch *et al*. (2000) suggested that *D13S158* (13q33.1) had linkage with a cholesterol-lowering gene. Arking *et al*. (2003) had found that the *KL-VS* allele of the *KLOTHO* gene was associated with early-onset CAD. Girelli *et al*. (2000) found that certain factor VII genotypes have a role in protection against myocardial infarction. In our study, locations 13q12-13, 13q13.1, 13q33.1 and 13q34 had no relationship with CAD, these differences may be due to the genetic heterogeneity in different populations.

One limitation of our present study is the area of the study population, as the sample size was small and only came from the Shandong province of China, which cannot represent all the Chinese population. Another limitation of the study is that we just found the susceptibility regions associated with CAD, but did not identify which genes were mutated and their influence on functions.

In conclusion, our results have confirm the association between chromosome 13 and CAD in a Chinese Han population in Shandong province and identified two regions, which may have susceptibility genes for CAD.

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