

Original Article

Association between selected tag SNPs in *NOX4* gene and diabetic nephropathy susceptibility

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Abstract: Aim: We aimed to select tag single nucleotide polymorphisms (SNPs) in *NOX4* gene, then process genotyping and explore the association between *NOX4* gene SNPs and haplotypes with diabetic nephropathy (DN) susceptibility in Chinese Han population. Methods: 120 DN patients and 153 healthy controls were enrolled in this study. GenBank of NCBI database was applied to determine the scope of human *NOX4* gene and its forward and reverse extending regions. Haploview software was used to construct haplotype blocks, screen out tag SNPs and analyze the haplotype and linkage disequilibrium (LD). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for genotyping. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were utilized to evaluate the DN susceptibility. Results: Block 1 was chosen as the study object in six haplotype blocks in human *NOX4* gene. *NOX4* rs1827428 was selected as the tag SNP according to r^2 and LOD values in Block 1. Frequencies of AA genotype and A allele in *NOX4* rs1827428 were obviously different between case and control groups, which indicated AA genotype and A allele were susceptible factors for DN (OR=6.789, 95% CI=1.454–31.667, $P=0.005$; OR=1.986, 95% CI=1.121–3.517, $P=0.017$). Conclusions: *NOX4* rs1827428 polymorphism was related to DN susceptibility. A-T-C haplotype in Block 1 is a susceptibility factor for DN. All of this suggested that, *NOX4* gene might increase the susceptibility of DN.

Keywords: *NOX4*, tag SNP, haplotype block, diabetic nephropathy

Introduction

With changes of human lifestyle, the number of patients with diabetes is increasing year by year. Diabetic nephropathy (DN), one of the most common chronic complications of diabetes, mainly refers to glomerulosclerosis which is caused by diabetes induced blood capillary lesion. It is characterized by nephrotic syndrome. DN seriously affects the survival and life quality of patients. Therefore, it is of particular importance to know about DN pathogenesis. Then find out a novel and effective method for DN prevention and treatment. But, the pathogenesis of DN is still not clear. Previous studies have revealed that oxidative stress, which involved reactive oxygen species (ROS), is closely related to DN occurrence and development [1-3].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can be activated by various stimuli of extracellular signals, and then medi-

ates the production of ROS. NADPH oxidase 4 (*NOX4*) is a catalytic subunit of NADPH oxidase complex. It acts as an oxygen sensor and catalyzes the reduction of molecular oxygen to multiple ROS. These ROS participate in numerous biological functions including signal transduction, cell differentiation and tumor cell growth. *NOX4* highly expressed in kidney and blood vessels [4-6], and also expressed in vascular endothelial cells, smooth muscle cells and fibroblasts [7-9]. Some studies indicate *NOX4* can regulate cell survival, proliferation, metastasis, senescence and differentiation [10-12]. While other researches demonstrate it can mediate endoplasmic reticulum stress [13], DNA damage [14].

Recent years, many studies evaluated *NOX4* polymorphisms, but most of them only focused on the relationship between several single nucleotide polymorphisms (SNPs) in *NOX4* gene and different diseases [15-17]. We still not find any study analyzing the whole *NOX4* gene

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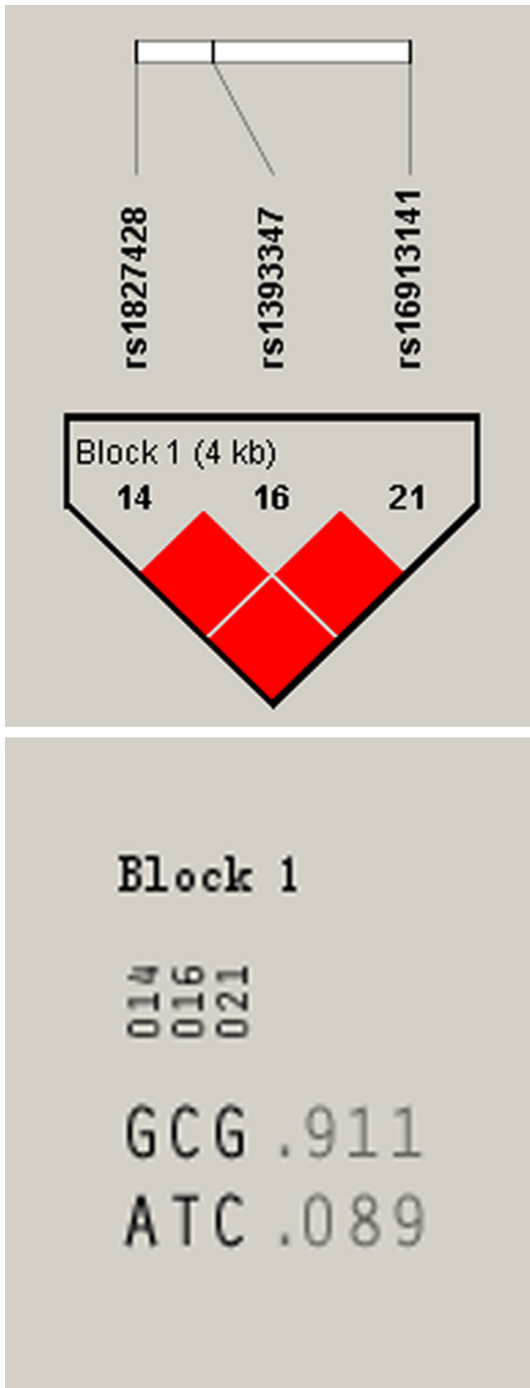


Figure 1. Linkage disequilibrium and haplotypes in Block 1.

through bioinformatics. Besides, no study explores the association between *NOX4* polymorphisms and DN risk on the whole genetic level. By analyzing the whole *NOX4* gene, constructing haplotype blocks and selecting the tag SNP, this study aimed to explain the association

between *NOX4* polymorphisms and DN risk, and offer a comprehensive understanding of the biological significance of genetic polymorphisms.

Materials and methods

Study population

We randomly selected 120 DN patients (82 in male, 38 in female) in Department of Endocrinology in Tai'an Central Hospital from June, 2008 to June, 2014. Meanwhile, 153 healthy check-up individuals (101 in male and 52 in female) were chosen as controls. Diabetes patients with 24 h urinary albumin excretion rate (UAER) greater than 20 $\mu\text{g}/\text{min}$ were considered as DN patients. The controls passed through the clinical and laboratory examinations and had no liver, kidney, endocrine, cardiovascular and cerebrovascular diseases. All the control subjects were matched with patient population by age and gender. The mean age of controls was 49.2, while the mean age of cases was 45.4. All participants were no kinship Chinese Han population. Informed consent signed by every participant. This study was approved by ethical society of Tai'an Central Hospital.

Acquisition of tag SNP

Research scope was ascertained through NCBI database, including *NOX4* gene and 10 kb-length extending regions. We also used SNP database (HapMap2 version) to obtain all the genotyping data of SNPs in researched scope. The Haploview 4.2 software was used to construct haplotypes and screen out the tag SNP. Tag SNP selection based on the following standards: upper limit of 95% confidence interval (95% CI) of the D' value was greater than 0.98 and lower limit of 95% CI was greater than 0.7; $r^2 > 0.8$. This standard suggested that the region hardly presents any genetic recombination and these SNPs can constitute a haplotype block [18-20].

DNA extraction and genotyping

10 mL peripheral vein blood was collected from every object who had a 12 hours fast. DNA extraction kit (Bio-light Biotechnology Co., Ltd) was used to extract DNA, according to instructions. Polymerase chain reaction-restriction fr-

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Table 1. Haplotype blocks in *NOX4* gene

Block	SNP	Region	Length (bp)
Block 1	rs1827428 rs1393347 rs16913141	89336138-89340246	4018

Table 2. D' value of SNPs in haplotype blocks of *NOX4* gene

SNP	SNP	D'	CL*	CU#
rs1827428	rs1393347	1.0	0.8	1.0
rs1827428	rs16913141	1.0	0.8	1.0
rs1393347	rs16913141	1.0	0.8	1.0

CL* represents lower limit of 95% CI of D' value between SNPs; CU# represents upper limit of 95% CI of D' value between SNPs.

Table 3. The r^2 and LOD values between SNPs in Block 1 of *NOX4* gene

Block	SNP	SNP	Mean value of r^2	Mean value of LOD
Block 1	rs1827428	rs1393347	1.0	9.32
		rs16913141		
	rs1393347	rs1827428	1.0	9.32
		rs16913141		
	rs16913141	rs1827428	1.0	9.32
		rs1393347		

Table 4. Haplotype frequencies of tag SNP in Block 1

Haplotype	Frequency (%)	Allele (tag SNP)
G-C-G	91.1	G (rs1827428)
A-T-C	8.9	A (rs1827428)

fragment length polymorphism (PCR-RFLP) was applied to analyze rs1827428 polymorphism in *NOX4* Block 1. Primer (synthesized by Shanghai Sangon Biotech Co., Ltd) sequences were as follows: forward 5'-TTACACTTCTGGTGCTTGAT-3' and reverse 5'-ATCAAGCACCAAGTGTA-3'. Each PCR system was carried out in a 50 μ L volume, containing 5 μ L 10 \times PCR buffer, 4 μ L 2.5 mmol/L dNTP, 2 μ L forward primer, 2 μ L reverse primer, 5 μ L template DNA, 0.6 μ L TaqDNA polymerase and complemented sterile double-distilled water. The amplification of PCR was initially proceeded predegeneration at 94°C for 5 min, followed by 35 cycles of 94°C degeneration for 50 s, 59°C annealing for 50 s and 72°C extension for 5 min and a final extension at 72°C for 5 min. 1 μ L restriction enzyme RsaI (Promega Company) was added to 15 μ L

PCR amplification products, then the mixtures were incubated at 37°C for 4 h. The digested fragments were treated with 2.5% agarose gel electrophoresis, EB staining and photographing under the ultraviolet lamp.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software package, with statistical significance in difference when $P < 0.05$. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were used to represent the DN susceptibility. Haploview software was utilized to select tag SNP and analyze the linkage disequilibrium (LD) and haplotypes of SNPs in *NOX4* gene. χ^2 test was applied to confirm whether allele frequencies were in accordance with Hardy-Weinberg equilibrium (HWE), with statistical significance of $P > 0.05$.

Results

The selection of tag SNP and haplotypes

In the HapMap database, there were 94 *NOX4* SNPs (not shown, $MAF > 0.05$) in Chinese Han population. According to the selection standards, we selected Block 1 among six haplotype blocks within the whole *NOX4* gene (**Figure 1**). Block 1 had 3 SNPs and with the length of 4 kbp, accounted for 3.3% of the whole genetic length (**Tables 1, 2**). With $r^2 = 1.0$ and $D' = 1.0$, rs1827428 was selected as the tag SNP among three SNPs in block1 in *NOX4* (rs1827428, rs1393347 and rs16913141, **Table 3**). Two representative haplotypes (G-C-G and A-T-C) were selected in Block 1 (**Table 4**).

Genotypes and alleles associated with DN

Genotypes and alleles distributions of *NOX4* rs1827428 in control group were accorded with HWE ($P > 0.05$). Genotypes and alleles distributional frequencies in case and control groups were measured by Chi-square test, and the results were shown in **Table 5**. Heterozygotes GA in *NOX4* rs1827428 did not show any significant difference between cases and controls ($P = 0.800$). However, the AA genotype and A

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Table 5. Distributions of genotypes and alleles in *NOX4* rs1827428

Genotype/Allele	Case (n=120)	Control (n=153)	χ^2	<i>P</i>	OR (95% CI)
Genotype					
GG	98	133	-	-	-
AG	12	18	0.064	0.800	0.905 (0.417-1.965)
AA	10	2	7.732	0.005	6.789 (1.454-31.667)
Allele					
G	208 (86.7)	284 (92.8)	-	-	-
A	32 (13.3)	22 (7.2)	5.697	0.017	1.986 (1.121-3.517)

allele frequencies were significantly higher in patients compared with controls, indicating AA genotype and A allele increased the susceptibility of DN (OR=6.789, 95% CI=1.454-31.667, *P*=0.005; OR=1.986, 95% CI=1.121-3.517, *P*=0.017). This result suggested that the A-T-C haplotype of *NOX4* block 1 or whole *NOX4* gene might increase the susceptibility of DN.

Discussion

DN is the most common microvascular complication of diabetes. It is also one of the main factors for death caused by diabetes. DN pathogenesis was related with various factors, including metabolic disorders, abnormality of glomerular hemodynamics, oxidation stress, genetic susceptibility and etc. NADPH oxidase is the main source of ROS. NADPH oxidase was initially found in neutrophils and mononuclear macrophages [21, 22]. In recent years, the homologs of phagocyte NADPH oxidase have been discovered in cytoplasmic membrane of non-phagocyte. All of them named NOX (NADPH oxidase) protein family. Seven kinds of NADPH oxidase have been identified in human, namely NOX1, NOX2, NOX3, NOX4, NOX5, Duox1 and Duox2 [4, 23-25]. Human *NOX4* gene, with the length of 165139 kb and containing 24 exons, is located on the eleventh chromosome q14.2-q21. NOX4 protein contains 578 amino acids and has 39% homology with NOX2 [5]. NOX4 may serve as a sensitive oxygen sensor, and initial studies indicate that *NOX4* is highly expressed in kidney tissues. NOX4 is a p22^{phox} dependent enzyme [26], and previous research enunciated that NOX4 and p22^{phox} were increased in distal tubule cells and glomeruli in diabetic rats [27]. Recently, many researches showed that lack of NOX4 may alleviate renal injury and afford a renoprotection in mice [28, 29].

Only a few domestic and foreign studies explored the association between *NOX4* polymorphisms and susceptibility to diseases. These studies simply focused on the relationship of a certain SNP with diseases. With the development of International HapMap Project, the number of SNPs has been found increasing from one million to three million. According to SNP map with high density in the HapMap database, it is possible to comprehensively observe and study a whole gene or a genome, which is also an international hotspot of research on SNPs. In face of huge data in databases, the study of haplotype blocks can screen out useful information quickly and accurately. This method can reduce the workload and avoiding the blind detection of SNPs.

In our study, we applied Haploview software effectively to analyze linkage disequilibrium of SNPs in *NOX4* gene, and construct *NOX4* gene haplotype blocks in Chinese Han population. Then select the haplotype Block 1 as tag block on the basis of more strict standards. Therefore, the constructed haplotype blocks were relatively accurate regions without the possibility of recombination. Optimum tag SNP *NOX4* rs1827428 selected from Block 1 could effectively represent the haplotypes in Chinese Han population. Then we evaluated the association between rs1827428 and DN risk. The result indicated that AA genotype and A allele enhance the DN susceptibility about 6.789 and 1.986 times, respectively. According to the HAPMAP database, 3 SNPs in Block 1 was completely linkage. Collectively, our data suggested that *NOX4* rs1827428 A allele and its corresponding haplotype A-T-C related to DN, and could increase the risk of DN. That was accorded with previous studies, which found out the association between *NOX4* gene and DN adopted other methods [28, 29]. Using this method, other

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cytokines and their receptors genetic sequences can be analyzed more completely. Association between DN risk with SNPs and haplotypes can be obtained and researched. Therefore, this study offers some beneficial help for analysis of target genes and select tags from the perspective of bioinformatics.

Although we obtained a meaningful result, but there also was many limitation in our study. Firstly, in this study only had one race, that cannot supply a comprehensive suitability. Secondly, functional verification was insufficient, lack of evidence to certify the association between NOX4 gene SNPs and DN risk. Finally, the linkage disequilibrium between many NOX4 SNPs was very low, only one SNP in Block 1 to represent the whole gene might inadequate. Perhaps, select a tag SNP from every blocks respectively might solve this problem. Thus, a well designed study was necessary to define the association between NOX4 SNPs and haplotype with DN risk. That contributed to certify DN pathogenesis, and provide novel method for DN diagnosis and treatment.

Disclosure of conflict of interest

None.

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