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Original Article

Gene polymorphisms of nitric oxide synthase (G-954C, G894T) and sorbitol dehydrogenase (C-1214G, G-888C) in Chinese patients with diabetic retinopathy combined with cystoid macular edema

Jian-Bin An*, Na-lei Zhou*, Jing-Xue Ma, Yan-Jun Gao, Li-Ya Liu

*Department of Ophthalmology, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei, China. *Equal contributors.*

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Abstract: Objectives: To investigate the association of gene polymorphisms including G-954C, G894T in nitric oxide synthase (NOS) and C-1214G, G-888C in sorbitol dehydrogenase (SDH) in type 2 diabetes mellitus (T2DM) patients with diabetic retinopathy (DR) combined with cystoid macular edema (CME) in China. Methods: 430 T2DM patients with DR combined with CME and 107 healthy volunteers were in this study. Clinical characteristics were collected and compared among control, non-DR (NDR), single DR (SDR) and proliferative DR (PDR) groups. Genotypes of G-954C, G894T in NOS and C-1214G, G-888C in SDH were determined by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and allele-specific PCR. Results: There were significant differences of the course of T2DM and insulin absorption between the T2DM patients with DR and those without ($P<0.05$). G-954C, G894T, C-1214G or G-888C was not associated with DR development in Chinese patients with T2DM. The combined GG-GC genotype of C-1214G–G-888C (OR 2.41, 95% CI 0.39-12.58) was correlated with DR, and the frequency decreased as the severity of DR aggravating, the same was to the GC-GC genotype (OR 3.06, 95% CI 1.12-8.59). Conclusions: G-954C, G894T, C-1214G or G-888C was not associated with DR development in Chinese patients with CME, while GC-GC and GG-GC genotypes in combining C-1214G with G-888C was associated with DR pathogenesis, providing DR detection, therapy and prognosis with gene targets.

Keywords: Nitric oxide synthase, sorbitol dehydrogenase, polymorphisms, diabetic retinopathy, cystoid macular edema

Introduction

Diabetic retinopathy (DR), as the microangiopathy, was one of the most serious complications of diabetes mellitus (DM) and the main disease causing blind. Many data showed that the generation and development of DR were not only related to the course of disease and the situation of blood glucose control, but also associated with gene polymorphisms [1].

Bitar [2] et al. considered that nitric oxide (NO) was related to the generation and development of DR, closely. Due to the short half-life of NO and the NO producing in the body under the catalysis of nitric oxide synthase (NOS), the researches were more on the mechanisms of NO in DR basing on the investigating NOS. G-954C located at the promoter region of inducible NOS (iNOS), whose expression was lost control under the pathological state [3]. G894T located at the 7th exon of endothelial NOS (eNOS), as the critical enzyme deciding the vessel wall NO level, with its polymorphisms related to cystoid macular edema (CME) [4].

Treins [5] et al. studied and found that the polyol pathway was the important role in glucose metabolism in tissue and cell consisted of aldose reductase (AR) and sorbitol dehydrogenase (SDH), which was correlated to the diabetic microangiopathy, closely. According to Szaflik [6] et al., there was association of the single nucleotide polymorphisms (SNPs) in SDH gene with DR, with the racial differences but without related reports. The SNPs of the promoter of

Table 1. Clinical characteristics of T2DM patients and healthy volunteers

Groups	Cases (N=590)	Sex	Age (X±SDs)
NDR	160	M: 109	68.3±10.5
		F: 51	
SDR	138	M: 82	66.5±11.2
		F: 56	
PDR	132	M: 77	70.5±9.8
		F: 55	
Control	107	M: 69	72.6±12.2
		F: 38	

Note: M, male; F, female; X±SDs, mean ± standard deviations.

the SDH gene were reported to be correlated to SDH expression in retinal cells in diabetes [7]. C-1214G located at -1214 position in SDH gene, while G-888C located at -888. Both of them were the SNPs in the region of the promoter in SDH gene.

In our study, we focused on the SNPs including G-954C, G894T in NOS and C1214G, G888C in SDH, in order to investigate the association of SNPs with DR combined with CME in Chinese patients and provide treatment of DR combined with CME with targets.

Material and methods

Patients and diagnosis

430 patients were diagnosed as T2DM according to criteria of American diabetes association (ADA) and world health organization (WHO) in 2014. 107 healthy people were taken as control. All the data of the patients and volunteers were collected from October 2007 to September 2015. Clinical assessment ethical approval was obtained from the Second Hospital of Hebei Medical University. Inform consent was obtained from each patient and healthy volunteer.

The 430 T2DM patients were received routine ophthalmoscopy, including routine ophthalmic examination included vision, intraocular pressure (IOP), anterior segment examination with slit lamp microscope, mydriasis examination with ophthalmoscopy and fluorescein angiography. 160 patients were without DR (NDR group). 270 patients were diagnosed as DR and divided into SDR group (138 patients with single DR) and PDR group (132 patients with proliferative DR). The basically clinical characteristics of T2DM patients and healthy volunteers were showed in **Table 1**. There was no significant difference of age and sex among the four groups.

The excluded criteria were followed: other ocular diseases; impaired glucose tolerance; family history of diabetes; cardiovascular and cerebrovascular disease; systemic diseases with metabolic disorder influenced by liver, kidney and incretion.

Genomic DNA extraction

5 mL of the peripheral blood were collected from all the patients and controls, and then stored at -20°C with EDTA (Thermo Fisher Scientific Inc. Shanghai, China). The genomic DNA was extracted with genomic DNA extraction kit (Thermo) according to the specification and then stored at -20°C.

PCR-RFLP

The primers of G-954C, G894T, C-1214G and G-888C genes were showed in **Table 2**. The PCR system was 20 μl of volume, totally, including 2 μl of the primers (1 μl of the forward and reverse primers each), 10 μl of the 2×PCR Mix (Thermo), 1 μl of the template cDNA. The PCR was carried on according to the condition showed in **Table 3**. The PCR products were analyzed by 2% agrose gel with gel imaging system (Bio-Rad Laboratories Inc. the USA).

Statistical analysis

SPSS 11.0 software was used for analysis of the data. The clinical characteristics of the patients and volunteers were showed as mean ± standard deviations (X±SDs). t-test was used for comparison of basically clinical characteristics among groups. The allele frequencies were analyzed by gene counting and genotypes were scored. Chi-square test was used for comparing the distribution differences of the genes among groups. The odds ratios (ORs) and 95% confidence intervals (CIs) were analyzed with logistic regression model. Hardy-Weinberg equilibrium was used for analysis the alleles and genotypes. A value of $P<0.05$ considered significant difference.

Table 2. Primers of G-954C, G894T, C-1214G and G-888C genes for PCR-RFLP

Gene	Primers
G-954C	F: 5'-TTGAGTTCGAGACCAGCATGGACAACATGGTG-3'
	R: 5'-TTGAGTTCGACACCAGCATGGACAACATGGTG-3'
G894T	F: 5'-AAGGCAGGAGACAGACAGTGGATGGA-3'
	R: 5'-CCAGTCAATCCCTTTGGTGCTCA-3'
C-1214G	F: 5'-TGTTGCCCAGGCTGGTGTTC-3'
	R: 5'-TGTTGCCCAGGCTGGTGTTG-3'
G-888C	F: 5'-CGCCCGGCCTCATGTCTTTT-3'
	R: 5'-TTGGGGTGGGGAATGTGAGG-3'

Note: F, forward sequence; R, reverse sequence.

Table 3. PCR conditions

Step	Temperature (°C)	Time	Cycle number
Predegeneration	95°C	1 min	
Degeneration	95°C	30 s	30 cycles
Annealing	62°C	30 s	
Extension	72°C	30 s	
Extension terminal	72°C	5 min	1 cycle
Storage	4°C	→∞	

Results

Clinical characteristics comparison in NDR group and SDR combined with PDR group

The clinical characteristics comparisons between the patients with DR and those without DR were showed in **Table 4**. There were significant differences of the course of T2DM and insulin absorption between the T2DM patients with DR and those without ($P<0.05$). There were no significant differences of SBP (systolic blood pressure), DBP (diastolic blood pressure), FBG (fasting blood glucose), HbAlc (glycosylated hemoglobin Alc), LDL (low density lipoprotein), HDL (high density lipoprotein), TG (triglyceride) and TC (total cholesterol) between in patients with DR and those without.

SNPs of G-954C, G894T, C-1214G and G-888C comparison among four groups

All the distributions of the genotypes were accorded to Hardy-Weinberg equilibrium. The distributions and frequencies of the genotypes and alleles of G-954C, G894T, C-1214G and G-888C in NDR, SDR, PDR and control groups were showed in **Tables 5** and **6**.

Comparing the genotype and allele frequency of G-954C, the GG genotype frequency in SDR and PDR groups was higher than in control and NDR groups. The CC genotype frequency in NDR, SDR, and PDR groups was lower than in control group. The G allele frequency in NDR, SDR and PDR groups were higher than in control group.

Comparing the genotype and allele frequency of G894T, the GG genotype frequency in PDR group was lower than in control, NDR and SDR groups. The GT genotype frequency in PDR group was higher than in control, NDR and SDR groups. The TT genotype frequency in NDR and PDR groups was lower than in control and SDR groups. The T allele frequency in NDR group was lower than in control, SDR and PDR groups.

Comparing the genotype and allele frequency of C-1214G, the CC genotype frequency in SDR and PDR group was higher than in control and NDR group. The CG genotype frequency in SDR and PDR group was lower than in control and NDR group, the same was to GG genotype frequency. The C allele frequency in SDR and PDR group was higher than in control and NDR group.

Comparing the genotype and allele frequency of C-888C, the GG genotype frequency in NDR group was lower than in control, SDR and PDR groups. The GC genotype frequency in control group was higher than in NDR, SDR and PDR groups. The CC genotype frequency in NDR group was higher than in control, SDR and PDR groups. The G allele frequency in NDR group was lower than in control, SDR and PDR groups.

Genotype frequencies of combining C-1214G with G-888C

Combing the C-1214C with G-888c, the genotype frequencies were showed in **Table 7**. The combined GG-GC genotype (OR 2.41, 95% CI 0.39-12.58) was correlated with DR, and the frequency decreased as the severity of DR aggravating. The combined GC-GC genotype (OR 3.06, 95% CI 1.12-8.59) was correlated with DR, and the frequency decreased as the severity of DR aggravating.

Table 4. Clinical characteristic comparisons between NDR group and SDR combined with PDR group

	DR (SDR and PDR) (N=270)	NDR (N=160)	t	P
Course of T2DM (years)	19.65±8.30	15.5±9.2	2.056	0.047*
SBP (mmHg)	141.77±22.56	142.08±20.45	1.067	0.198
DBP (mmHg)	87.04±10.75	85.29±10.86	0.886	0.379
FBG (mM)	12.34±8.31	10.47±9.44	1.205	0.287
HbAlc (mM)	9.90±4.15	9.25±3.02	0.439	0.672
LDL (mM)	2.67±1.02	3.05±0.95	-0.580	0.402
HDL (mM)	1.30±0.69	1.12±0.64	1.582	0.174
TG (mM)	1.92±2.50	1.42±1.67	0.695	0.390
TC (mM)	5.03±1.20	4.98±1.38	1.193	0.289
Insulin absorption (n/%)	212/57.3	46/28.8	1.120	0.038*

Note: SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbAlc, glycosylated hemoglobin Alc; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride; TC, total cholesterol; *P<0.05.

Table 5. The genotype frequencies of G-954C, G894T, C-1214G and G-888C in NDR, SDR, PDR and control groups

Genes	Genotypes	Groups (N=430) (n/%)			
		Control (n=107)	NDR (n=160)	SDR (n=138)	PDR (n=132)
G-954C	GG	72/67.3	106/66.3	96/69.6	92/69.7
	GC	26/24.3	45/28.1	37/26.8	36/27.3
	CC	9/8.4	9/5.6	5/3.6	4/3.0
G894T	GG	77/72.0	131/81.9	108/78.3	89/67.4
	GT	25/23.4	28/17.5	24/17.4	42/31.8
	TT	5/4.7	1/0.6	6/4.3	1/0.8
C-1214G	CC	11/10.3	20/12.5	46/33.3	46/34.8
	CG	66/61.7	98/61.3	68/49.3	66/50.0
	GG	28/26.2	42/26.3	24/17.4	20/15.2
G-888C	GG	19/17.8	14/8.8	33/23.9	32/24.2
	GC	46/43.0	53/33.1	50/36.2	47/35.6
	CC	52/48.6	93/58.1	55/40.0	53/40.2

Table 6. The allele frequencies of G-954C, G894T, C-1214G and G-888C in NDR, SDR, PDR and control groups

Genes	Alleles	Groups (N=860) (n/%)			
		Control (n=214)	NDR (n=320)	SDR (n=276)	PDR (n=264)
G-954C	G	170/79.4	257/80.3	229/83.0	220/83.3
	C	44/20.6	63/19.7	47/17.0	44/16.7
G894T	G	179/83.6	290/90.6	240/87.0	220/83.3
	T	35/16.4	30/9.4	36/13.0	44/16.7
C-1214G	C	88/41.1	138/43.1	160/58.0	158/59.8
	G	126/58.9	182/56.9	116/42.0	106/40.2
G-888C	G	84/39.3	81/25.3	116/42.0	111/42.0
	C	130/60.7	239/74.9	160/58.0	153/58.0

Discussions

DR was found to be associated with the SNPs of retinoid-X receptor (RXR) gene [8], transcription factor 7-like 2 (TCF7L2) gene [9] and so on. In our study, we focused on the association of NOS and SDH gene SNPs with DR, including G-954C, G894T, C-1214G and G-888C. By analyzing the genotype and allele frequencies, we found that the SNPs of G-954C, G894T, C-1214G and G-888C were not associated with DR, singly. Combined C-1214G and G-888C, the genotype frequencies, including GG-GC and GC-GC, were associated with DR development, which decreased as the severity of DR aggravating.

In previous study, NOS2 G-954C polymorphism was related to NOS activity and the production of NO [10], with modifying binding of constitutive acting DNA binding protein to the SNPs site. According to the research of Warpeha [11] et al. NOS2 G-954C played a role in prevention from DR, but the mechanism was still unclear. In our study, we did not find the role of NOS2 G-954C playing in DR development of Chinese patients with T2DM.

NOS2 G894T was found to be associated with DR development in Egyptian patients with T2DM [12]. However, in our study, no relationship was found between G894T and DR development in Chinese patients with T2DM. This phenomenon might be the regional difference with many factors influencing, such as diet, climate and so on.

SDH was one of the enzymes in the second step of polyol pathway, with the polymorphism related with the etiology of DR [7]. Both C-1214G and G-888C were the polymor-

Table 7. Genotype frequencies of combing C-1214G with G-888C

Genotypes (C-1214G–G-888C)	Group (N=430) (n/%)				OR (95% CI)
	Control (n=107)	NDR (n=160)	SDR (n=138)	PDR (n=132)	
GG-GG	13/12.1	22/13.8	17/12.3	17/12.9	1.15 (0.34-3.50)
GG-GC	14/13.1	13/8.1	4/2.9	3/2.3	2.41 (0.39-12.58)
GG-CC	2/1.9	5/3.1	3/2.2	4/3.0	1.82 (0.13-20.04)
GC-GG	36/33.6	50/31.3	47/34.1	44/33.3	0.90 (0.41-1.79)
GC-GC	28/26.2	42/26.3	15/10.9	12/9.1	3.06 (1.12-8.59)
GC-CC	0/0	0/0	3/2.2	3/2.3	-
CC-GG	10/9.3	18/11.3	25/18.1	25/18.9	0.49 (0.15-1.52)
CC-GC	3/2.8	8/5.0	17/12.3	17/12.9	0.37 (0.10-1.43)
CC-CC	1/0.9	2/1.3	7/5.1	7/5.3	0.27 (0.08-2.84)

phism sites of SDH gene. There were few reports about SDH C-1214G in DR or T2DM, the same was to SDH G-888C. In our study, G-888C was not related to DR in Chinese patients, which was similar to the result found in Caucasian-Brazilians [13], but opposite to the study of Szaflik [14] et al. focusing on the patients in the region of Poland.

Szaflik [14] et al. combined the C-1214G with G-888C and found that only GC-GC genotype was related to the patients with non-proliferative DR. In our study, we also combined the C-1214G with G-888C, but we found that except for GC-GC genotype, GG-GC genotype was also related to DR development in Chinese patients. As the severity of DR aggravating, the frequencies of GC-GC genotype and GG-GC genotype decreased, significantly, which indicated that the GC-GC and GG-GC genotypes were associated with DR development.

In conclusion, one polymorphism site, G-954C, G894T, C-1214G or G-888C, was not associated with DR development in Chinese patients with CME, while GC-GC and GG-GC genotypes in combining C-1214G with G-888C was associated with DR pathogenesis, significantly. These results provided DR detection, therapy and prognosis with gene targets. However, there was deficiencies in our study, such as the sample only focusing on the patients in the region of bei he. The further investigations on the mechanism of NOS2 and SDH SNPs related to DR in patients with T2DM was needed lager samples size from different regions.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jing-Xue Ma, Shijiazhuang, Heping West Road, No. 215, The Second Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei, China. Tel: +86-311-6600-2561; Fax: +86-311-6600-2561; E-mail: majingxue_l@163.com

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