

# Association of polymorphisms of angiotensin I converting enzyme 2 with retinopathy in type 2 diabetes mellitus among Chinese individuals

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## Abstract

**Aims** To examine the association of Angiotensin I converting enzyme 2 (*ACE2*) gene polymorphisms and retinopathy in a Chinese type 2 diabetes mellitus (T2DM) cohort.

**Methods** A total of 743 T2DM participants were involved in this study including 408 female and 335 male cases. Female cases were divided into two groups: diabetes without retinopathy (DNR group,  $n = 171$ ) and with retinopathy (DR group,  $n = 237$ ), the latter was further subclassified into non-proliferative DR (NPDR group,  $n = 121$ ) and proliferative DR (PDR group,  $n = 116$ ). Male cases were assigned to DNR group ( $n = 153$ ) and DR group ( $n = 182$ ) which was further grouped into NPDR group ( $n = 86$ ) and PDR group ( $n = 96$ ). Two single nucleotide polymorphisms (SNPs; rs2074192 and rs714205) in *ACE2* gene were genotyped.

**Results** In female cases, the frequency of genotypes TT in rs2074192 and CC in rs714205 were higher in DR and PDR group than in DNR group ( $P < 0.05$ ). The frequency of alleles T in SNP rs2074192 and C in SNP rs714205 was higher in DR group ( $P < 0.05$ ) and PDR group ( $P < 0.05$ ) than in DNR group. The frequency of allele T in SNP rs2074192 was higher in PDR group ( $P = 0.04$ ) than in NPDR group. The frequency of haplotype TC and CG was higher in DR and PDR groups, respectively ( $P < 0.05$ ). No positive results were found in male cases.

**Conclusions** Our results revealed that SNPs rs2074192 and rs714205 in *ACE2* gene were associated with the susceptibility of DR and PDR.

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## Introduction

Diabetic retinopathy (DR), one of the most common chronic ocular complications of type 2 diabetes mellitus (T2DM), often causes blindness in diabetic patients worldwide. Hyperglycemia and prolonged duration of diabetes have been deemed to be the main causes of DR.<sup>1</sup> However, some individuals develop DR in a shorter period despite having a tight metabolic control which indicates that other factors may have a role in the development of DR.<sup>2</sup> In the past few years, many factors, including endothelial nitric oxide synthase, receptor for advanced glycation end products, vascular endothelial growth factor and angiotensin converting enzyme were demonstrated to be involved in the development of DR.<sup>3–6</sup>

Angiotensin II (AngII), the main active peptide within the renin–angiotensin system (RAS), has been conformed to have a key role in the pathogenesis of T2DM by inhibiting the secretion of insulin, promoting the generation of inflammation and fibrosis, or producing insulin resistance.<sup>7–11</sup> It is reported that AngII participates in the progress of retinopathy by inducing the synthesis of vascular endothelial growth factor, platelet-derived growth factor and connective tissue growth factor.<sup>12</sup> Blockade of the RAS provides beneficial effects in terms of delaying the development and progression of diabetes and DR.<sup>13,14</sup>

AngI converting enzyme 2 (*ACE2*), a novel component of RAS, as well as an ACE homolog,

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was reported to be able to degrade AngII to Ang-(I–VII) which exerts opposing actions to those of AngII.<sup>15,16</sup> ACE2 is highly expressed in normal kidney, heart, and retina.<sup>15,17,18</sup> Previous studies on single nucleotide polymorphisms (SNPs, rs2074192 and rs714205) in ACE2 gene and diabetic nephropathy or hypertension were conducted,<sup>19–21</sup> however, only SNP rs2074192 was reported to be associated with diabetic hypertension in male individuals, no association was found between the two SNPs in ACE2 gene and diabetic nephropathy in either men or women. It is not clear whether ACE2 gene variants influence the retinopathy progress in T2DM cohort, and whether gender is involved. Herein, we sought to clarify the association of polymorphisms rs2074192 and rs714205 in ACE2 gene with retinopathy in a Chinese T2DM cohort.

## Materials and methods

### Participants

A case-control study was conducted in 743 Chinese cases with T2DM from Qilu Hospital of Shandong University including 408 female cases and 335 male cases. Since ACE2 gene is located on the X chromosome, female and male cases were analyzed, respectively. Female cases were divided into two groups: (1) diabetes without retinopathy (DNR) group comprised 171 patients with DNR and (2) DR group consisted 237 patients with DR, which was further subclassified into nonproliferative DR (NPDR) group with 121 patients and proliferative DR (PDR) group with 116 patients. Male cases were also assigned to DNR group containing 153 patients and DR group involving 182 DR patients, which was further grouped into NPDR group with 86 patients and PDR group with 96 patients. The diagnosis of T2DM was according to clinical features and the guidelines of the Expert Committee Report of the American Diabetes Association.<sup>22</sup> Retinopathy status was graded according to the Early Treatment Diabetic Retinopathy Study.<sup>23</sup> All participants were carefully assessed to ensure meeting the included criteria. The study protocol was approved by the Institutional Review Boards of Qilu Hospital of Shandong University and adhered to the tenets of the Declaration of Helsinki. All subjects gave their informed consent before anonymously participating in the study.

### Genotyping

Genomic DNA was extracted from peripheral venous blood using the DNA Purification kit (TIANamp Blood DNA Kit, Tiangen Biotech, Beijing, China). ACE2 genotyping was performed using PCR-ligase detection reaction technology. The primer pairs used was as

follows: rs2074192, 5'-ACTTTGCCCTTAAACACAGC-3', 5'-AAGTGTGGAAATGTATAAAT-3'; rs714205, 5'-ATCTTGTGGTCTGAGAATGC-3', and 5'-TCCATGAGTTCTAGCCAGAC-3'. The PCR amplification was performed in the GeneAmp PCR system 9600 (Applied Biosystems, Foster City, CA, USA) in a 20- $\mu$ l reaction system and the mixture were denatured at 94 °C for 15 min and then 35 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 7 min. LDR was performed in a total volume of 10  $\mu$ l containing 1  $\mu$ l PCR product, 0.05  $\mu$ l Taq DNA ligase, 1  $\mu$ l probe mix, 1  $\mu$ l Taq DNA ligase buffer, and 6.95  $\mu$ l deionized water. The LDR conditions were 2 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 2 min at 50 °C. The fluorescent products of ligase detection reaction were differentiated by ABI sequencer 377 (Applied Biosystems).

### Statistical analysis

Clinical characteristics were compared using the student's *t*-test for continuous variables and Pearson's  $\chi^2$ -test for categorical variables. All frequency and genotype distributions of studied SNPs were tested for Hardy–Weinberg equilibrium (HWE). Distribution of allele, genotype, and haplotype frequencies of each group were estimated using  $\chi^2$ -test. Statistical analyses were conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Pair-wise linkage disequilibrium (LD) and haplotype frequency estimations were obtained using <http://analysis.bio-x.cn>,  $r^2$  and  $D'$  measures LD for the two investigated SNPs, and those haplotypes that exhibited a frequency <0.03 were ignored in analysis. Logistic regression was used to adjust the risk of DR for age, gender, blood glucose, HbA1c level, and duration of disease. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to estimate the strength of the association. *P*-values <0.05 were considered nominally significant. Data were shown as mean  $\pm$  SD.

## Results

Table 1 shows that significant differences existed in the duration of diabetes, systolic blood pressure, the level of blood urea nitrogen, and total cholesterol in DR group than in DNR group in the Chinese T2DM cohort ( $P < 0.05$ ). However, no statistically significant differences were observed in age, body mass index, the level of fasting plasma glucose, hemoglobin A1c, and triacylglycero between the DR and DNR groups.

The ACE2 gene is X linked, herein the analyses in this study were performed on female and male subjects separately. In the female cases, the genotype distribution of the two SNPs rs2074192 and rs714205 among female cases are given in Table 2, they were in HWE in DNR and

DR groups ( $P > 0.05$ , respectively). The frequency of genotype TT in SNP rs2074192 was significantly increased in DR group (OR = 1.65; 95% CI = 1.01–2.71,

**Table 1** Clinical characteristics of type 2 diabetic patients with retinopathy (DR) and without retinopathy (DNR)

	DR	DNR	P
<i>n</i> (male/female)	419 (182/237)	324 (153/171)	
Age (years)	62.4 ± 12.0	60.2 ± 11.7	0.13
Duration of DM (years)	13.6 ± 7.3	10.7 ± 7.0	0.001
FPG (mmol/l)	8.9 ± 3.8	9.1 ± 3.0	0.34
HbA1c (%)	9.3 ± 2.9	8.6 ± 2.0	0.07
BMI (kg/m <sup>2</sup> )	25.6 ± 4.2	26.2 ± 4.8	0.30
TG (mmol/l)	2.0 ± 1.2	2.2 ± 1.8	0.48
BUN (mmol/l)	10.1 ± 7.6	5.9 ± 3.1	<0.001
TC (mmol/l)	6.4 ± 1.8	5.1 ± 1.6	<0.001
<i>BP (mm Hg)</i>			
SBP	135 ± 17	123 ± 21	<0.001
DBP	78 ± 11	76 ± 10	0.10 < $P < 0.20$

Abbreviations: BMI, body mass index; BP, blood pressure; BUN, blood urea nitrogen; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; *n*, number of subjects; SBP, systolic blood pressure; TC, total cholesterol; TG, triacylglycerol. Data are mean ± SD.

$P = 0.046$ ) and PDR group (OR = 1.97; 95% CI = 1.12–3.47,  $P = 0.02$ ) compared with DNR group, respectively. Moreover, genotype CC in rs2074192 was significantly lower in PDR group (OR = 0.56; 95% CI = 0.32–0.98,  $P = 0.04$ ) than in NPDR group. No significant difference was detected on the genotype distribution of SNP rs2074192 between NPDR and DNR groups. Compared with DNR group, the frequency of genotype CC in SNP rs714205 was demonstrated significantly higher in DR group (OR = 1.61; 95% CI = 1.004–2.59,  $P = 0.047$ ) and PDR group (OR = 1.84; 95% CI = 1.07–3.18,  $P = 0.03$ ), respectively. However, no positive results were shown on this genotype in the comparison between groups NPDR and DNR, PDR and NPDR, respectively.

The frequencies of alleles at both loci in female T2DM cases are shown in Table 2. Compared with DNR group, the frequency of allele T in rs2074192 locus was significantly higher in DR group (OR = 1.49; 95% CI = 1.12–1.98,  $P = 0.006$ ) and PDR group (OR = 1.81; 95% CI = 1.29–2.87,  $P = 0.0006$ ), respectively. Moreover, allele T frequency in SNP rs2074192 was found to increase the risk of PDR compared with NPDR (OR = 1.47; 95%

**Table 2** Frequencies of genotype and allele in female patients

ACE2 loci	Genotype allele	DNR (n = 324); n (%)	Retinopathy					
			All DR (n = 419)		NPDR (n = 207)		PDR (n = 212)	
			n (%)	P <sup>a</sup>	n (%)	P <sup>b</sup>	n (%)	P
rs 2074192	Genotype							
	CC	69 (0.41)	72 (0.30)	<b>0.02</b>	44 (0.36)	0.4	28 (0.24)	<b>0.03<sup>c</sup></b> <b>0.04<sup>d</sup></b>
	CT	69 (0.41)	104 (0.44)	0.61	50 (0.41)	1	54 (0.47)	0.38 <sup>c</sup> 0.42 <sup>d</sup>
	TT	29 (0.17)	61 (0.26)	<b>0.046</b>	27 (0.22)	0.3	34 (0.29)	<b>0.02<sup>c</sup></b> 0.22 <sup>d</sup>
	Allele							
C	207 (0.62)	248 (0.52)	<b>0.006</b>	138 (0.57)	0.23	110 (0.47)	<b>0.0006<sup>c</sup></b> <b>0.04<sup>d</sup></b>	
T	127 (0.38)	226 (0.48)		104 (0.43)		122 (0.53)		
rs 714205	Genotype							
	CC	33 (0.20)	67 (0.28)	<b>0.047</b>	31 (0.26)	0.23	36 (0.31)	<b>0.03<sup>c</sup></b> 0.36 <sup>d</sup>
	CG	79 (0.47)	114 (0.48)	0.83	57 (0.47)	0.99	57 (0.49)	0.73 <sup>c</sup> 0.75 <sup>d</sup>
	GG	56 (0.33)	56 (0.24)	<b>0.03</b>	33 (0.27)	0.27	23 (0.20)	<b>0.01<sup>c</sup></b> 0.18 <sup>d</sup>
	Allele							
	C	145 (0.43)	248 (0.52)	<b>0.01</b>	119 (0.49)	0.15	129 (0.56)	<b>0.004<sup>c</sup></b> 0.16 <sup>d</sup>
G	191 (0.57)	226 (0.48)		123 (0.51)				

Abbreviations: DNR, diabetes without retinopathy; DR, diabetic retinopathy; NPDR, nonproliferative DR; PDR, proliferative DR.

$\chi^2$ -Test was used to compare the genotype and allele frequencies between DR and DNR subjects.

$P$ -values < 0.05 considered to be statistically significant are shown in bold.

<sup>a</sup>All DR vs DNR.

<sup>b</sup>NPDR vs DNR.

<sup>c</sup>PDR vs DNR.

<sup>d</sup>PDR vs NPDR.

CI = 1.02–1.14,  $P = 0.04$ ). In rs714205 locus, the frequency of allele C was significantly higher in DR group ( $P = 0.01$ ) and PDR group ( $P = 0.004$ ) compared with DNR group respectively, the strength of their association with DR and PDR were indicated as OR = 1.45, 95% CI = 1.09–1.92 and OR = 1.65, 95% CI = 1.18–2.31, respectively.

The two investigated SNPs were in LD with each other (DR vs DNR:  $r^2 = 0.83$ ,  $D' = 1.00$ ; PDR vs DNR:  $r^2 = 0.80$ ,  $D' = 1.00$ ; NPDR vs DNR:  $r^2 = 0.85$ ,  $D' = 1.00$ ). Haplotype frequencies of the two loci among female T2DM subjects are summarized in Table 3. Of the haplotypes constructed, three were found to be common, and were included in subsequent analysis. Significantly increased frequency of haplotype TC was seen in DR (OR = 1.49; 95% CI = 1.12–1.98,  $P = 0.006$ ) and PDR groups (OR = 1.81; 95% CI = 1.29–2.54,  $P = 0.0006$ ) compared with DNR, respectively. Meanwhile, reduced frequency of haplotype CG were found in DR (OR = 0.68; 95% CI = 0.52–0.90,  $P = 0.008$ ) and PDR groups (OR = 0.60;

95% CI = 0.43–0.84,  $P = 0.003$ ). No significant differences were found in NPDR compared with DNR.

No significance was observed on the frequency distribution analyses of genotypes and alleles at both loci among male T2DM subjects (Table 4).

### Discussion

Some studies have been conducted to report the association between polymorphisms (SNPs, rs2074192 and rs714205) in ACE2 gene and diabetic nephropathy or hypertension.<sup>19–21</sup> It is not clear whether ACE2 gene variants influence the retinopathy progress in T2DM cohort. In this study, we analyzed these two SNPs in the ACE2 gene in T2DM patients.

This study showed a strong correlation of SNPs rs2074192 and rs714205 polymorphism in ACE2 gene with DR and PDR in Chinese female T2DM patients, not in male T2DM patients. The ACE2 gene is located on

**Table 3** Analysis of haplotype frequency for rs2074192 and rs714205 in female patients

Haplotypes rs2074192/rs714205	DNR n (%)	Retinopathy					
		All DR		PDR		NPDR	
		n (%)	P <sup>a</sup>	n (%)	P <sup>b</sup>	n (%)	P <sup>c</sup>
C/C	22 (4.6)	16 (4.8)	0.92	7 (3.0)	0.29	15 (6.2)	0.46
C/G	226 (47.7)	191 (57.2)	<b>0.008</b>	103 (44.4)	<b>0.003</b>	123 (50.8)	0.13
T/C	226 (47.7)	127 (38.0)	<b>0.006</b>	122 (52.6)	<b>0.0006</b>	104 (43.0)	0.23

Abbreviations: DNR, diabetes without retinopathy; DR, diabetic retinopathy; NPDR, nonproliferative DR; PDR, proliferative DR.

Frequency >0.03 in both DR and DNR groups has been involved to calculate the odds ratio and 95% confidence interval.

P-values <0.05 considered to be statistically significant are shown in bold.

<sup>a</sup>P: DNR vs DR.

<sup>b</sup>P: DNR vs PDR.

<sup>c</sup>P: DNR vs NPDR.

**Table 4** Frequencies of allele in male patients

ACE2 loci	Genotypes allele	DNR (n = 324) n (%)	Retinopathy					
			All DR (n = 419)		NPDR (n = 207)		PDR (n = 212)	
			n (%)	P <sup>a</sup>	n (%)	P <sup>b</sup>	n (%)	P
rs 2074192	Male							
	C	92 (0.61)	111 (0.61)	0.95	56 (0.65)	0.56	55 (0.57)	0.53 <sup>c</sup> 0.28 <sup>d</sup>
rs 714205	T	58 (0.39)	71 (0.39)		30 (0.35)		41 (0.43)	
	Male							
	C	58 (0.39)	73 (0.40)	0.79	30 (0.35)	0.56	43 (0.45)	0.34 <sup>c</sup> 0.17 <sup>d</sup>
	G	92 (0.61)	109 (0.60)		56 (0.65)		53 (0.55)	

Abbreviations: DNR, diabetes without retinopathy; DR, diabetic retinopathy; NPDR, nonproliferative DR; PDR, proliferative DR.

$\chi^2$ -Test was used to compare the genotype and allele frequencies between DR and DNR subjects.

<sup>a</sup>All DR vs DNR.

<sup>b</sup>NPDR vs DNR.

<sup>c</sup>PDR vs NPDR.

<sup>d</sup>PDR vs NPDR.

chromosome Xp22 and consists of 18 exons and 17 introns, which infers its gender characteristics.<sup>24</sup> Therefore, we analyzed the two SNPs in female and male, respectively. In the female group, the genotypes TT or CC and alleles T or C in SNPs rs2074192 or rs714205 were demonstrated as risk genotypes or alleles in DR and PDR; however, minor homozygous genotype CC in SNP rs2074192 was shown protective on PDR individuals. Moreover, strong LD was detected between the two loci. The haplotype TC comprised by the risk alleles of the two SNPs was significantly higher in DR group, especially in PDR group, whereas the protective haplotype CG was significantly over represented in DNR group. It is a crucial finding that SNPs rs2074192 and rs714205 in the *ACE2* gene were associated with the susceptibility to DR and PDR in the Chinese female T2DM cohort. The minor homozygous genotype in SNP rs2074192 can reduce the risk of PDR. We also found that TC or CG may be the risk or protective haplotypes of DR subjects, especially PDR subjects.

To the best of our knowledge, this is the first study to investigate the association of *ACE2* gene polymorphism with DR in T2DM. It is demonstrated that *ACE2* cleaves AngII to form Ang-(I–VII) with a high catalytic efficiency; whereafter, Ang-(I–VII) exerts opposing actions to those of AngII by inhibiting vasoconstriction and cell proliferation and consequently protect the retina.<sup>16,25</sup> The reduction of serum level of Ang-(I–VII) may reduce the protective function on retina and cause the occurrence and development of DR. In this study, we found that *ACE2* gene variants were probably involved in the pathogenesis of DR and PDR in the Chinese female T2DM cohort. The possible reason may be that the variants rs2074192 and rs714205 located on the intron area of *ACE2* gene which may affect the expression of *ACE2* gene by regulating the splicing of mRNA and eventually results in the decrease of serum level of Ang-(I–VII).

As we know, gender and sex hormones can probably affect the components of the RAS,<sup>26</sup> *ACE2* is one of the components of RAS gene that is located on X chromosome.<sup>27</sup> The escapes of X inactivation are reported on many genes may contribute to the sexual differences of *ACE2* gene in this Chinese T2DM cohort.<sup>27</sup>

In conclusion, the present study demonstrates that the *ACE2* gene polymorphisms rs2074192 and rs714205 are correlated with DR in the Chinese female T2DM cohort. The two SNPs can increase the susceptibility of DR and PDR. The minor homozygous genotype at SNP rs2074192 can reduce the risk of PDR. Moreover, the risk and protective haplotypes comprised by the two SNPs were found in DR. Further researches focusing on the exact biological mechanism and other possible factors underlying the association of the *ACE2* gene with DR in T2DM individuals are needed to be confirmed in the future.

## Summary

### What was known before

- Previous studies on single nucleotide polymorphisms (SNPs, rs2074192 and rs714205) in *ACE2* gene and diabetic nephropathy or hypertension were conducted.

### What this study adds

- This is the first study to report the association of *ACE2* gene polymorphisms with diabetic retinopathy in Chinese type 2 diabetes mellitus individuals.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

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