

Correlations of AQP4 expression and polymorphism with diabetic retinopathy

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Abstract. – **OBJECTIVE:** The aim of this study was to investigate the relationships of aquaporin 4 (AQP4) rs200498749, rs149465 and rs650217 polymorphisms and gene expression with diabetic retinopathy (DR).

PATIENTS AND METHODS: A total of 400 patients with diabetes mellitus (DM) treated in our hospital were enrolled in this study. All subjects were divided into two groups, including DM group (n=200, without DR) and DR group (n=200, with DR). The polymorphisms rs200498749, rs149465 and rs650217 of AQP4 gene were analyzed in the two groups. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect gene expression, and statistical analysis was performed in combination with clinical data.

RESULTS: The distribution of alleles of AQP4 rs650217 ($p=0.015$) in DR group was different from that in DM group, and the frequency of T allele was significantly higher in DR group than DM group. The distribution of genotypes of AQP4 rs149465 ($p=0.000$) and rs650217 ($p=0.000$) showed statistically significant difference between DR group and DM group. The frequency of AA genotype of polymorphism rs149465 and CT genotype of polymorphism rs650217 was significantly higher in DR group than DM group. Besides, there was a difference in the distribution of recessive models of AQP4 rs149465 ($p=0.023$) and rs650217 ($p=0.014$) between DR group and DM group. DR group exhibited remarkably lowered frequency of AA + AT recessive model of the polymorphism rs149465 and raised frequency of CT + TT recessive model of the polymorphism rs650217. Similarly, a difference was found in the distribution of haplotypes CAT ($p=0.014$) and CTC ($p=0.003$) of AQP4 rs200498749, rs149465 and rs650217 between DR group and DM group. The polymorphism rs200498749 of AQP4 gene was significantly correlated with AQP4 gene expression ($p<0.05$). Meanwhile, the expression of AQP4 gene was clearly higher in patients with CC genotype in DR group ($p<0.05$). AQP4 polymorphism rs200498749 was related to fasting blood glucose ($p=0.000$) and hemoglobin A1c (HbA1c) ($p=0.000$) in DR group, and polymor-

phism rs650217 had an association with serum creatinine level ($p=0.034$). AQP4 polymorphisms rs149465 ($p=0.023$) and rs650217 ($p=0.042$) were correlated with clinical stage in DR group. In addition, the proportion of patients with AA genotype of rs149465 at stage VI and with TT genotype of rs650217 at stage I rose significantly ($p<0.05$).

CONCLUSIONS: AQP4 gene polymorphism has a potential relationship with the susceptibility and progression of DR.

Key Words:

Diabetic retinopathy, Polymorphism, AQP4.

Introduction

Diabetes mellitus (DM) is one of the most common diseases of the endocrine system. In recent years, the incidence rate of DM increases greatly, and it has become one of the major health issues hindering the development of global economy. Currently, there are 400-500 million DM patients worldwide^{1,2}. Numerous studies have indicated that one of the leading causes of DM-induced body damage is the development of complications including ocular complications, diabetic foot, and cardio-cerebrovascular complications³. As a severe ocular complication, diabetic retinopathy (DR) is a major cause of acquired blindness in adults^{4,5}. Therefore, discovering the pathogenesis of DR is of great significance to reduce eye damage in patients.

Currently, it has been proven that gene polymorphism is a factor affecting the susceptibility to many diseases^{6,7}. Aquaporin 4 (AQP4) is one of the important molecules affecting the flow of water molecules inside and outside cells. It is also able to affect the progression of various diseases⁸⁻¹⁰. Previous studies have suggested that AQP4 gene polymorphism may

be a susceptible factor affecting DM and DR progression. In this study, therefore, 400 DM patients were selected as research objects. Based on the presence of DR, these patients were divided into two groups. The difference in the distribution of alleles, genotypes and haplotypes of AQP4 polymorphisms rs200498749, rs149465, and rs650217 was analyzed. In addition, the relationships of AQP4 polymorphisms rs200498749, rs149465, and rs650217 and gene expression with DR were analyzed in combination with AQP4 gene expression, clinical indicators and patient's clinical stage.

Patients and Methods

General Data

A total of 400 patients diagnosed with DM as per the diagnostic criteria for DM (proposed by the World Health Organization Expert Committee on Diabetes Mellitus in 1999) in our hospital over the past three years were selected as research objects. Specific inclusion criteria were as follows: patients with typical symptoms of DM (polyuria, polydipsia, polyphagia and emaciation) and random blood glucose ≥ 11.1 mmol/L or fasting blood glucose ≥ 7.0 mmol/L. All subjects were divided into two groups, including DM group (n=200, without DR) and DR group (n=200, with DR). DR was diagnosed *via* fundus examination by a doctor with intermediate professional title or above. There were no statistically significant differences in the general data of patients (gender and age) between the two groups ($p > 0.05$). This investigation was approved by the Ethics Committee of Shandong Shanxian Central Hospital.

Sample Collection and Genomic Deoxyribonucleic Acid (DNA) Extraction

Peripheral blood (5-6 mL) was sampled by nurses on duty from patients in DM group and DR group, respectively. Collected samples were centrifuged at 3500 rpm for 5 min within 1 h, and stored at 4°C for extraction of genomic DNA within one week. Thereafter, the middle karyocytes were carefully collected into new Eppendorf (EP) tubes using a pipette tip, followed by DNA extraction in strict accordance with a peripheral blood genomic DNA extraction kit (Invitrogen, Carlsbad, CA, USA). Briefly, karyocytes were collected, added with proteinase K and mixed, followed by still standing and mixture with anhydrous ethanol. Next, flocculent precipitates were extracted using an adsorption column, rinsed and

added with elution buffer. After centrifugation, genomic DNA was obtained.

Determination of AQP4 Polymorphisms rs200498749, rs149465 and rs650217

The regions of AQP4 polymorphisms rs200498749, rs149465 and rs650217 were amplified by polymerase chain reaction (PCR). The primers were designed using Primer Premier 5 software. Primers for each polymorphic region were as follows: rs200498749 forward: "AGCAGTCACAGCGGAATTTCT", reverse: "TCTGTTCCACCCAGTTGATG", rs149465 forward: "TCAGCATCGCCAAGTCTGTC", reverse: "CTGGGAGGTGTGACCAGATAG", rs650217 forward: "CATGGAAATCTTACCGCTGGT", reverse: "TCAGTCCGTTTGAATCACAG". All sequences amplified were sent to Nanjing Biotechnology Co., Ltd. (Nanjing, China) for DNA sequencing.

Measurement of AQP4 Gene Expression

Real-time quantitative polymerase chain reaction (RT-qPCR) was conducted to detect the expression of AQP4. Total RNAs were extracted from peripheral blood karyocytes in DM and DR groups through TRIzol method. Subsequently, extracted RNA was reversely transcribed into stable complementary deoxyribose nucleic acids (cDNAs) (Invitrogen, Carlsbad, CA, USA). The expression of AQP4 gene was detected *via* qPCR using a 25 μ L system (1 μ L of forward primer, 1 μ L of reverse primer, 2 μ L of the template cDNAs, 12.5 μ L of 2 \times Mix, and double distilled water supplemented the volume to 25 μ L). Primers for AQP4 gene were: AQP4 forward: ATCAGCATCGCTAAGTCCGTC, reverse: GAGGTGTGACCAGGTAGAGGA; GAPDH forward: AGGTCGGTGTGAACGGATTTG; reverse: TG-TAGACCATGTAGTTGAGGTCA. With GAPDH as endogenous control, the relative expression level of AQP4 was calculated by 2^{-DDCt} method¹³.

Detection of Clinical Indicators and Clinical Correlation Analysis

Fasting blood glucose, glycated hemoglobin A1c (HbA1c), and serum creatinine in DR group were detected in the biochemical room of the Department of Clinical Laboratory of our hospital. The fully automatic biochemical detector utilized was subjected to daily quality control before use. Based on the severity of DR, patients in DR group were classified into 6 stages, namely, stage I (mild non-hyperplasia), stage II (moderate non-hyperplasia), stage III (severe non-hyperplasia), stage IV (early hyperplasia), stage V (fibroplasia) and stage VI (advanced hyperplasia).

Table I. Distribution of alleles of AQP4 polymorphisms rs200498749, rs149465 and rs650217.

Polymorphism	Allele	DM group	DR group	OR	95% CI	χ^2	p
rs200498749	C	190 (0.475)	173 (0.432)	0.84	0.63-1.11	1.45	0.227
	T	210 (0.525)	227 (0.568)				
rs149465	A	201 (0.502)	227 (0.568)	1.29	0.98-1.71	3.39	0.060
	T	199 (0.497)	173 (0.432)				
rs650217	C	237 (0.593)	203 (0.507)	0.71	0.53-0.93	5.83	0.015
	T	163 (0.407)	197 (0.492)				

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for statistical processing. Differences between two groups were analyzed by using the Student's *t*-test. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). To test the population homogeneity of the study subjects, the allele frequencies were tested against Hardy-Weinberg equilibrium by the χ^2 -test. Polymorphisms were analyzed online using the SHEsis website (<http://analysis.bio-x.cn/myAnalysis.php>). $p < 0.05$ was considered statistically significant.

Results

Distribution of Alleles of AQP4 Gene Polymorphisms rs200498749, rs149465 and rs650217 in the Two Groups

All allele frequencies did not deviate from Hardy-Weinberg equilibrium. The distribution of alleles of AQP4 polymorphisms rs200498749, rs149465 and rs650217 in the two groups was shown in Table I. The distribution of alleles of AQP4 rs650217 ($p=0.015$) in DR group was different from that in DM group, and the frequency

of T allele was significantly higher in DR group than DM group.

Distribution of Genotypes of AQP4 Polymorphisms rs200498749, rs149465 and rs650217 in the Two Groups

The genotype distribution of AQP4 gene polymorphisms rs200498749, rs149465 and rs650217 (Table II) showed that the distribution of genotypes of AQP4 rs149465 ($p=0.000$) and rs650217 ($p=0.000$) exhibited a statistically significant difference between DR group and DM group. The frequency of AA genotype of polymorphism rs149465 and CT genotype of polymorphism rs650217 was higher in DR group than DM group.

Analysis of AQP4 Polymorphisms rs200498749, rs149465 and rs650217 in the Two Groups

Based on the analysis of AQP4 gene polymorphisms rs200498749, rs149465 and rs650217 (Table III), there was a difference in the distribution of recessive models AQP4 rs149465 ($p=0.023$) and rs650217 ($p=0.014$) between DR group and DM group. DR group had significantly lowered frequency of AA + AT recessive model of the polymorphism rs149465 and raised frequency of CT + TT recessive model of the polymorphism rs650217.

Table II. Distribution of genotypes of AQP4 polymorphisms rs200498749, rs149465 and rs650217.

Polymorphism	Genotype	DM group	DR group	χ^2	p
rs200498749	CC	51 (0.255)	46 (0.230)	1.62	0.444
	CT	88 (0.440)	81 (0.405)		
	TT	61 (0.305)	73 (0.365)		
rs149465	AA	35 (0.175)	81 (0.405)	45.01	0.000
	AT	131 (0.655)	65 (0.325)		
	TT	34 (0.170)	54 (0.270)		
rs650217	CC	79 (0.395)	43 (0.215)	18.03	0.000
	CT	79 (0.395)	117 (0.585)		
	TT	42 (0.210)	40 (0.200)		

Table III. Analysis of AQP4 polymorphisms rs200498749, rs149465 and rs650217.

	Polymorphism	Genotype	DM group	DR group	χ^2	p
Dominant model	rs200498749	CC + CT	139 (0.795)	127 (0.635)	4.15	0.126
		TT	61 (0.305)	73 (0.365)		
	rs149465	AA + AT	166 (0.830)	144 (0.730)	4.22	0.121
		TT	34 (0.170)	54 (0.270)		
	rs650217	CC + CT	158 (0.790)	160 (0.800)	3.41	0.182
		TT	42 (0.210)	40 (0.200)		
Recessive model	rs200498749	CC	51 (0.255)	46 (0.230)	3.48	0.176
		CT + TT	149 (0.745)	154 (0.770)		
	rs149465	AA	35 (0.175)	81 (0.405)	7.56	0.023
		AA + AT	165 (0.725)	119 (0.595)		
	rs650217	CC	79 (0.395)	43 (0.215)	8.52	0.014
		CT + TT	121 (0.605)	157 (0.785)		

Table IV. Haplotype analysis of AQP4 polymorphisms rs200498749, rs149465 and rs650217.

Haplotype	DM group	DR group	OR	95% CI	χ^2	p
CAC	60.57 (0.151)	55.52 (0.139)	0.903	0.609-1.339	0.257	0.612
CAT	30.83 (0.077)	51.91 (0.130)	1.786	1.117-2.854	5.989	0.014
CTC	60.15 (0.150)	33.18 (0.083)	0.511	0.326-0.801	8.821	0.003
CTT	38.45 (0.096)	32.38 (0.081)	0.828	0.508-1.352	0.569	0.451
TAC	59.37 (0.148)	63.21 (0.158)	1.077	0.733-1.583	0.143	0.706
TAT	50.23 (0.126)	56.35 (0.141)	1.142	0.759-1.718	0.406	0.524
TTC	56.91 (0.142)	51.08 (0.128)	0.883	0.588-1.325	0.364	0.546
TTT	43.49 (0.109)	56.35 (0.141)	1.344	0.881-2.051	1.893	0.169

Haplotype Analysis of AQP4 Polymorphisms rs200498749, rs149465 and rs650217

Haplotype analysis of AQP4 polymorphisms rs200498749, rs149465 and rs650217 was shown in Table IV. A difference was found in the distribution of the haplotypes CAT ($p=0.014$) and CTC

($p=0.003$) of AQP4 polymorphisms rs200498749, rs149465 and rs650217 between DR group and DM group.

Associations of AQP4 Polymorphisms rs200498749, rs149465 and rs650217 With Gene Expression

The correlations of AQP4 polymorphisms rs200498749, rs149465 and rs650217 with gene expression (Figures 1-3) revealed that AQP4 gene polymorphism rs200498749 was evidently correlated with AQP4 gene expression ($p<0.05$). Meanwhile, the expression of AQP4 gene was remarkably higher in patients with CC genotype in DR group ($p<0.05$).

Correlations of AQP4 Gene Polymorphisms rs200498749, rs149465 and rs650217 With Clinical Indicators

As shown in Table V, AQP4 polymorphism rs200498749 was correlated with fasting blood glucose ($p=0.000$) and HbA1c ($p=0.000$) in DR group. In addition, polymorphism rs650217 had an association with serum creatinine level ($p=0.034$).

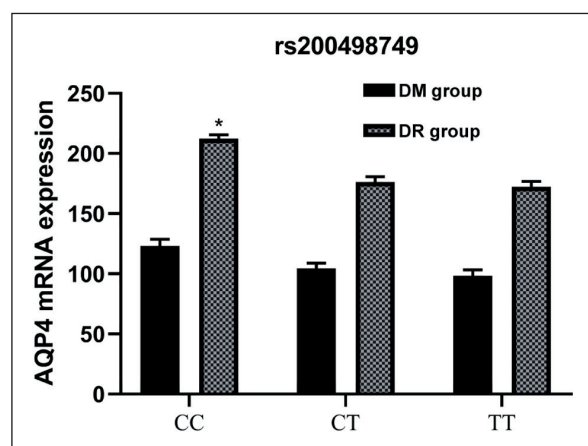


Figure 1. Relationship between AQP4 polymorphism rs200498749 and gene expression (* $p<0.05$ in ANOVA).

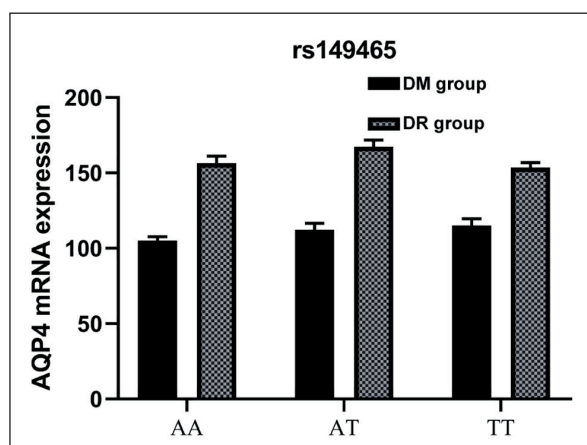


Figure 2. Association between AQP4 polymorphism rs149465 and gene expression.

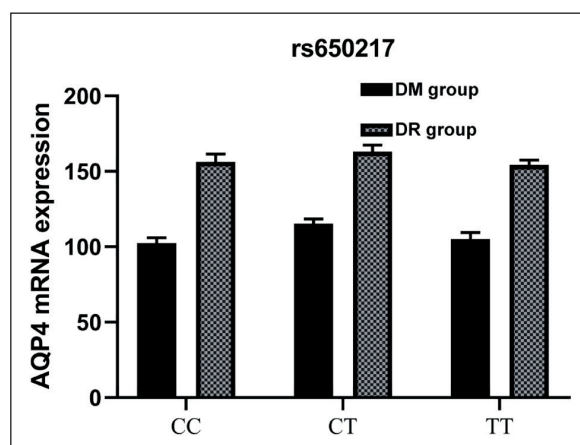


Figure 3. Correlation between AQP4 polymorphism rs650217 and gene expression.

Relations of AQP4 Polymorphisms rs200498749, rs149465 and rs650217 With Clinical Stage in DR Group

The correlations of AQP4 polymorphisms rs200498749, rs149465 and rs650217 with clinical stage in DR group were shown in Figures 4-6. AQP4 polymorphisms rs149465 ($p=0.023$) and rs650217 ($p=0.042$) were correlated with clinical stage in DR group. The proportions of stage VI patients with AA genotype of rs149465 and stage I patients with TT genotype of rs650217 rose notably ($p<0.05$).

Discussion

DR is one of the common complications of DM, the number of which accounts for about 20-30% of DM patients. It has been found that DR

has a strong relationship with country, region and race^{12,13}. There are mainly two types of DR, i.e., proliferative DR and non-proliferative DR. Statistics have shown that the incidence rate of non-proliferative DR is 10-15% higher than that of proliferative DR. The pathogenesis of DR is mainly correlated with the course and progression of primary diseases, as well as other concurrent cardiovascular diseases including hypertension and hyperlipemia¹⁴. Changes in genetic materials (such as the deletion, insertion and translocation of gene fragments) in DM patients are also important risk factors for DR¹⁵. As a vital factor triggering changes in genetic materials, gene polymorphism has been proven to be associated with the development of DM and its complications^{16,17}. Hence, the exploration of the association between gene polymorphism and the development of DR is of great significance for discovering its pathogenesis.

Table V. Correlations of AQP4 polymorphisms rs200498749, rs149465 and rs650217 with clinical indicators.

Polymorphism	Genotype	Fasting blood glucose (mmol/L)		HbA1c (%)		Serum creatinine (μmol/L)	
		DR group	p	DR group	p	DR group	p
rs200498749	CC	10.82±0.74	0.184	8.2±1.2	0.274	125±12	0.174
	CT	11.45±1.93		7.9±1.1		135±14	
	TT	10.21±1.26		8.0±0.9		133±17	
rs149465	AA	13.87±1.42	0.000	9.3±1.6	0.000	132±22	0.112
	AT	10.32±1.65		7.2±0.8		138±10	
	TT	10.25±1.22		7.6±0.9		124±31	
rs650217	CC	11.74±2.67	0.165	8.1±1.0	0.317	126±8	0.034
	CT	11.17±1.84		8.3±0.8		178±13	
	TT	10.24±1.11		8.0±1.2		130±12	

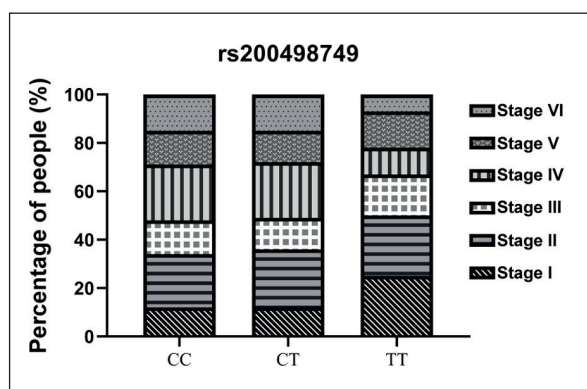


Figure 4. Relation between AQP4 polymorphism rs200498749 and clinical stage in DR group.

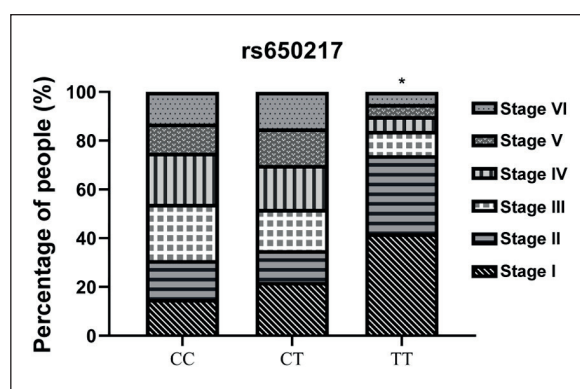


Figure 6. Relationship between AQP4 polymorphism rs650217 and clinical stage in DR group (* $p < 0.05$ in ANOVA).

AQP4, a member of the AQP family, plays a vital role in AQP selection in the plasma membrane of many cells¹⁸. AQP4 protein is the main AQP in the brain, which is of great importance in brain fluid balance. Moreover, AQP4 gene is able to encode variable splicing transcripts in different sub-types, thereby exerting different functions¹⁹. Current studies have shown that AQP4 gene expression and protein activity can affect the progression of various diseases. AQP4 gene polymorphism has also been verified to be capable of affecting the susceptibility to such diseases as cerebral hemorrhage^{20,21}. As to promotion on DR in DM patients, the comparison of AQP4 gene polymorphism between DM group and DR group in this study showed that the distribution of alleles of AQP4 rs650217 ($p=0.015$) in DR group was different from that in DM group. The frequency of T allele was significantly higher in DR group than that in DM group. The distribution of genotypes of AQP4 rs149465 ($p=0.000$) and rs650217 ($p=0.000$) showed a difference between DR

group and DM group, and the frequency of AA genotype of polymorphism rs149465 and CT genotype of polymorphism rs650217 was significantly higher in DR group. These findings suggested that AQP4 gene polymorphism was one of the causes for increased incidence rate of DR, serving as a DR-susceptible factor. In addition, AQP4 gene polymorphism could be used to predict the incidence rate of DR in DM patients.

Besides, the analysis in combination with two genotypes of the same polymorphism showed that there was a difference in the distribution of recessive models of AQP4 rs149465 ($p=0.023$) and rs650217 ($p=0.014$) between DR group and DM group. DR group exhibited significantly lowered frequency of AA + AT recessive model of the polymorphism rs149465 and raised frequency of CT + TT recessive model of the polymorphism rs650217 in recessive model. Haplotype analysis demonstrated that the distribution of the haplotypes CAT ($p=0.014$) and CTC ($p=0.003$) of AQP4 polymorphisms rs200498749, rs149465 and rs650217 in DR group was different from that in DM group. These results demonstrated once again that AQP4 gene polymorphism had an evident effect on the development of DR. Since the incidence rate of DR is remarkably elevated in people with specific AQP4 gene haplotypes like CAT, more close attention should be paid to such people during the diagnosis and treatment of DM. Moreover, such a complication should be prevented in advance.

Furthermore, the relationship between AQP4 gene polymorphism and gene expression was analyzed in this study. The results revealed that there was an evident relationship between polymorphism rs200498749 and gene expression ($p < 0.05$). The expression of AQP4 gene was

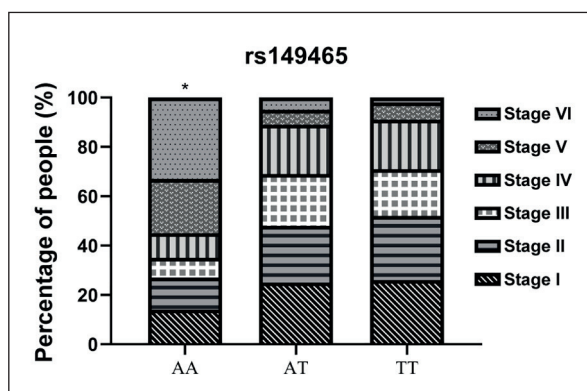


Figure 5. Association between AQP4 polymorphism rs149465 and clinical stage in DR group (* $p < 0.05$ in ANOVA).

clearly higher in patients with CC genotype in DR group. This indicated that AQP4 gene polymorphism might affect the expression of AQP4 gene to regulate the expression of downstream genes, thereby affecting the complications of DM.

Additionally, the analysis combined with clinical data denoted that AQP4 polymorphism rs200498749 was correlated with fasting blood glucose ($p=0.000$) and HbA1c ($p=0.000$) in DR group. Polymorphism rs650217 had an association with serum creatinine level ($p=0.034$). AQP4 polymorphisms rs149465 ($p=0.023$) and rs650217 ($p=0.042$) were correlated with clinical stage in DR group. In addition, the proportions of stage VI patients with AA genotype of rs149465 and stage I patients with TT genotype of rs650217 rose notably.

Conclusions

Shortly, AQP4 gene polymorphism is associated with the progression of DR, which can act as one of the auxiliary indexes for analyzing DR progression.

Conflict of Interests

The authors declare that they have no conflict of interests.

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