**Effect of SNPs in protein kinase Cz gene on gene expression in the reporter gene detection system**

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**Abstract**

AIM: To investigated the effects of the SNPs (rs411021, rs436045, rs427811, rs385039 and rs809912) on gene expression and further identify the susceptibility genotypes of type 2 diabetes.

METHODS: Ten allele fragments (49 bp each) were synthesized according to the 5 SNPs mentioned above. These fragments were cloned into luciferase reporter gene vector and then transfected into HepG2 cells. The activity of the luciferase was assayed. Effects of the SNPs on RNA splicing were analyzed by bioinformatics.

RESULTS: rs427811T allele and rs809912G allele enhanced the activity of the reporter gene expression. None of the 5 SNPs affected RNA splicing.

CONCLUSION: SNPs in protein kinase Cz (PKCZ) gene probably play a role in the susceptibility to type 2 diabetes by affecting the expression level of the relevant genes.

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**INTRODUCTION**

Type 2 diabetes is a highly heterogeneous chronic disease characterized by metabolic disorder of blood glucose, its onset involves a number of susceptibility genes. Since 1996, locating and cloning the predisposing genes of type 2 diabetes, as well as the functional investigation, has become one of the hot spots worldwide in type 2 diabetes research. Based on genomic screening technology, it was reported firstly among Western population in succession that type 2 diabetes susceptibility genes located on different chromosomes[1-23]. The susceptibility genes were localized on chromosome 9 in Chinese population[24]. According to the case-control analysis in the region of l36.33-l36.23, our research group found that one SNP locus, rs436045 in protein kinase Cz (PKCZ) gene, was linked to type 2 diabetes in Chinese population, and the haplotype block has been identified. While analyzing the haplotype which consists of the 5 SNPs (rs411021, rs436045, rs427811, rs385039, rs809912), we noticed that, in the case group, the haplotype CGTAG showed a significantly higher frequency than that in control group, whereas the frequency of haplotype TAGGA decreased significantly (P<0.01, OR = 1.625), it implied that the changes of those haplotypes related to the onset of type 2 diabetes in Chinese[25]. However, it is still unclear weather haplotypes play a role during the episode of the disease.

To determine the biological function of those haplotypes, we investigated their influence on gene expression by bioinformatics approach and reporter gene activity determination system, which would provide a basis for further research.

In the previous work, we found that the 5 SNPs at the introns of PKCZ gene located in the same haplotype block in case group, and the haplotype they formed was clearly associated with type 2 diabetes mellitus. In order to determine the susceptibility loci associated with type 2 diabetes, we performed functional analysis on 5 SNPs.

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**MATERIALS AND METHODS**

**Identification of SNPs in the coding region of PKCZ gene**

Coding region (from exon 4 to exon 13 or from rs1878745 to rs262642) of PKCZ gene was investigated for SNPs (cSNP) by sequencing. Ten unrelated type 2 diabetic patients and 10 control subjects from Han population in China were enrolled in a case-control study. Primers were designed by Primer 3.0 program (http://zeno.well.ox.ac.uk:8080/gitbin/primer3 www.cgi) and each PCR product was limited within about 500 base pairs. The sequencing results from ABI377 sequencer were analyzed through PhredPhrap/consed program to identify functional SNPs.

**Analysis of the effect of 5 intron SNPs on mRNA splicing**

The distance from the SNP to the splicing point in exon was determined based on the published genome sequence. According to this information, we preliminarily estimated whether the SNP site influences gene splicing.

**Search of the information on PKC family member**

The location and sequence of other PKC family members were obtained by means of bioinformatics. Then, differences splicingosomes from other family members residing in the sequence of PKCZ were analyzed.

**Analysis of the introns where 5 SNPs located**

Each SNP and the intron sequence were analyzed, and then the amino acid was compared with the data in cDNA database (www.sanbi.ac.za) to reveal the sequence homology. The open reading frames in this sequence were analyzed, and then the amino acid was compared with the data in cDNA database (www.sanbi.ac.za) to reveal the sequence homology.
blasts using the (www.ncbi.nlm.nih.gov) protein database in search of the sequence homology.

**Effects of SNPs on gene expression by transient transfection**

Ten alleles corresponding to the 5 SNPs in *PKCZ* gene were cloned into pGGL3-promoter vector in the direction from 5’ to 3’ (Table 1). Meanwhile, HepG2 cells were cultured with DMEM (Gibco, LOS Angeles, USA) containing 100 mL/L fetal bovine serum. Then, the cells (1.5×10^5 - 2×10^5) were transfected with pGGL3-promoter vector (1 uL) or recombinant vector with Lipofectamine transfection reagent (Promega, Madison, USA). The transfection rate was assayed by using pRL-SV40 DNA (100 ng, Promega, Madison, USA) as an internal control. Forty-eight hours post transfection, the luciferase activity was determined by the Dual-Luciferase® Reporter Assay System using pRL-SV40 as an internal control.

| Fragment name            | Sequence                                      |
|--------------------------|-----------------------------------------------|
| rs809912G-forward      | 5’ ggggtaccgcagcatcctc eac gcccatctccac 3’ |
| rs809912G-reverse       | 3’ gttgtcgaggggtg g gggtaaagaggtagcttagaag 5’ |
| rs809912A-forward       | 5’ gsggtacccagctcctgtaag a atttggctcaagct 3’ |
| rs809912A-reverse       | 3’ gggtacccgagcatcctc eac gcccatctccac 5’ |
| rs436045A-forward       | 5’ ggggtaccgcagctctgtaag a atttggctcaagct 3’ |
| rs436045A-reverse       | 3’ tggctgcagctc t aacaccgtccacagcttagaag 5’ |
| rs436045G-forward       | 5’ ggggtaccgcagcatcctgtaag a atttggctcaagct 3’ |
| rs436045G-reverse       | 3’ tggctgcagctc eac accacgcagcttagaag 5’ |
| rs427811T-forward       | 5’ ggggtaccgcagctctgtaag a atttggctcaagct 3’ |
| rs427811T-reverse       | 3’ ggggtaccgcagctctgtaag a atttggctcaagct 5’ |
| rs385039G-forward       | 5’ ggggtacccagctcctc eac gcccatctccac 3’ |
| rs385039G-reverse       | 3’ caaatgtcttcgatg c aacattgtggacgagatctagaag 5’ |
| rs385039A-forward       | 5’ ggggtacccagctcctc eac gcccatctccac 3’ |
| rs385039A-reverse       | 3’ caaatgtcttcgatg a acattgtggacgagatctagaag 5’ |
| rs411021C-forward       | 5’ ggggtaccgcagctctgtaag a atttggctcaagct 3’ |
| rs411021C-reverse       | 3’ caaatgtcttcgatg c aacattgtggacgagatctagaag 5’ |
| rs411021T-forward       | 5’ ggggtaccgcagctctgtaag a atttggctcaagct 3’ |
| rs411021T-reverse       | 3’ ggggtaccgcagctctgtaag a atttggctcaagct 5’ |

Table 1: Sequence of 49-bp fragments containing each allele of 5 SNPs

**RESULTS**

**SNPs in the coding region of PKCZ gene**

While seeking for functional SNPs by sequencing the exons around the 13 intron SNPs discovered in the previous work, we found no new ones except for the rs1878745 corresponding to NCBI database. It suggested that the disease loci probably did not exist in the coding region.

**Influence of positive SNP on the PKCZ gene expression**

To locate the disease SNP, we investigated the effect of the 5 positive SNPs (rs411021, rs436045, rs427811, rs385039, and rs809912) lying in the same haplotype block on PKCZ gene expression. The influence of the 5 SNPs over RNA splicing was evaluated since all the 5 SNPs lay in the introns. The distance of the SNPs from the upstream and downstream of the splicing site are respectively as the following: rs411021 (3 535 bp, 5 283 bp), rs436045 (4 770 bp, 4 048 bp), rs427811 (8 729 bp, 89 bp), rs385039 (1 629 bp, 57 bp), and rs809912 (>2 kb, 2 057 bp). Those are comparatively long distant to 5’ splice donor site, 3’ receptor site and the internal vertex, suggesting that they have little association with pre-mRNA splicing. In addition, we estimated if differential splicing occurs between *PKCZ* gene and other PKC family members. Although there are at least 11 family members besides *PKCZ*, none of them locate on chromosome 1, which negates the ‘differential splicing supposition’. The location of introns where 5 SNPs located was analyzed. As a first step, we compared the intron sequence around the loci of each of the 5 SNPs with the data in cDNA database (www.sanbi.ac.za) in order to reveal the sequence homology. Result showed that the introns had no coding function because neither cDNA sequence homology nor protein sequence homology by ORF analysis was found. But this result needs to be further confirmed by Northern blotting. And finally, the effects of the SNPs on gene expression were investigated. Transfected HepG2 cell containing pGGL3-promoter reporter gene vector was used to detect the activity of the reporter gene that could reflect indirectly whether the fragment inserted affected gene expression. Statistical analysis showed a significant difference between the two SNPs of rs4278111 and rs809912. In rs4278111, the reporter gene activity of T allele was 1.5 times that of G allele, while in rs809912, in G allele it was 1.7 times that of A allele (Table 2). Therefore, these two SNPs will probably affect the expression level of *PKCZ* gene.

| Construct                  | Relative luciferase activity | P       |
|----------------------------|------------------------------|---------|
| pGL3-promoter              | 0.3533±0.040                |         |
| pGL3-rs411021C            | 0.5167±0.064                | 0.928   |
| pGL3-rs411021T            | 0.5100±0.102                |         |
| pGL3-rs436045A            | 0.3433±0.051                | 0.363   |
| pGL3-rs436045G            | 0.3767±0.023                |         |
| pGL3-rs427811T            | 0.6233±0.064               | 0.023   |
| pGL3-rs427811G            | 0.4433±0.068                | 0.029   |
| pGL3-rs385039A            | 0.3500±0.044                |         |
| pGL3-rs385039G            | 0.3467±0.015                | 0.907   |
| pGL3-rs809912A            | 0.1800±0.017               | 0.009   |
| pGL3-rs809912G            | 0.3033±0.042                |         |

*P<0.05 in comparison contrast and pGGL3-promoter vector.

**DISCUSSION**

*PKCZ* is a member of serine/threonine protein kinase family, belonging to atypical PKC, and independent of both calcium and diacylglycerol (DAG) [26]. It is insensitive to PKC inhibitors and cannot be activated by phorbol ester. *PKCZ* protein is thought to function downstream of phosphatidylinositol 3-kinase (PI 3-kinase) in insulin signaling pathway and plays a role in promoting the translocation and activation of Glut4 from the cytosol to membranes which will accelerate the glucose transport in skeletal muscle and adipocytes [27-30]. In addition, *PKCZ* can induce negative feedback to the signaling pathway through phosphorylating IRS-1 [31, 32]. Insulin-stimulated glucose transport is defective in type 2 diabetes mellitus, and this defect can be ameliorated via correcting PRKC-zeta/lambda activation defect [33], suggesting that the transport deficiency is at least partly associated with the activation defect of *PKCZ*. Our previous research showed that *PKCZ* is related to susceptibility to type 2 diabetes mellitus in Chinese population. If so, whether genetic polymorphism of *PKCZ* gene will influence the pathways associated with blood glucose regulation by affecting its gene expression, and increase the susceptibility to this disease ultimately? Based on bioinformatics research and reporter gene activity determination system, our data provide first evidence that intron SNP loci in *PKCZ* gene affect gene expression. Horikawa [34] has reported that gene expression was under the influence of the 3 intron SNPs in *CAPN10* gene, the
susceptibility gene of type 2 diabetes in Mexican American. Such kind of result was also reported by other groups, for example, an SNP in COL1NI gene can change the binding site of transcription factor Sp1 thereby influencing the gene expression, resulting in the decline of bone density as well as osteoporosis[13].

In our experiment, we found the two alleles (rs427811T and rs809912G) that had a relatively high frequency in type 2 diabetic patients could improve the reporter gene expression, apparently in conflict with our predicted result. This phenomenon might be explained by the hypothesis that PKCZ gene was involved in other signaling pathways and its relation to the disease was more complicated than we had estimated. Till now, there have been no reports that PKCZ gene expression is changed in the tissues of type 2 diabetic patients. But PED/PEA-15, a substrate of PKC, was reported to increase tissue insulin resistance of type 2 diabetes. Our next goals are to determine whether PKCZ interacts with PED/PEA-15 in insulin signaling pathway, and whether PED/PEA-15 or its analogue is involved in the inhibition of the insulin stimulated glucose transport via another signal pathway.

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