Protein kinase C/ζ (PRKCZ) Gene is associated with type 2 diabetes in Han population of North China and analysis of its haplotypes

Yun-Feng Li, Hong-Xia Sun, Guo-Dong Wu, Wei-Nan Du, Jin Zuo, Yan Meng, Fu-De Fang

Abstract

AIM: To identify the susceptible gene(s) for type 2 diabetes in the previously mapped region, 1p36.33-p36.23, in Han population of North China using single nucleotide polymorphisms (SNPs) and to analyze the haplotypes of the gene(s) related to type 2 diabetes.

METHODS: Twenty three SNPs located in 10 candidate genes in the mapped region were chosen from public SNP domains with bioinformatic methods, and the single base extension (SBE) method was used to genotype the loci for 192 sporadic type 2 diabetes patients and 172 normal controls, implying a possible association with the disease. Interestingly, there are 5 serial makers in the p terminal region, 1p36.33-36.23, showed the linkage, which strongly suggests that there might be susceptible genes residing in this region[2].

In order to clone the susceptible genes in the 1p36.33-36.23 region, we conducted a linkage relative study by using single nucleotide polymorphism (SNP), and observed that three SNPs extending about 7kb were in the same haplotype block and there was a significant difference in their haplotype frequencies between case and control groups, which further proves that the PRKCZ gene is a susceptible gene for type 2 diabetes.

INTRODUCTION

Type 2 diabetes is a highly heterogeneous multifactorial disease with both genetic and environmental determinants and an uncertain mode of inheritance. It is characterized by hyperglycaemia due to defects in insulin secretion and action[1]. Now there are 143 millions people with the disease and more than 15 millions diabetic patients in China. In addition, the prevalence of diabetes is still increasing. The belief that type 2 diabetes has strong genetic determinants is based on several lines of evidence, including the high concordance rate among MZ twins[2,3], the marked difference in disease rate between populations[4-6], and the close correspondence between admixture rate and disease prevalence in hybrid populations[7,8]. In addition, there are evidences for major gene(s) influencing diabetes or its specific clinical manifestations, such as glucose concentration, 2-h postprandial insulin level, and age at onset of diabetes[9-11]. However, the mode of inheritance of type 2 diabetes appears to be variable across populations, suggesting a complex genetic mechanism underlying the disease.

In our previous genome-wide screening, we detected the possible susceptibility gene loci located on chromosomes 1, 12, 18 and 20 in Han population of North China. The 4 regions on chromosome 1 (1p36, 1p31, 1q22, 1q42-43) showed strong evidences of linkage with type 2 diabetes. Interestingly, there are 5 serial markers in the p terminal region, 1p36.33-36.23, showed the linkage, which strongly suggests that there might be susceptible genes residing in this region[2].

RESULTS: Among the 23 SNPs, 8 were found to be common in Chinese Han population. Allele frequency of one SNP, rs436045 in the protein kinase C/ζ gene (PRKCZ) was statistically different between the case and control groups (P<0.05). Furthermore, haplotypes at five SNP sites of PRKCZ gene were identified.

CONCLUSION: PRKCZ gene may be associated with type 2 diabetes in Han population in North China. The haplotypes at five SNP sites in this gene may be responsible for this association.

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for sex and age, were enrolled in a case-control study. The criteria for diagnosis of diabetes mellitus conformed to those of World Health Organization. Informed consent was obtained from each subject, and the study was performed with the approval of the Ethical Committee of Peking Union Hospital. Genomic DNA was isolated from the blood samples by conventional phenol and chloroform methods. Their final concentrations were all adjusted to 20 ng/µl.

**SNP-searching in the 1p36.33-36.23 region**

23 SNPs in 10 genes located in or near the 1p36.33-36.23 region were selected from the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP) for genotyping. All these genes were either glucose metabolism-related or lipid metabolism-related or involved in signal transduction pathways.

**Primer design**

The Primer3.0 program (http://zeno.well.ox.ac.uk:8080/eztbin/primer3_www.cgi) was used to design three primers to every SNP site. One pair of primers was used to amplify the fragments including the SNP site from genomic DNA. The third primer was designed to carry out the single base extension (SBE) reaction,[13] and this primer should be near the upstream of the SNP site, and could be used to anneal with the template. We then carried out a multiplex polymerase chain reaction (PCR). We designed eight different SBE primers according to different SNPs. The primers’ lengths were 18, 22, 26, 30, 34, 38, 42 and 46 bp, respectively, with Tm between 60 °C-80 °C.

**PCR and purification of the products**

The touch-down PCR was carried out. The reaction system was 10 µl mixture containing 50 ng genomic DNA, 3 mMol/L Mg²⁺, 0.3 mMol/L dNTP, 1 U AmpliTaq Gold. The reaction conditions were denaturation at 94 °C for 12 min, then 15 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 40 s, with the annealing temperature being decreased 0.5 °C every cycle. After 15 cycles, the reaction conditions were denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 40 s, for 25 cycles, then extension at 72 °C for 10 min. The excess primers and dNTPs were removed by adding exonuclease 1 (1 U), USB, OHIO, USA) and calf intestine alkaline phosphatase (1.5 U, Boehringer Mannheim, Germany) to the PCR reaction mixture and incubating it at 37 °C for 1 h, and then at 95 °C for 15 min to inactivate the enzymes.

**SBE reaction and identification of genotypes**

SBE reaction was carried out on the purified PCR products using SBE primer (100 nm), Joe-ddATP (30 nm), Fam-ddGTP (30 nm), Tamra-ddCTP (30 nm), Rox-ddUTP (150 nm) and Thermosequenase (1 U, Amersham Pharmacia, USA). The reaction conditions were denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 60 °C for 40 s, for a total of 35 cycles, followed by extension at 60 °C for 3 min.

One µl of SBE products in loading buffer (2 µl) was electrophoresed in the ABI377 sequencers. The length of the products was compared with differently colored luciferin marking a DNA complex with different lengths (i.e.19, 23, 27, 31, 35, 39, 43 and 47 bp). The SNP’s genotype was determined by the color and length of each line. Then the PCR products were sequenced to check the SBE genotypes.

**Statistical analysis**

Hardy-Weinberg equilibrium[14] was tested for each genotyped locus. Using SPSS10.0 program, we compared the difference in allele frequency between cases and controls with χ² test.

**SNP genotype in PRKCZ gene and haplotype analysis**

The SNPs in PRKCZ gene were found from the NCBI SNP database according to gene name and 16 SNPs upstream and downstream of rs436045 were selected and genotyped. For SNP genotyping in PRKCZ gene and analysis of the haplotypes, the association analysis was carried out as follows: (1) Hardy-Weinberg equilibrium analysis; (2) The allele association analysis using SPSS10.0 program; (3) The linkage disequilibrium analysis and haplotype analysis were performed to find the haplotype related to type 2 diabetes. Genotyping results were chosen from the 24 normal controls to calculate the number of individuals with different haplotypes using the Phase program. The above results were transformed into a FASTA file analyzed using DnaSP3.5 program (http://www.bio.ub.es/~julio/DnaSP.html). The degree of linkage disequilibrium between each SNP pair (D’ and r²) and the minimum number of recombination events and their locations were calculated in order to determine the haplotype block structure of these loci. The number of haplotypes and the number of individuals with different haplotypes in each haplotype block in both case and control groups were calculated by the DnaSP3.5 program again to search for the disease-associated haplotypes.

**RESULTS**

**Results of 23 SNPs in both case and control groups analyzed by SPSS program**

Of the 23 candidate SNPs tested, one was failed to be amplified from genomic DNA (rs586965) and three were heterozygous in all the samples tested (rs5251, rs15431 and rs15854). We deduced that these three were false SNPs due to paralogous sequences. In the remaining 20 candidates, 7 SNPs were homozygous in all the samples (rs91350, ss91351, rs9117, rs5259, rs228691, rs228677 and rs170633), suggesting that they might not be true polymorphisms. Four SNPs (rs1801131, rs14311, rs5254 and rs609805) had minor allele frequencies of less than 15 % and thus were discarded. The remaining 8 SNPs (rs1801133, rs436045, rs228648, rs11740, rs262669, rs228669, rs170629 and rs161825) were genotyped in both case and control groups. Their minor allele frequencies ranged from 26.3 % to 43.16 % and all belonged to the transition type. All the SNPs were studied in the Hardy-Weinberg equilibrium. SPSS analysis showed that the allele frequency of one SNP, namely rs436045 in an intron of PRKCZ gene, was statistically different between case and control groups (Table 1).

**Results of SNP genotype in PRKCZ gene and haplotype analysis**

Sixteen SNPs in the upstream and downstream of rs436045 in PRKCZ gene were selected, and genotyped. Then the results were analyzed. (1) Hardy-Weinberg equilibrium analysis. (2) The allele frequencies analysis, χ² analysis showed the difference between the case and controls (Table 2). (3) Linkage disequilibrium analysis and haplotype analysis were used to find the haplotype related to type 2 diabetes.

To search for the disease-associated haplotypes, haplotypes were constructed by linkage disequilibrium mapping in the region. The results were analyzed using DnaSP3.5. The recombination analysis showed that the minimum number of recombination events was three and they were detected between [rs1878745, rs1467217], [rs1467217, rs1401126], [rs1401126, rs411021]. From the results, we believed that 10 SNPs from rs411021 to rs262642 in 13 loci were in the linkage disequilibrium. They were in the same haplotype block. Further analysis on the frequencies of haplotypes formed by alleles of the 10 loci in case and control groups showed that a more
significant difference existed in the frequencies of different haplotypes (Table 3). There were mainly 4 haplotypes in the control group, accounting for 98.3% of the total, suggesting that these loci were in the same haplotype block. But there were many more different haplotypes with variable frequencies in the case group. Analysis using the DnaSP3.5 program showed that recombination events existed between [rs809912, rs262669], [rs262669, rs262662], [rs262662, rs381664], [rs381664, rs262650] and [rs262650, rs262642], suggesting that only rs411021, rs436045, rs427811, rs385039 and rs809912 were in the same haplotype block in the case group (Figure 1). The haplotypes containing these five loci were further analyzed in the two groups. The results showed that there were mainly two haplotypes and their frequencies were significantly different in the two groups (Table 4).

### Table 1 SPSS analysis results of genotyped SNPs in case and control group

| SNP        | Allele | Case | Control | Frequency of allele | P value | OR     |
|------------|--------|------|---------|---------------------|---------|--------|
| rs11740    | C      | 129  | 245     | 0.978               | 0.345   |        |
|            | T      | 110  | 208     | 0.346               |         |        |
| rs161285   | A      | 216  | 140     | 0.917               | 0.393   |        |
| rs170629   | G      | 261  | 119     | 0.710               | 0.313   |        |
| rs228648   | A      | 113  | 297     | 0.029               | 0.276   |        |
| rs262669   | G      | 244  | 502     | 0.351               |         |        |
| rs436045   | A      | 264  | 110     | 0.455               | 0.294   |        |
| rs1801133  | G      | 224  | 502     | 0.351               |         |        |

### Table 3 Difference in frequencies of haplotypes formed by alleles of the 10 loci in case and control groups

| Haplotypes         | Frequency (%) |
|--------------------|---------------|
| CGTAGTCCTGC        | 63.5          |
| TGGATGCCAC         | 22.5          |
| CGTAGTCTGC         | 7.8           |
| TGGATCCAT          | 4.5           |
| CGTAGCTGC          | 0.8           |
| CGGAGTCTGC         | 0.4           |
| CAGAGTCCAT         | 0.4           |
| CGTAGGCTTCA        | 0             |
| CGTAGTTGGT         | 0             |
| CGTAGTCTTAT        | 0             |

### Table 4 Difference in frequencies of haplotypes formed by alleles of the 5 loci in case and control groups

| Haplotypes         | Frequency (%) |
|--------------------|---------------|
| CGTAG              | 81.0%         |
| TGGAG              | 22.5%         |
| GTGAG              | 0.5%          |
| TAGGG              | 0.2%          |
| CGGAG              | 0.3%          |
| TAGGGAG            | 0.3%          |
| TGGAGG             | 0.3%          |

**DISCUSSION**

In our previous genome-wide screening, we detected the possible susceptible gene loci located on chromosomes 1, 12, 18 and 20 in Han people of North China. Especially, the four regions on chromosome 1 (1p36, 1p31, 1q22 and 1q42-43) showed strong evidence of linkage with type 2 diabetes. Interestingly, five serial makers in the p terminal region, 1p36.33-36.23, showed the linkage, strongly suggesting that there may be susceptible
among about 50 kilobasepairs region, and the recombination there was a slight difference between case and control groups. So we suggested that PRKCZ might be a susceptible gene for type 2 diabetes.

Protein kinase C zeta (PRKCZ) is a member of the PKC family of serine/threonine kinases, which consists of at least 10 structurally related enzymes that have been implicated in a variety of cellular processes. PRKCZ gene belongs to the αPKC subfamily and is thought to function downstream of phosphatidylinositol 3-kinase (PI 3-kinase) in the insulin signal pathway and to contribute to the translocation of the protein encoded by GLUT4. The activated PRKCZ products can accelerate glucose transport during insulin action on rat skeletal muscle and adipocytes. In addition, PRKCZ may participate in a negative feedback pathway by phosphorylating insulin receptor substrate-1 (IRS-1) and impairing its ability to activate phosphatidylinositol 3-kinase in response to insulin. Insulin-stimulated glucose transport is defective in type 2 diabetes, and this defect is ameliorated by thiazolidinediones and lowering of blood glucose by chronic insulin therapy or short-term fasting. Rosiglitazone treatment, insulin treatment, and fasting can reverse the defects in PRKCZ-zeta/lambda activation by insulin in GK rat muscles and adipocytes and increase glucose transport in GK rat adipocytes, suggesting that insulin-sensitizing modalities may similarly improve defects in insulin-stimulated glucose transport at least partly by correcting defects in insulin-induced activation of PRKCZ-zeta/lambda[14]. The above may explain our results.

It is the essential prerequisite for localizing genes associated to disease on the base of the multitude to study linkage disequilibrium (LD) model in detail of the multitude. Now there are still some controversies on the LD capacity. The computer imitating[22] and the experience data[23] all showed that the LD would elongate several kilo basepair near some common SNP, while some other data showed that the LD would elongate more sometimes beyond 100 kilo basepairs[24-26]. Some new research data showed that the LDs would exist in the genomic DNA as the block structure, they would be broken up by the recombination spot[27-30]. Understanding the LD structure is very important for LD analysis, and for carrying out studies on disease-related mutations, population genetics, and the human genomes project. Haplotype blocks are very important for LD. Once a haplotype block is identified in some sequences, different alleles based on it can be selected for LD analysis. So, haplotype block is a very effective method to test the genomic DNA fragments associated with diseases.

Blocks are defined according to the genetic content, not according to how the information is produced and why it exists. Thus, there is no strictly limit in the definition of blocks, which may have different according to different population. Now there are no very accurate methods to construct haplotype blocks. When we constructed the blocks, we classified a set of SNPs, which have a high linkage disequilibrium but have no recombination, into one block under the condition of the capacity of LD and the recombination spots for every two SNPs.

In order to further study PRKCZ associated with diseases, a set of SNPs located in the upstream and downstream from rs436045 in PRKCZ gene were selected to conduct a case-control study and LD analysis. The results showed that the frequencies of many sites’ alleles were significantly different between case and control groups. LD analysis and recombination analysis were further carried out, and the results showed that there was a slight difference between case and control groups. The LD capacity was very high between rs411021 to rs262642 among about 50 kilobasepairs region, and the recombination frequency was very low, suggesting that these SNPs existed in one haplotype block. In cases, however, the LD capacity was very high only in five SNPs from rs411021 to rs809912, within the about 7 kilobasepairs region. The haplotypes containing these five loci were further analyzed in the two groups. The results showed that there were mainly two haplotypes with frequencies very different between the two groups. The frequency of CGTAG haplotype was significantly increased while that of TAGGA haplotype decreased in the case group (P<0.01), suggesting that CGTAG and TAGGA might be disease-associated haplotypes. In conclusion, PRKCZ gene may be associated with type 2 diabetes in Han population of North China. The haplotypes at five SNP sites in this gene may be responsible for this association.

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