RETRACTED ARTICLE: Association of the polymorphisms in FOXO1 gene and diabetic nephropathy risk

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ABSTRACT

Purpose: This study was aimed to study the hypothesis that forkhead box O1 (FOXO1) gene rs17446614 and rs17592236 single nucleotide polymorphisms (SNPs) influenced the development of diabetic nephropathy (DN).

Methods: This study included 138 DN patients and 149 healthy controls. Controls were matched with the patients in age and gender. The method of polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) was used to detect FOXO1 gene polymorphisms. Haplotype software was conducted to analyze the linkage disequilibrium and haplotypes of FOXO1 gene polymorphisms. Relative risk of DN was expressed by odds ratios (ORs) and 95% confidence intervals (95% CIs), then the results were adjusted by clinical characteristics of the study subjects using logistic regression analysis. Subgroup analysis was performed according to gender.

Results: AA genotype of rs17446614 SNPs was significantly associated with the risk of DN (P = 0.037, adjusted OR = 5.412, 95% CI = 1.103–26.559), especially in female (OR = 8.700, 95% CI = 1.08–75.062, P = 0.021), FOXO1 rs17446614 A allele positively associated with the development of DN (P = 0.027, adjusted OR = 1.680, 95% CI = 1.060–2.662, particularly in women (OR = 2.033, 95% CI = 1.070–3.749, P = 0.028). A-C haplotype formed by FOXO1 gene rs17446614 and rs17592236 SNPs was significantly associated with the increased risk of DN (P = 0.011, OR = 1.850, 95% CI = 1.146–2.986).

Conclusion: FOXO1 gene rs17446614 SNP, and the A-C haplotype of rs17446614 and rs17592236 polymorphisms were risk factors for the development of DN.

Introduction

Diabetic nephropathy (DN) is one of the most common microvascular complications of diabetes mellitus (DM). It also is the cause of end-stage renal disease (ESRD) [1,2]. The characteristics of DN mainly include nephrotic syndrome and glomerular sclerosis which is caused by microvascular pathological changes in the kidney. In the early stage, it often has no symptoms. During the advanced stages, these disorders usually lead to proteinuria, hypertension and other symptoms. With the progress of therapy methods in DM patients, the mortality of it is decreasing. However, diabetes complications with cardiovascular diseases and kidney diseases has become the main reasons for death and disability in diabetic patients [3,4]. DN occurs both in type 1 and type 2 diabetes (T1DM and T2DM). However, the pathogenesis of DN is not clear. Epidemiology studies showed that the occurrence of DN is affected by the combined actions of multiple factors [5,6]. Among these factors, genetic factors play a crucial role in DN onset, and many genes are found [7,8].

Forkhead box O1 (FOXO1) is a member of the forkhead box (FOX) family which belongs to transcription factors. FOXO1 is widely expressed in mammals. In humans, this protein is encoded by the FOXO1 gene which is located in chromosome 13q14.1. FOXO1 could combine with a promoter by shuttling into the nucleus and activate the transcription activity of the target genes. FOXO1 directly or indirectly mediates many biological responses. Recent researches found that FOXO1 involves in the regulation of proliferation and the response for oxidative stress [9–11]. In addition, other researches found that FOXO1 regulates adipogenesis and glucose metabolism [12,13]. It is demonstrated that FOXO1 involves in the development of DM by regulated the glycolysis and gluconeogenesis [14].

A rat model analysis showed that FOXO1 is also related to kidney injury [15]. Persistent DM results in many complications, such as DN. So we assumed that FOXO1 is associated with the development of DN. In the present study, we selected two single nucleotide polymorphisms (SNPs) of FOXO1 gene (rs17446614 and rs17592236) to verify the hypothesis in the Chinese Han population.

Materials and methods

Research subjects

Our study was approved by the Ethics Committee of The Third Hospital of Hebei Medical University. Written informed consent was obtained from all included patients, and the study was conducted under the principles of the Declaration of Helsinki.

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consents were provided by all of the participants. Controls were in line with the cases in terms of age and gender. All of the subjects were Chinese Han population and had no genetic relationship.

287 DM patients were diagnosed by two pathologists in The Third Hospital of Hebei Medical University according to the previous criteria [16]. 138 DN patients who had no other complications were recruited as case group. DM patients without any complications were enrolled in the control group. All of the patients had no histories of hypertension, ischemic cardiomyopathy and chronic heart failure and tumours.

**Sample collection**

3 ml venous blood sample was collected from each subject after 8 h fasting period. The samples were put into the anticoagulative tube with EDTA 2Na. Genomic DNA was extracted by the conventional chloroform/isoamyl alcohol method, and then stored at −20 °C for future use.

**Genotyping method of FOXO1 gene polymorphisms**

PCR primers of rs17446614 and rs17592236 were designed by Primer Premier 5.0 software, based on the sequence of FOXO1 gene (Table 1). Primer sequences were synthesized by Sangon Biotech (Shanghai, China).

PCRs reaction used a 25 μl system, including 2 μl templates DNA, 1.5 μl primer mixture, 2.5 μl 10 × Buffer, 0.8 μl Taq DNA polymerase, 2 μl dNTPs and 16.2 μl ddH2O. PCR reaction program was as follows, initial degeneration at 94 °C for 5 min; then followed by 35 cycles of degeneration at 94 °C for 55 s, annealing at 59 °C for 45 s, extension at 72 °C for 60 s and the final extension at 72 °C for 10 min. PCR products were digested by restriction enzyme and examined by 2% agarose gel electrophoresis.

**Statistical analysis**

Hardy–Weinberg equilibrium (HWE) test was performed to assess the genotype distributions both in cases and controls. Direct counting method was utilized to calculate the genotype and allele frequencies. Linkage disequilibrium and haplotypes between polymorphisms were checked by Haploview software. Association of FOXO1 gene polymorphisms with the risk of DN was represented by odds ratios (ORs) with 95% confidence intervals (95% CIs). P-values of <.05 revealed statistical significance. All of the calculations were compiled by SPSS 18.0.

### Table 1. Primer sequences of FOXO1 gene rs17446614 and rs17592236 SNPs.

| SNP          | Primer sequences                          |
|--------------|-------------------------------------------|
| rs17446614   | 5′-TACATAAAAAACATAATGAATTT-3′             |
|              | 5′-AACTTAATAGTTTATGTATAGTA-3′            |
| rs17592236   | 5′-CTTATTTCTTATAACACACACTG-3′            |
|              | 5′-CAGTGGTGTGTTATAAAGATAAAG-3′           |

### Table 2. The clinical characteristics of subjects.

| Variables                  | Cases, n = 138 | Controls, n = 149 | P     |
|----------------------------|----------------|-------------------|-------|
| Age (year)                 | 61.51 ± 8.99   | 60.08 ± 8.96      | .177  |
| Gender (%)                 |                |                   | .308  |
| Male                       | 75 (54.35%)    | 72 (48.32%)       |       |
| Female                     | 63 (45.65%)    | 77 (51.68%)       |       |
| BMI (kg/m²)                | 26.35 ± 3.82   | 25.41 ± 3.19      | <.01  |
| Smoking (%)                | 43 (31.16%)    | 29 (19.46%)       | .022  |
| Smoking (%)                | 38 (27.54%)    | 31 (20.81%)       | .182  |
| History of hypertension (%)| 13.84 ± 4.49   | 12.81 ± 4.14      | .043  |
| SBP (mmHg)                 | 135.44 ± 26.31 | 124.69 ± 16.38    | <.01  |
| DBP (mmHg)                 | 79.46 ± 13.24  | 78.31 ± 12.42     | <.01  |
| TC (mmol/L)                | 4.06 ± 1.36    | 4.23 ± 1.54       | .382  |
| HDL-C (mmol/L)             | 0.93 ± 0.26    | 0.99 ± 0.31       | .026  |
| LDL-C (mmol/L)             | 2.43 ± 1.02    | 2.38 ± 0.96       | .513  |
| TG (mmol/L)                | 1.58 ± 0.31    | 1.51 ± 0.22       | .038  |

BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; TG: triglyceride.

**Results**

**The clinical characteristics of subjects in the case and control groups**

In this study, the demographic and clinical characteristics of subjects were investigated and recorded. The average age of the cases and controls were 61.51 ± 8.99 and 60.08 ± 8.93, respectively. The case group contained 75 males and 63 females, the number of men and women in the control group was 72 and 77, respectively. There was no significant difference between the case and control groups in age and gender (P > .05). we found that BMI (body mass index), smoking, duration of diabetes and the history of hypertension might be the influence factors for DN development (P < .05), but not drinking (P > .05). SBP and DBP in the DN patients were significantly higher than that in DM patients (P < .01). The concentration of HDL-C (high-density lipoprotein-cholesterol) and TG (triglyceride) were obviously between the two groups (P < .05), but not LDL-C (low-density lipoprotein-cholesterol) or TC (total cholesterol). The detailed data are shown in Table 2.

**Association of FOXO1 gene polymorphisms with the DN risk**

The distribution of FOXO1 polymorphisms in the study population is displayed in Figure 1. Frequencies of rs17446614 GG, GA and AA genotypes were 66.67%, 27.54% and 5.80% in cases and 76.51%, 22.15% and 1.34% in controls, respectively (Table 3). AA genotype frequency was significantly different between case and control groups (AA vs. GG: OR = 1.680, 95% CI = 1.716–23.911). FOXO1 rs17446614 A allele carriers were frequently observed in DN patients than in DM patients (P = .047), indicating a statistical association with the risk of DN (OR = 4.957, 95% CI = 1.027–23.911). FOXO1 rs17446614 A allele carriers were frequently observed in DN patients than in DM patients (P = .019). The difference showed that A allele might increase the risk of DN was represented by odds ratios (ORs) with 95% confidence intervals (95% CIs). P-values of <.05 revealed statistical significance. All of the calculations were compiled by SPSS 18.0.
For rs17592236 polymorphism, the frequencies of CC, CT and TT genotypes were 44.20%, 42.75% and 13.04% in DN patients and 45.64%, 41.61% and 12.75% in DM patients, respectively. However, all of the genotypes and alleles had no distinct differences between case and control groups (Table 3, \( P > .05 \)).

In addition, the subgroup analysis based on gender was also performed to estimate the relationship between FOXO1 polymorphisms with DN risk. The distributions of FOXO1 rs17446614 and rs17592236 SNPs in control and case groups according to the individuals’ gender are shown in Figure 2. Analysis results summarized in Table 4 demonstrated that rs17446614 AA genotype (OR = 8.700, 95% CI = 1.008–75.062, \( P = .021 \)) and A allele (OR = 2.003, 95% CI = 1.070–3.749, \( P = .028 \)) predicted a high risk of DN in females. Meanwhile, the close association was not observed among men (\( P > .05 \) for all). Additionally, FOXO1 rs17592236 SNP did not show any significant correlation with DN risk neither in men nor in women (\( P > .05 \) for all).

### Table 3. Association between FOXO1 gene polymorphisms with DN risk.

| SNP     | Case n = 138 (%) | Control n = 149 (%) | \( P \) | OR (95% CI) | \( P^a \) | ORa (95% CI) |
|---------|------------------|---------------------|------|-------------|--------|-------------|
| Rs17446614 |                  |                    |      |             |        |             |
| GG      | 92 (66.67)       | 114 (76.51)        | –    | –           | –      | –           |
| GA      | 38 (27.54)       | 33 (22.15)         | .197 | 1.427 (0.830–2.452) | .287 | 1.347 (0.778–2.330) |
| AA      | 8 (5.80)         | 2 (1.34)           | .047 | 4.957 (1.027–23.911) | .037 | 5.412 (1.103–26.559) |
| G       | 222 (80.43)      | 261 (87.59)        | –    | –           | –      | –           |
| A       | 54 (19.57)       | 37 (12.42)         | .019 | 1.716 (1.089–2.704) | .027 | 1.680 (1.060–2.662) |
| Rs17592236 |                |                    |      |             |        |             |
| CC      | 61 (44.20)       | 68 (45.64)         | –    | –           | –      | –           |
| CT      | 59 (42.75)       | 62 (41.61)         | .816 | 1.061 (0.646–1.743) | .944 | 1.018 (0.615–1.684) |
| TT      | 18 (13.04)       | 19 (12.75)         | .884 | 1.056 (0.508–2.195) | .959 | 1.020 (0.482–2.157) |
| C       | 181 (65.58)      | 198 (66.44)        | –    | –           | –      | –           |
| T       | 95 (34.42)       | 100 (33.56)        | .827 | 1.039 (0.736–1.468) | .942 | 1.013 (0.714–1.437) |

*aRepresented the values of \( P \) and OR were adjusted by clinical parameters.

For rs17592236 polymorphism, the frequencies of CC, CT and TT genotypes were 44.20%, 42.75% and 13.04% in DN patients and 45.64%, 41.61% and 12.75% in DM patients, respectively. However, all of the genotypes and alleles had no distinct differences between case and control groups (Table 3, \( P > .05 \)).

Discussion

The main finding of the present study was that FOXO1 gene rs17446614 and rs17592236 SNPs were positively associated with the development of DN. Müssig et al. indicated that AA genotype of FOXO1 gene rs17446614 might increase the glucose level and associated with the T2DM development in German [17]. But it was not explored in DN patients. In the present study, we found that AA genotype and A allele of rs17446614 SNP might increase 5.412 and 1.680 times risk of DN, respectively. There is no study focusing on FOXO1 gene rs17592236 polymorphism and the risk of DN. In the present study, we failed to find any association between this SNP and the development of DN. But Tan et al. showed that the CT + TT genotypes of rs17592236 decreased the risk of hepatocellular carcinoma [18]. DN is caused by the combined effects of multiple factors, but not the single factor. So, we explored the interaction between the two FOXO1 gene polymorphisms and found linkage disequilibrium existed between these two SNPs, and the A-C haplotype was associated with 1.850 times increased risk of DN. After interacting with the rs17592236 C allele, the effect of rs17446614 A allele was increased. So we suggested that FOXO1 gene rs17446614 and rs17592236 SNPs are significantly related to the risk of DN.

### Haplotype analysis of FOXO1 gene rs17446614 and rs17592236 SNPs

Linkage disequilibrium was found between FOXO1 gene rs17446614 and rs17592236 SNPs (\( D’ = 1.0, r^2 = .05 \)). Four haplotypes were constituted by these two SNPs. But only three haplotypes (G–C, G–T and A–C) were found in the present study (Table 5). Among them, frequencies of G–T and A–C haplotypes were higher in cases than that in controls. But the difference of G–T haplotype was not significant (\( P > .05 \)). Compared with G–C haplotype, A–C haplotype significantly associated with the risk of DN (\( P = .011 \), OR = 1.850, 95% CI = 1.146–2.986).
FOXO1 is a member of O subfamily of FOX transcription factors. FOXO1 is an element of the PI3K-Akt signaling pathway. It mainly expresses in hepatocytes, adipocytes, pancreatic β cells and vascular endothelial cells [19–22]. The activity of FOXO1 is primarily regulated by phosphorylation, acetylation, methylation and ubiquitination. Phosphorylation of FOXO1 is performed by Akt. When FOXO1 is in the inactive cytosolic phosphorylated state, it will stimulate the proliferation and cell survival [23]. Another function of FOXO1 is the resistance for oxidative stress. An in vitro study which was executed by Ning et al. showed that in cardiomyocytes, FOXO1 might induce autophagy against the oxidative stress [11]. In addition, it was suggested that FOXO1 inhibitors are probably used as novel medicines for obesity and metabolic disorders [12,24]. Inhibitions of FOXO1 will active the PI3K-Akt pathway, and then regulate the glycogen synthesis and gluconeogenesis [25]. FOXO1 control the glycometabolism through mediating the expression of metabolic enzymes which catalyze key steps [26]. Mutation or deregulation of FOXO1 can result in pathophysiological conditions, such as DM [27].

DM is a chronic dysmetabolic syndrome in the whole body. It will lead to various chronic injuries and dysfunctions of organs, such as nephropathy, neuropathy and retinopathy. Microvascular complication is one of the causes of death and disability in DM patients. In recent years, with the improvement of living standards, the changes of lifestyle and the increase of ageing population, the incidence of DM has risen rapidly. Thus the prevalence of DN has increased. It takes a heavy toll on the economics of society and family. In order to diagnose and treat the DN in the early stage, it is necessary to identify the genetic factors that contribute to the disease. In this study, we performed a subgroup analysis based on gender to investigate the genetic association of FOXO1 polymorphisms with DN risk. The results are presented in Table 4 and Table 5.

Table 4. Genetic influences of FOXO1 polymorphisms with DN risk based on individuals’ gender.

| SNP          | Case (n, %) | Control (n, %) | P     | OR (95% CI) |
|--------------|-------------|----------------|-------|-------------|
| Male         |             |                |       |             |
| Rs17446614   |             |                |       |             |
| GG           | 52 (69.33%) | 56 (77.78%)    | –     | –           |
| GA           | 21 (28.00%) | 15 (20.83%)    | .290  | 1.508 (0.703–3.232) |
| AA           | 2 (2.67%)   | 1 (1.39%)      | .527  | 2.154 (0.190–24.464) |
| G            | 125 (83.33%)| 127 (88.15%)   | –     | –           |
| A            | 25 (16.67%) | 17 (11.81%)    | .234  | 1.494 (90.769–2.902) |
| Rs17592236   |             |                |       |             |
| CC           | 32 (42.67%) | 34 (47.22%)    | –     | –           |
| CT           | 33 (44.00%) | 29 (40.28%)    | .592  | 1.209 (0.604–2.421) |
| TT           | 10 (13.33%) | 9 (12.50%)     | .750  | 1.181 (0.425–3.280) |
| C            | 97 (64.67%) | 97 (67.36%)    | –     | –           |
| T            | 53 (35.33%) | 48 (33.33%)    | .252  | 0.741 (0.444–1.238) |
| Female       |             |                |       |             |
| Rs17446614   |             |                |       |             |
| GG           | 40 (63.49%) | 58 (70.75%)    | –     | –           |
| GA           | 17 (26.98%) | 18 (23.03%)    | .426  | 1.369 (0.630–2.974) |
| AA           | 6 (9.52%)   | 7 (8.70%)      | .021  | 8.700 (1.008–75.062) |
| G            | 97 (76.98%) | 134 (87.87%)   | –     | –           |
| A            | 29 (23.02%) | 20 (12.13%)    | .028  | 2.003 (1.070–3.749) |
| Rs17592236   |             |                |       |             |
| CC           | 29 (46.03%) | 34 (47.22%)    | –     | –           |
| CT           | 26 (41.27%) | 33 (43.75%)    | .828  | 0.924 (0.452–1.886) |
| TT           | 8 (12.70%)  | 10 (13.33%)    | .905  | 0.938 (0.327–2.690) |
| C            | 84 (66.67%) | 101 (70.41%)   | –     | –           |
| T            | 42 (33.33%) | 53 (29.59%)    | .849  | 0.953 (0.579–1.567) |

Table 5. Haplotype analysis between rs17446614 and rs17592236 SNPs.

| Locus1–Locus2 | Case (n=276 (%)) | Control (n=298 (%)) | P     | OR (95% CI) |
|---------------|------------------|---------------------|-------|-------------|
| G–C           | 127 (46.01%)     | 161 (54.03%)        | –     | –           |
| G–T           | 95 (34.42%)      | 100 (33.56%)        | .317  | 1.204 (0.936–1.734) |
| A–C           | 54 (19.57%)      | 37 (12.42%)         | .011  | 1.850 (1.146–2.986) |

Figure 2. Subgroup analysis based on gender was performed to investigate the genetic association of FOXO1 polymorphisms with DN risk. NS: not significant; *P < .05.
necessary to certify the pathogenesis of DN. A recent study on an animal model revealed that under high glucose conditions, FOXO1 could inhibit the proliferation of mesangial cells [28]. To a certain extent, FOXO1 decreases podocyte injury. However, FOXO1 expression and activity are reduced in the kidney of diabetic rats [29]. Polymorphisms in the FOXO1 gene might act as a reason for these changes.

In spite of confirming the hypothesis, many limitations existed in this study. Firstly, the small sample size will affect the accuracy of the present results. Secondly, it is believed that DN is the result of combinations among multiple genetic and environmental factors, but not a single factor [30–32]. Therefore, other factors should be considered in future studies. Additionally, our results may be helpful in selecting individuals with a high risk of DN. Prevention strategies were necessary for these susceptible individuals. Whether the FOXO1 gene could be employed as a biomarker for early diagnosis of DN remained unclear. Further investigations are required.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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