

ORIGINAL ARTICLE

Genome-wide linkage and association scans for pulse pressure in Chinese twins

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Elevated pulse pressure (PP) is associated with cardiovascular disorders and mortality in various populations. The genetic influence on PP has been confirmed by heritability estimates using related individuals. Recently, efforts have been made by mapping genes that are linked to the phenotype. We report the results of our gene mapping studies conducted in the Chinese population in mainland China. The genome-wide linkage and association scans were carried out on 63 middle-aged dizygotic twin pairs using high-density markers. The linkage analysis identified three significant linkage peaks (all with a single point $P < 1e^{-05}$) on chromosome 11 (LOD core 4.06 at 30.5 cM), chromosome 12 (LOD score 3.97 at 100.7 cM) and chromosome 18 (LOD score 4.01 at 70.7 cM), with the last two peaks closely overlapping with linkage peaks reported by two American studies. Multiple regions with suggestive linkages were identified, with many of the peaks overlapping with published linkage regions. The genome-wide association analysis detected a suggestive association on chromosome 4 (rs17031508, $P < 8.34e^{-08}$) located within a wide region of suggestive linkage. Our results provide some evidence for genetic linkages and associations with PP in the Chinese population. Further investigation is warranted to replicate the findings and to explore the susceptibility loci or genes for PP.

Hypertension Research (2012) 35, 1051–1057; doi:10.1038/hr.2012.90; published online 5 July 2012

Keywords: association analysis; Chinese twins; genome-wide; linkage analysis; pulse pressure

INTRODUCTION

Pulse pressure (PP) measures the change in blood pressure during a contraction of the heart. Elevated PP has been associated with cardiovascular disorders and mortality.¹ Several anthropometric factors have been shown to affect PP. For example, aging results in increased stiffness and loss of elasticity in the aorta and major artery, leading to increased PP.² In addition, body size (and consequently, gender) affects PP by altering the magnitude and timing of wave reflection.^{3,4} Recently, the age and gender effects have been shown to be cross-cultural.⁵

In addition to anthropometric factors, genetic epidemiology studies using family and twin data have provided verifiable evidence for a low to modest genetic contribution to PP with heritability estimates ranging from 0.13 using twins in the United Kingdom,⁶ 0.21⁷ and 0.24⁸ in European pedigrees, to 0.35 and 0.54 in American Caucasian⁹ and African¹⁰ families, respectively. An even higher heritability estimate of 0.63 was reported by a Swedish study.¹¹ A recent analysis of our Chinese twin data showed a heritability estimate of 0.45 (unpublished data), which is consistent with the American and African data. These results indicate that, similar to all

other complex traits, the etiology of PP can be complicated due to the involvement of both genetic and environmental dissections.¹²

The existence of a genetic contribution has warranted efforts to elucidate genes that affect PP, especially in recent years. For example, genetic marker-based linkage studies have identified multiple loci harboring susceptibility genes for PP in different populations, including Caucasians,^{9,13,14} Mexicans,¹⁵ American Indians¹⁶ and African Americans.¹⁷ The verification of susceptibility loci requires replication studies within and across populations to consolidate linkage results, which is especially important given the fact that inconsistent linkage results were reported by different studies.¹²

Twins are of special interest for genetic studies of complex diseases or phenotypes because, as naturally matched pairs, the confounding effects of a large number of potentially causal factors are matched when comparing twins who share them. In addition to heritability estimates, twins also serve as valuable samples for gene mapping advantaged by low confounding and low probability of non-paternity, which results in increased power.¹⁸ We report herein the results from the first extensive twin-based genetic epidemiology study on PP in the Chinese population, where hypertension has become the most

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Received 21 November 2011; revised 17 April 2012; accepted 18 April 2012; published online 5 July 2012

common risk factor for cardiovascular and cerebrovascular diseases, which are among the top three leading causes of death in China. Genome-wide gene mapping was conducted using high-density single-nucleotide polymorphism (SNP) markers, enabling both linkage and association analyses across the genome. The results from our study will be compared with those reported in the literature, and the significance of our findings will be discussed.

METHODS

Twin samples

All of the twins were taken from the most recent wave of twin sampling for heritability study on multiple phenotypes associated with metabolic syndrome conducted by the Qingdao Twin Registry^{19,20} at the Qingdao Center for Disease Control and Prevention in 2008 (Table 1). Twins were sampled through the local disease control network and residence registry. After receiving written consent, the twins were invited to a clinical investigation if both co-twins were alive. Those who were pregnant, breastfeeding, had known diabetes and/or cardiovascular disease, or were regularly taking any medications within 1 month before participation were excluded, and incomplete twin pairs were discharged. The zygosity of like-sex twin pairs was determined by DNA testing using 16 short tandem repeat DNA markers.

Phenotypes

Blood pressure was measured in a sitting position following standard procedure using a mercurial table stand model sphygmomanometer. Systolic blood pressure was calculated as Korotkoff phase I (appearance of sound), and diastolic blood pressure was calculated as Korotkoff phase V (disappearance of sound). Three repeated measurements were taken for each subject, with at least 1 min between each measurement. The mean of these three measurements for systolic or diastolic blood pressure was used for analyses. PP was taken as the difference between the systolic and diastolic blood pressures. Body mass index (BMI) was calculated by taking one subject's weight (in kg) and dividing it by the subject's height (in meters) squared. All measurements greater than 3 standard deviations above or below the mean were assigned as missing values. In Table 1, we present the descriptive statistics for monozygotic (MZ) and dizygotic (DZ) twins and their phenotypes separately. The generalized estimating equation model²¹ was used to adjust the effect of age, sex and BMI on PP, all of which showed high statistical significance. The adjusted PP (that is, the residuals) was used for calculating the intra-class correlation coefficient²² and for the heritability estimation. In Table 1, the intra-class correlation coefficient in MZ twins is over two times higher than that in the DZ twins, suggesting the existence of genetic effects. In fact, results from our twin modeling showed that the model with additive genetic (A) and unique environment (E) effects (that is, the AE model) was the best model with a heritability estimate of 45%, meaning that nearly half of the variation in PP is determined by genetic factors. These data form the basis for subsequent gene mapping.

DNA samples and genotyping

From the 241 pairs of DZ twins, 63 pairs were randomly selected for gene mapping using both linkage and association analyses. Whole-blood samples were taken for extracting leukocytes for DNA purification and genotyping using standard procedures. We used the Affymetrix Genome-Wide Human SNP Array 6.0 featuring ~900 000 SNP markers enabling high-resolution genome-wide analyses. Detailed information about the array can be found at the manufacturer's website (www.affymetrix.com). Genotyping was performed by the Affymetrix service provider, Shanghai Biochip, in China using purified DNA samples. High-quality genotyping was performed with a SNP calling rate ranging from 93.4 to 99%. Before data analysis, we introduced an error detection procedure (*-error*) implemented in the free linkage software package Merlin.²⁰ The procedure finds genotypes that provide information about gene flow in a pedigree that contradicts information provided by the other pedigrees. The procedure considers all available data simultaneously such that accuracy for each pedigree can be improved. The procedure retrieves a list of unlikely genotypes, which are then removed from the data using the Merlin

Table 1 Descriptive, regression and correlation analyses of twin data

	MZ, 389 pairs		DZ, 241 pairs	
	Mean	s.d.	Mean	s.d.
Age, years	37.36	9.29	36.26	9.29
BMI, kg m ⁻²	23.89	3.04	23.82	3.16
SBP, mm Hg	118.08	15.52	120.05	15.30
DBP, mm Hg	80.07	11.15	81.43	10.75
PP, mm Hg	38.80	8.77	39.44	9.32
<i>GEE for PP</i>	<i>Coefficient</i>	<i>P-value</i>	<i>Coefficient</i>	<i>P-value</i>
Age, year	0.003	3.85e-04	0.004	1.69e-03
Sex, m = 1; f = 2	-0.099	3.62e-10	-0.081	7.99e-05
BMI, kg m ⁻²	0.222	1.94e-04	0.226	4.69e-03
ICC*	rMZ	<i>P-value</i>	rDZ	<i>P-value</i>
	0.42	5.00e-07	0.19	3.06e-03

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; DZ, dizygotic; GEE, generalized estimating equation; ICC, intra-class correlation coefficient; PP, pulse pressure; MZ, monozygotic; SBP, systolic blood pressure.
*ICCs in MZ and DZ twins are statistically significantly different with a *P*-value of 1.90e-03 (*Z* = 3.10).

Table 2 Results of error detection by chromosome

Chromosome	Number of SNPs	Number of valid SNPs	Number of SNPs with genotyping error	Error rate* (%)
1	73 249	72 301	948	1.29
2	75 893	75 023	870	1.14
3	62 251	61 474	777	1.25
4	57 532	56 614	918	1.59
5	57 879	57 249	630	1.09
6	57 838	57 228	610	1.05
7	48 327	47 815	512	1.06
8	49 839	49 267	572	1.15
9	42 658	42 288	370	0.87
10	49 510	49 112	398	0.80
11	45 920	45 520	400	0.87
12	43 787	43 400	387	0.88
13	35 089	34 677	412	1.17
14	28 882	28 546	336	1.16
15	26 728	26 516	212	0.79
16	28 636	28 335	301	1.05
17	21 327	21 117	210	0.98
18	27 170	26 867	303	1.12
19	12 312	12 190	122	0.99
20	23 480	23 295	185	0.79
21	12 906	12 764	142	1.10
22	12 003	11 832	171	1.42
Total	893 216	883 430	9786	1.10

Abbreviation: SNP, single-nucleotide polymorphism.

*Percentage of SNPs excluded from analysis according to defined criteria.

command *pedwipe*. In Table 2, we show the information about error detection for each chromosome separately. As shown in the table, the SNP genotyping error rate for each chromosome is very low with a mean of only 1.1%. The criteria for quality control and SNP filtering include a *P*-value for defining the Hardy-Weinberg equilibrium, which was set to $\geq 1e^{-04}$, the SNP call rate set to $\geq 95\%$ and the sample call rate set to $\geq 95\%$.

Table 3 Significant and suggestive linkage peaks for pulse pressure

<i>Chromosome location</i>	<i>Closest marker</i>	<i>Reference SNP</i>	<i>LOD score</i>	<i>Point P-value</i>	<i>Genome significance</i>	<i>Published reference no.</i>
<i>Chromosome 1</i>						
90.6 cM	SNP_A-2061574	rs11584915	3.05	9.0e-05	No	
196.9 cM	SNP_A-8711523	rs12118925	3.54	3.0e-05	No	16
<i>Chromosome 3</i>						
32 cM	SNP_A-2179383	rs9869761	2.86	1.4e-04	No	12
93.9 cM	SNP_A-8508966	rs1387735	2.50	3.0e-04	No	
152 cM	SNP_A-2051216	rs838610	3.60	2.0e-05	No	
<i>Chromosome 4</i>						
116 cM	SNP_A-1916183	rs2850413	3.08	8.0e-05	No	
<i>Chromosome 5</i>						
83.3 cM	SNP_A-2158948	rs33417	2.57	3.0e-04	No	
<i>Chromosome 6</i>						
78.3 cM	SNP_A-8667531	rs9357726	2.75	2.0e-04	No	12
143.9 cM	SNP_A-1941963	rs9494756	3.19	6.0e-05	No	
<i>Chromosome 7</i>						
57.7 cM	SNP_A-8399705	rs6973458	3.24	6.0e-05	No	
74.4 cM	SNP_A-4247483	rs10270531	3.41	4.0e-05	No	13; 31
102.5 cM	SNP_A-8635038	rs7781900	3.18	7.0e-05	No	
<i>Chromosome 8</i>						
26.5 cM	SNP_A-8293021	rs17216024	2.79	2.0e-04	No	
<i>Chromosome 9</i>						
159.5 cM	SNP_A-8464500	rs11103695	3.49	3.0e-05	No	
<i>Chromosome 10</i>						
134.5 cM	SNP_A-1832075	rs7067647	2.80	2.0e-04	No	
<i>Chromosome 11</i>						
30.5 cM	SNP_A-8636879	rs11603765	4.06	1.0e-05	Yes	
<i>Chromosome 12</i>						
100.7 cM	SNP_A-8445857	rs2085866	3.97	1.0e-05	Yes	12
<i>Chromosome 15</i>						
14.8 cM	SNP_A-8688089	rs12592159	2.70	2.0e-04	No	
115.6 cM	SNP_A-8676706	rs7177487	2.32	5.0e-04	No	13
<i>Chromosome 16</i>						
3.1 cM	SNP_A-2181571	rs4984954	2.87	1.4e-04	No	
<i>Chromosome 17</i>						
75.3 cM	SNP_A-1842000	rs17645899	3.32	5.0e-05	No	
<i>Chromosome 18</i>						
70.7 cM	SNP_A-2005638	rs2576052	4.01	1.0e-05	Yes	31
<i>Chromosome 20</i>						
86.1 cM	SNP_A-2133602	rs16997979	2.38	5.0e-04	No	
<i>Chromosome 22</i>						
5.6 cM	SNP_A-1970673	rs5746419	3.04	9.0e-05	No	

Bold entries indicate linkages that reached genome-wide significance.

Genome-wide linkage analysis

We applied the variance components method for non-parametric linkage analysis²³ to our genome-wide SNP marker data on the DZ twins. Data were analyzed with the linkage package Merlin.²⁴ The variance components²⁵ ($-vc$) procedure was used to test for linkage of PP with age, sex and BMI incorporated as covariates.

Although our high-density SNP map increases the linkage information content that is important in the non-parametric linkage analysis,²⁶ it could also result in a linkage disequilibrium (LD) among adjacent SNPs. When parental phase information is missing, LD can lead to an overestimation of the number of alleles shared identical-by-descent and thus inflate the LOD score estimates.²⁷ To accommodate LD, we adopted a SNP-dropping strategy similar to Cho and Dupuis²⁷ by picking up SNPs for linkage analysis at approximately every 5 cM. According to their simulation study, this stringent strategy efficiently eliminates the LOD score inflation due to LD in the case of dense markers.

The genome-wide significance of the identified linkage peaks was assessed by applying the threshold criterion provided by Lander and Kruglyak²⁸ with a score of 2.2 for suggestive linkages and 3.6 for genome-wide significant linkages.

Genome-wide association analysis

The high-density SNP markers enable us to perform a genome-wide association study (GWAS) on PP. To this end, the score test ($-fastAssoc$) for the family-based association analysis implemented in Merlin was used, with age, sex and BMI included as covariates. The family-based association test allows phenotype correlation among family members (in this case, the twin pair) by introducing a kinship coefficient in the variance component-based

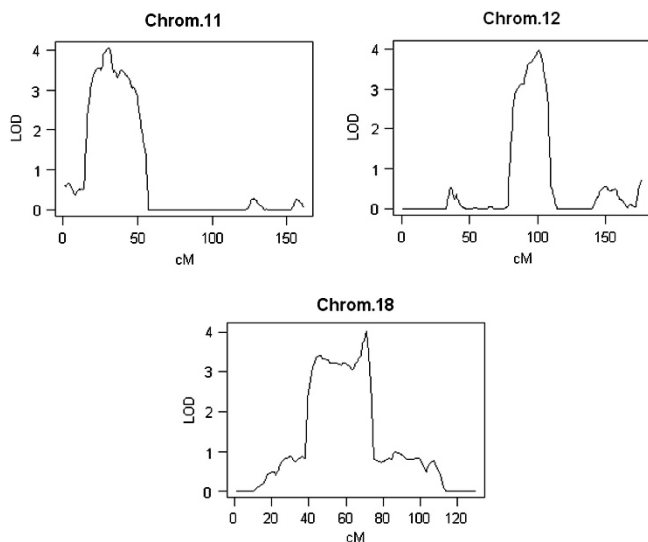


Figure 1 The significant linkage peaks identified on chromosomes 11, 12 and 18 with LOD score estimates of 4.06, 3.97 and 4.01, respectively.

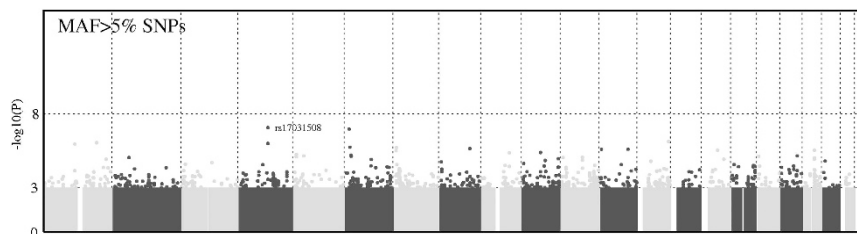


Figure 2 Manhattan plot for GWAS of pulse pressure. The strongest association is shown by SNP rs17031508 (102 779 313 bp) on chromosome 4. A full color version of this figure is available at the *Hypertension Research* journal online.

approach.²⁹ Association analysis was performed assuming additive genetic effects and SNPs with minor allele frequency <5% were dropped. The genome-wide significance level was set stringently to $P < 5e^{-08}$.

RESULTS

Genome-wide linkage analysis

With age, sex and BMI incorporated as covariates, our whole-genome linkage scan (Table 3) identified three significant linkage peaks on chromosome 11 (LOD score of 4.06, linkage region from 26 to 33 cM), chromosome 12 (LOD score of 3.97, from 93 to 104 cM) and chromosome 18 (LOD score of 4.01, from 68 to 72 cM) (Figure 1, Table 3), together with multiple suggestive linkage peaks with LOD scores over 3 on chromosome 1 (3.05 from 81 to 100 cM, 3.54 from 170 to 242 cM), chromosome 3 (3.6 from 141 to 170 cM), chromosome 4 (3.08 from 72 to 128 cM), chromosome 6 (3.19 from 129 to 163 cM), chromosome 7 (3.24 from 55 to 64 cM, 3.41 from 68 to 88 cM, 3.18 from 95 to 116), chromosome 9 (3.49 from 154 to 167 cM), chromosome 17 (3.32 from 61 to 103 cM) and chromosome 22 (3.04 from 1 to 31 cM).

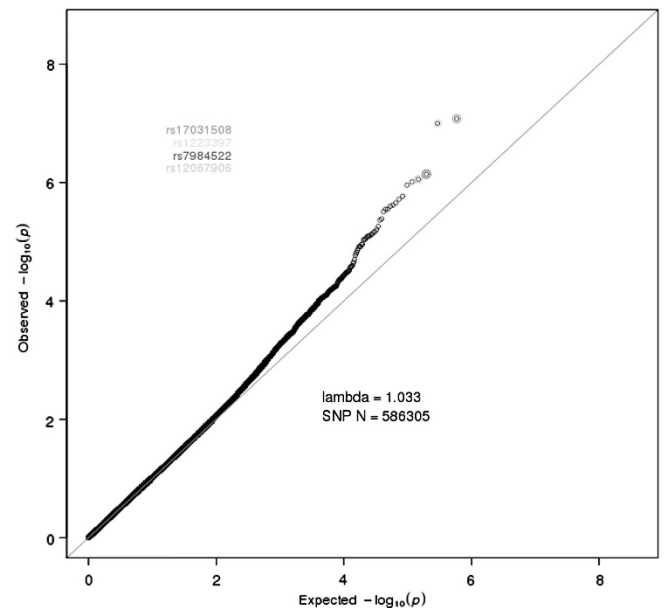


Figure 3 Quantile-quantile (Q-Q) plot for data in Figure 3. Relative importance is shown for the top SNPs that deviate from the middle line between the x axis and y axis. A full color version of this figure is available at the *Hypertension Research* journal online.

Table 4 Detailed information for the top significant SNPs

Chromosome	SNP	Base pair	Affymetrix ID	cM	Closest genes	Distance to genes (BP)	P-value	Effect-allele	Effect-size	Allele frequency
1	rs12067906	192 431 998	SNP_A-2232678	198,2338	RGS21 PPP3CA	95 583 291 937	8,76E-07	1	1.556	0.945
4	rs17031508	102 779 313	SNP_A-8589212	112,3186	BANK1 PPP3CA	151 473 300 105	8,34E-08	2	0.888	0.841
4	rs4580657	102 787 481	SNP_A-8690649	112,3249	BANK1	143 305	9,74E-07	1	1.034	0.881
6	rs1223397	13 378 924	SNP_A-8537269	32,41495	PHACTR1	0	1,04E-07	1	1.706	0.911
13	rs7984522	108 145 660	SNP_A-4249816	115,9055	MYO16	0	7,21E-07	1	2.447	0.944

Abbreviation: SNP, single-nucleotide polymorphism.

Genome-wide association study

Figure 2 is the Manhattan plot for our GWAS results. As a measure for genomic control, an inflation factor Λ was estimated to be 1.033, suggesting sample homogeneity. The SNP showing the strongest association (rs17031508 at 102 779 313 bp, \sim 112.3 cM) resides in the middle of chromosome 4 with a P -value of $8.34e^{-08}$. Another SNP (rs1223397 at 13 378 924 bp, \sim 32.41 cM) on chromosome 6 showed a P -value of $1.04e^{-07}$. The data in Figure 2 are also plotted in a quantile–quantile plot (Figure 3) such that the relative importance of SNPs can be easily perceived. With the stringent level for defining genome-wide significance ($P < 5e^{-08}$), no significant SNP was found in our GWAS, although SNP rs17031508 is close to significance. In Table 4, we listed the top five SNPs displaying high levels of association with detailed information concerning SNP IDs, reference number, chromosome location and frequency.

DISCUSSION

We have conducted the first extensive genetic epidemiology study on PP in the Chinese population using both linkage and association approaches. Our linkage and association analyses identified multiple genomic regions harboring susceptibility loci for PP, together with multiple SNPs, showing suggestive evidence of genome-wide association. Although each of the two approaches focused on different aspects of the genetic architecture of PP (that is, linkage mainly captures rare variants with large effects, while association detects common variations with small effects), overall, our results emphasize the genetic control over hemodynamic conditions.

Three linkage peaks that reside separately on chromosomes 11, 12 and 18 have been found to show genome-wide significance (LOD score > 3.6) with their LOD score estimates of ~ 4 . It is surprising that among the three significant linkage peaks, two have been confirmed to show linkage in other populations. In a multipoint linkage scan performed on large and extended Utah pedigrees, Camp *et al.*³¹ reported linkage evidence on chromosome 12q at 109 cM, which is covered by our linkage region on chromosome 12 in Figure 1. In another study based on American families, Bielinski *et al.*¹³ detected a significant linkage for PP on chromosome 18 at 71 cM; this was the most significant linkage in their study. It is interesting that this is exactly the location for our linkage peak on chromosome 18 (at 70.75 cM, Figure 1). Considering that there has been a limited number of whole-genome linkage scans conducted on PP in the literature, it is very unlikely that these results are coincidences.

Multiple suggestive linkages were detected by our linkage scan (Table 3). We assume that their trustworthiness increases with their

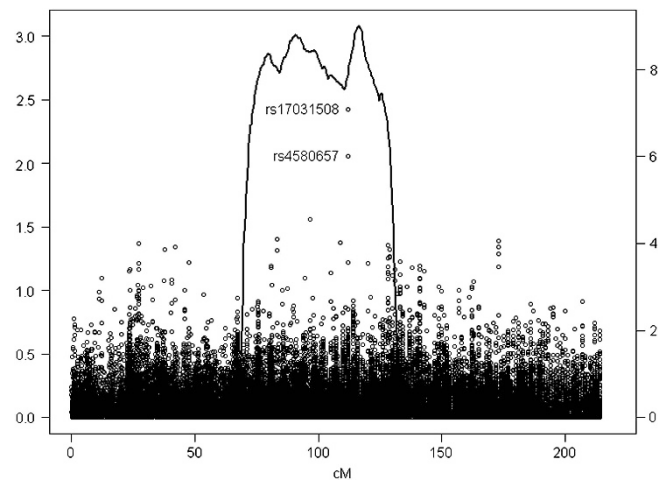


Figure 4 Linkage and association plots superimposed on chromosome 4. The two topmost SNPs detected by the association analysis reside in a broad region showing suggestive linkage in the middle of chromosome 4.

LOD score magnitudes, which seems to be reasonable considering the fact that four suggestive peaks with LOD scores > 3 overlap with reported linkages in previous studies. The highest suggestive linkage peak on chromosome 1 lies in the same region where Sherva *et al.*¹⁷ reported a suggestive linkage at 215 cM. This region is where the G protein-coupled receptor gene resides, which is involved in the signaling cascades that regulate blood pressure during changes in cardiac output.¹⁷ The highest suggestive linkage peak on chromosome 7 at 74.37 cM coincides with a linkage peak at 75 cM reported by Bielinski *et al.*¹³ and is in the vicinity of a peak at 71 cM in the same region reported by DeStefano *et al.*¹⁵ The peak regions on chromosomes 17 and 22 cover linkage peaks for PP reported by Bielinski *et al.*¹³ (at 89 cM on chromosome 17 and 11 cM on chromosome 22). In addition, their study also detected linkages on chromosomes 1 (106 cM), 3 (172 cM) and 7 (75 cM), which are located in the vicinity of our suggestive regions. All of these results emphasize the importance of our suggestive linkages.

The performances of SNP and traditional microsatellite markers in linkage analysis have been compared by Schaid *et al.*³² and Evans and Cardon,³³ who consistently reported a higher information content from the dense map of SNP markers than the from the microsatellite markers spaced at one marker per ~ 10 cM. Moreover, it was reported that linkage mapping using dense SNP markers identified more linkage peaks with narrower widths than did the traditional

markers.³² This conclusion coincides with our multiple suggestive scores, many of which overlap with linkage peaks detected by published large studies.

This study performed the first GWAS on PP in mainland Chinese. Although our sample size is relatively small, important associations have been identified. It is interesting to note that the SNP of the strongest association (rs17031508) is located within a wide region of suggestive linkage in the middle of chromosome 4 (Figure 4). Another nearby SNP (rs4580657, only 8168 bp away) also showed a strong association, although weaker than rs17031508. These results emphasize the importance of the corresponding region on chromosome 4. In fact, one of the closest genes to the two SNPs is the *PPP3CA* gene, which is known as the calcineurin gene. This gene has been reported to be associated with cardiac phenotypes, including stroke volume, cardiac output, and left ventricular hypertrophy in Chinese young adults.³⁴ The second SNP of top rank association, rs1223397, resides in *phosphatase and actin regulator 1 gene (PHACTR1)* on chromosome 6. Recently, variations in this gene have been associated with early-onset myocardial infarction³⁵ and with coronary heart disease.³⁶ Considering our limited sample size, further replication studies in Chinese and other ethnic populations are needed to reconfirm our identified associations and to explore the genetic correlation between PP and the risk of cardiac disorders. Finally, we note that the quantile–quantile plot in Figure 3 departs relatively early from the line of identity, which could mean a systematic effect due to the population substructure in our sib-pair data that is not completely accounted for in our analysis. Despite this, the large deviations from the reference line by the top SNPs show clear evidence of association.

Zhao *et al.*³⁷ reported significant effects of dietary factors, including salt intake, on blood pressure differences between Chinese in the north (higher salt intake) and in the south (lower salt intake). As a limitation of the study, salt intake was not measured and thus cannot be adjusted; however, individual adjustment on a population factor can only have a limited effect within the same population. In contrast, our models for gene mapping that are based on twin pairs could help to match the effects of shared environmental factors (for example, family dietary habits). After all, it is encouraging to note that our results based on Chinese data are consistent with those from studies on other ethnic populations, which provides new support to the conclusion that the effects of these factors are cross-cultural.⁵

Linkages and associations identified by us, especially those overlapping closely with previous studies, encourage further investigations with the aim of identifying genetic variations that affect PP using more focused linkage and association approaches on large samples. Meanwhile, considering the limited sample size used in the study, the interpretation of our findings, especially the multiple suggestive linkages and associations of borderline significance (for example, rs17031508), should be performed with caution because the validity of these loci requires additional replication studies to be conducted, particularly in Chinese and Asian populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This project was funded by the European Foundation for the Study of Diabetes, the 2007 Research Project: A genome-wide linkage analysis on metabolic/intermediate phenotypes predisposing to type II diabetes in the Chinese population and by the National Natural Science Foundation of China (grant no. 30872170).

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