GENE POLYMORPHISMS OF WATER AND GLYCEROL CHANNELS AQUAPORIN 7 AND 9 IN OBESE AND/OR DIABETIC PATIENTS

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This study was presented as a poster at the 37th Congress of the International Union of Physiological Sciences 21-26 July 2013, Birmingham, UK.

ABSTRACT

PURPOSE: Adipose tissue is a major source of glycerol which is one of the substrates for gluconeogenesis. Glycerol production and its effluence from adipose tissue to the liver are key to regulate lipid and glucose homeostasis. Aquaporin 7 (AQP7) is an aquaglyceroporin that plays as the adipose glycerol channel. In addition, aquaporin 9 (AQP9) is also the specific glycerol channel in the liver. In this study, we investigated AQP7 (A953G, V59L) and AQP9 (T279A, C43T) polymorphisms in patients with obese and/or type 2 diabetes mellitus (T2DM).

METHODS: A hundred and ninety-eight volunteers divided into 3 groups: Group 1 (n=49, BMI<20 kg/m²) non-diabetic and non-obese subjects, group 2 (n=60, BMI≥30 kg/m²) non-diabetic, obese patients and group 3 (n=89, BMI≥30 kg/m²) patients with obesity and T2DM. After collecting venous blood samples, insulin, glycerol and blood lipid profiles were measured by using ELISA kit and immunoassay technique. Polymorphisms of A953G and V59L for AQP7 and T279A and C43T for AQP9 were studied by real-time polymerase chain reaction.

RESULTS: Glycerol and insulin levels were higher in obese and type 2 DM groups than in the control group. (p<0.001, p<0.001 respectively). AQP7 (A953G, V59L) and AQP9 (T279A, C43T) gene polymorphisms were similar in all study groups (p>0.005).

CONCLUSIONS: AQP7 (A953G, V59L) and AQP9 (T279A, C43T) gene polymorphisms seemed to be ineffective in the pathophysiology of obesity and type 2 diabetes.

Key words: Aquaporins, diabetes mellitus, obesity, polymorphism, Real-time PCR.

INTRODUCTION

Glycerol production and its flow from adipose tissue to liver are the key regulators of lipid and glucose homeostasis. So far, 13 different types of water channels are identified. Some of them (AQP3, AQP7, AQP9 and AQP10) also provide membrane transport of glycerol (1). Aquaporin 7 (AQP7) is an aquaglyceroporin that plays as the adipose glycerol channel. In addition aquaporin 9 (AQP9) is also the specific glycerol channel in the liver. (2) It was shown that AQP7 is expressed in adipose tissue more than in other tissues. Moreover, AQP7 also exists in heart, testicle, epididymis, gastrointestinal tract, iskeletal muscles, kidney

and internal ear (3). The relationship between functional deficiency of AQP7 and T2DM disease and obesity was confirmed (4). It was observed that AQP7 in adipose tissue was suppressed by insulin and increased by epinephrine. Triglycerides in adipose tissue are hydrolyzed during fasting and long-term exercise and resulting free fatty acids and glycerol are released into circulation. The transport of glycerol from cell membrane is conducted by means of AQP7. AQP7 increases in fasting whereas it reduces in satiety (5). AQP7 knockout (AQP7-KO) mice with deleted AQP7 genes in adipose tissue are available today (6). It is observed that body weights of AQP7-KO mice are similar with control group in the first 12 weeks of life, but their weight and adipose tissue mass increase with age although their food intake is the same as in the control group. It was reported that triglyceride synthesis and degradation rate

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were not affected. Glycerol and triglyceride content in adipose tissue boost the activity of glycerolkinase but a three-fold reduction occurs in glycerol release from adipocytes (6). The reduction of glycerol permeability of membrane leads to the increase in intracellular glycerol concentration and in glycerol-3-phosphate formation through the activation of glycerolkinase which has a low activity in a normal adipose tissue. Raised intracellular glycerol-3-phosphate is used as a substrate in triglyceride synthesis and leads to progressive triglyceride accumulation (7). AQP9 is expressed in liver, spleen, leukocyte and lungs in humans (8, 9). Glycerol which is released from adipose tissue by lipolysis permeates to liver and is used as a substrate for gluconeogenesis. AQP9 is considered to be the only glycerol channel in liver (10). AQP9 mRNA level increases in fasting state while it reduces by food intake (11). Miranda et al. (23) found a positive correlation between the levels of AQP7 adipose tissue and AQP9 liver in their studies that investigated glycerol metabolism and metabolic disorders.

Obesity and related abnormalities, including type 2 diabetes and dyslipidemia, are becoming epidemic, thus representing major health care problems. Although these abnormalities have a clear genetic component, the involved genes are mostly unknown.

In this study, we investigated AQP7 (V59L, A953G) and AQP9 (C43T, T276A) polymorphisms in obese and/or type 2 diabetes mellitus (T2DM) patient compared with healthy people.

MATERIAL AND METHODS

Patient population

Approval of local ethics committee was obtained before the study. 198 volunteers were recruited from patients attending the Trakya University Faculty of Medicine Department of Internal Diseases. Group 1; (n=49, M/F=18/31), non-diabetic people with normal weight ($BMI < 25 \text{ kg/m}^2$); Group 2; (n=60, M/F=29/31), non-diabetic obese people ($BMI \geq 30 \text{ kg/m}^2$) Group 3; (n=89, M/F=33/56), obese people diagnosed with T2DM ($BMI \geq 30 \text{ kg/m}^2$) (Table 1).

Blood Assays

Plasma samples were obtained by venipuncture after an overnight fast. The measurement of fasting blood glucose, cholesterol, triglyceride, HDL and LDL was conducted via "Clinical Chemistry System ADVIA 1800" device (Siemens-Germany) and serum insulin was measured with "immunoassay system UniCel

DxI 800" device (Beckman Coulter, USA). In the light of Elisa method, the levels of serum glycerol was measured by using "Free Glycerol Assay kit" (K630-100, Biovision, USA).

Real-Time PCR

DNA isolation was obtained from 2 ml blood samples taken from the patient and control groups into the tubes with EDTA by using "High Pure PCR Template Preparation Kit" (K11796828001 Roche, Germany). AQP7 V59L "LightSNIP rs4008659" (Roche Diagnostics (6). AQP7 A953G "LightSNIP rs29889924" (Roche Diagnostics) (26), AQP9 C43T "LightSNIP rs77284866" (Roche Diagnostics AQP9 T279A "LightSNIP rs18673080" (Roche Diagnostics) were used. "LightCycler FastStart DNA Master PLUS HybProbe" kit (Applied Science Roche, Germany) was used for amplification. The analysis of Real-time PCR in the reaction volume of 2µl and 25µl from the obtained DNA samples was conducted on "LightCycler2.0" (Roche, Germany) device.

Statistical analysis

Data were presented as means $\pm$ standard deviations. Descriptive data were reflected as frequency and percentage. The conformity of measurement variables to normal distribution was evaluated by using One-Sample Kolmogorov Smirnov test. To compare the groups, quantitative variables which were parametric were analysed with ANOVA and the ones non-parametric were analysed with Kruskal-Wallis. In the post hoc tests, Tukey test or Mann Whitney U test was used regarding the conformity. For qualitative variables, chi-square test was used. In the comparison of different frequencies of AQP7 and AQP9 polymorphism between the groups, chi-square and crosstabs were used. A p value lower than 0.05 was accepted as statistically significant.

RESULTS

In the comparison regardless of the polymorphism characteristics of groups, a significant difference in plasma glycerol and insulin levels was observed (**Table 1**). The lowest glycerol and insulin levels were observed in healthy control group. This difference was considered to stem from the increase in insulin resistance depending on obesity. High density lipoprotein (HDL) and low density lipoprotein (LDL) levels showed similar characteristics among groups ($p > 0.05$). Polymorphisms belonging to A953G and V59L gene sites of AQP7 were given in **Table 2**. When A953G polymorphism results which

were detected by nucleotide transformation in AQP7 “Promoter” sites were examined, AG genotype was seen in 50-60% of individuals forming the groups, AA genotype was seen as the second frequent one (approximately 25-30%) and GG genotype was seen as the third frequent one (approximately 15%). A significant difference among the groups could not be found. A953G gene polymorphisms of AQP7 were compared among the groups in terms of plasma glycerol and insulin levels (**Figure 1a and 1b**). Although there observed a significant difference among the groups when their plasma glycerol levels were compared, no statistically significant difference was seen among different genotypes based on A953G polymorphisms ($p>0.05$). Similarly, when plasma insulin levels were compared among the groups, they were found higher in Group 2 and Group 3 than in Group 1. However, no statistically significant difference was observed among these groups when they were compared

to A953G polymorphisms. In terms of V59L polymorphisms, CC genotype was identified in all individuals forming Group 1 and Group 3 (100%) whereas GC genotype was detected only in one individual (1%) and CC genotype was identified in the rest of the individuals in Group 2 (99-100%). GG genotype was detected in none of the participants in terms of V59L. The polymorphisms of T279A and C43T genotypes of AQP9 gene were given in **Table 3**. Regarding T279A polymorphism, the majority of the cases in three groups showed GG genotype (90-95%). GA genotype was seen in fewer cases (5-10%). AA genotyping was not observed in three groups. T279A polymorphisms of AQP9 gene was compared among the groups in terms of plasma glycerol and insulin values (**Figure 1c and 1d**). A statistically significant difference was not found in this comparison ($p>0.05$). In terms of C43T polymorphism, it was seen that all of the individuals showed TT genotype (100%).

Table 1. Demographic, antropometric and biochemical features of the Groups

	CONTROL N=49	OBESE N=60	T2DM+OBESE N=89	P value
Age, year	36.6 ±10.8	47.1 ±10.5	53.7 ± 8.5	<0.001
Gender, M/F	18/31	29/31	33/56	>0.05
BMI, kg/m ²	22.7 ±1.6	31.4 ±1.8	31.7±1.4	<0.001
Glucose*, mg/dL	90.0 ±9.1	97.6 ± 10.2	148.2 ±66.2	<0.001
COL, mg/dL	175.0 ±36.7	196.8 ±37.1	185.7±37.0	<0.02
TG, mg/dL	114.3 ±65.1	150.8 ±84.8	190.9 ±100.1	<0.001
HDL, mg/dL	51.5 ±13.1	49.2 ±13.4	46.0 ±12.6	>0.05
LDL, mg/dL	115.4 ±33.0	132.6 ± 34.8	124.5 ± 35.4	>0.05
Glycerol, µmol/mL	0.68 ±0.28	0.79 ±0.38	0.97 ± 0.44	<0.001
Insulin, µIU/mL	7.21 ±6.61	13.71 ±17.03	18.21 ± 21.88	<0.001

All the date was presented as mean=standard deviation. Abbreviations: BMI, body mass index; COL, cholesterol; TG, triglyceride; HDL, High density lipoprotein; LDL, low density lipoprotein. * Fasting blood glucose. Data were presented as means ± SD.

Table 2. The distribution of different AQP7 polymorphisms in Groups

	CONTROL N=49	OBESE N=60	T2DM+OBESE N=89
AQP7*			
A953G			
AA, n(%)	13(27.1%)	16(28.6%)	15(28.8%)
AG, n(%)	28(58.3%)	29(51.8%)	29(55.8%)
GG, n(%)	7(14.6%)	11(19.6%)	8(15.4%)
V59L			
CC n(%)	49(100%)	48(99%)	63(100%)
GC n(%)	0(0%)	1(1%)	0(0%)
GG n(%)	0(0%)	0(0%)	0(0%)

*Regarding the distribution between groups, no statistical significance was found on 2 gene areas which were related with AQP7 and were included in the study.

Table 3. The distribution of different AQP9 polymorphisms in Groups

	CONTROL N=49	OBESE N=60	T2DM+OBESE N=89
AQP9*			
T279A			
GG n(%)	42(91.3%)	19(95%)	60(90.9%)
AG n(%)	4(8.7%)	1(5%)	6(9.1%)
AA n(%)	0(0%)	0(0%)	0(0%)
C43T			
TT n(%)	48(100%)	56(100%)	84(100%)
TA n(%)	0(0%)	0(0%)	0(0%)
AA n(%)	0(0%)	0(0%)	0(0%)

* Regarding the distribution between groups, no statistical significance

Figure 1. Glycerol and insulin levels were similar regarding AQP7 A953G and AQP9 T279A polymorphism groups.

DISCUSSION

In this study, A953G and V59L genotypes of AQP7 gene and T279A and C43T genotypes of AQP9 gene in obese and/or diabetic patients did not reflect a different distribution compared to the control group patients who were not obese and diabetic. Aquaporins are channels that facilitates the transport of water across the cell membrane (1). So far, 13 aquaporin subtypes have been identified in humans. Among them, types 3, 7, 9 and 10 are subcategorized as aquaglyceroporins, which transport glycerol as well as water (18). The production of glycerol and the transfer of

glycerol from adipose tissue to liver is the key regulator of glucose homeostasis. The trafficking of glycerol from adipose and hepatic tissue is mainly mediated by 2 aquaglyceroporins channel proteins, AQP7 and AQP9 (4). Both aquaglyceroporins act in a coordinated manner. It is reflected that AQP7 is highly found in adipose tissue rather than other tissues and It is also seen in testicle, epididymis, gastrointestinal tract, skeletal muscle, heart, kidney and internal ear. It is also demonstrated that AQP9 is found in liver, leucocytes, lung and spleen (20-22).

Obesity and metabolic changes that cause to increased morbidity and mortality in T2DM diseases are related with functional changes that are seen on adipose tissue of obese people (12, 13). For the permanency of homeostasis, adipose tissue plays a crucial role on supporting the individual with energy in time of hunger and helps her/him to stay alive in time of long hunger (14, 15). It carries out this by hydrolyzing TGs and supporting the circulation with glycerol and free fatty acid (16). The current studies in this field have demonstrated that pathogenesis of metabolic syndrome is related with structural disorder of adipose tissue (17, 19).

In animal studies, feeding state reduces mRNA expression of the adipose AQP7 and results in a reduction of glycerol release from adipocytes (15). Feeding also reduces liver AQP9 mRNA expression and glycerol-induced gluconeogenesis (2). Miranda et al. (23) found a positive correlation between the levels of AQP7 adipose tissue and AQP9 liver in their studies that investigated glycerol metabolism and metabolic disorders. It is thought that AQP9 tract in liver is the only glycerol tract. The studies are mostly investigations of AQP7 and AQP9 gene expressions (24, 25).

Prudente et al. (26) examined A953G polymorphisms of AQP7 gene which is glycerol tract of obesity and the metabolic disorders that are related with obesity. According to these findings, the investigation of V59L genotype polymorphism of AQP7 and T279A and C43T genotype polymorphisms of AQP9 on obese patients with T2DM in this study is the preliminary in the field. For A953G polymorphism that was diagnosed with nucleotide change of AQP7 gene in promoter areas, the frequency of AA, AG and GG genotypes was found on GROUP 1 as AA 13 (%27.1), AG 28 (%58.3) and GG 7 (%14.6), on GROUP 2 as AA 16 (% 28.6), AG 29 (%51.8) and GG 11 (%19.6) and on GROUP 3 as AA 15 (%28.8), AG 29 (% 55.8) and GG 8 (%15.4). Regarding the V59L polymorphisms, CC genotype was detected on all the individuals in Group 1 and Group 3 (100%). However, GC genotype was detected on only one individual in Group 2, while CC genotype was found on the rest of Group 2. Regarding the V59L, GG genotype found on none of the participants. Regarding the distribution of polymorphisms of both areas that were determined for AQP7 gene, no significant difference was found between the groups. Polymorphisms of T279A and C43T genotypes that are belong to second examined gene area AQP9 were investigated. Regarding T279A,

while GG polymorphism was the most frequently detected one (90-95%), AG was found less frequently (5-10%). AA polymorphism was found on none of the participants in three groups. As for the polymorphisms that are belong to C43T area, while TT genotype was found on all of the participants, TA and AA genotypes were not found on any participants in three groups. As a result, no significant difference was found regarding the distribution of AQP9, T279A and C43T polymorphisms between the groups. In the study of Ceperuelo-Mallafre et al. (25) on extremely obese women, glycerol plasma levels and AQP7 mRNA were found as significantly low. Nevertheless, a correlation could not be found between AQP7 gene and glycerol in circulation. After nutrition, plasma insulin concentration increases. In regulatory sites of AQP7 and AQP9 genes, AQP7 specific to adipose tissue and AQP9 specific to liver are suppressed by means of negative insulin response element. Interestingly, it was observed that AQP7 in adipose tissue and AQP9 in liver increased although obese db/db rats with insulin resistance had hiperinsulinemia (27). The increase in AQP7 causes the increase in plasma glycerol concentration. The increase in glycerol concentration in circulation leads to the increase in AQP9 and the production of hepatic glucose (28). Regardless of polymorphism characteristics of the groups, blood glycerol and insulin values were compared in our study. Glycerol levels were found significantly high in Group 2 (Obese) and in Group 3 (DM+Obese) compared to Group 1 (Control) ($p<0.001$). Similarly, plasma insulin levels were seen within normal limits in Group 1 whereas they were found in higher values in Group 2 and Group 3 ($p<0.001$). The increase in glycerol and insulin values in Group 2 and Group 3 was considered to stem from the increase in insulin resistance based on obesity. These results support Rodriguez et al.'s study (11). High plasma insulin levels are considered to increase AQP7 and AQP9 in addition to the existence of insulin resistance. Current findings entail the consideration of deterioration in the regulation of AQP7 specific to adipose tissue and AQP9 specific to AQP9 in obesity and T2DM disease. In the study conducted by Prudente et al. (26), A953G single nucleotide transformation polymorphisms were detected in promoter site of AQP7 gene and it was displayed that A953G genotype in AQP7 promoter site was related to T2DM disease in 977 Caucasians. It has been found that AQP7 expression in adipose tissue was lower in individuals with -953G variant. Besides, the

risk of obesity was increased in T2DM. BMI was found to be higher in females with XG (AG+GG) than in AA genotype (26).

Although plasma insulin values were found significantly high in Group 2 and Group 3 compared to Group 1, there was no significant difference when they were compared to A953G polymorphisms. On the other hand, when the results were examined thoroughly, the insulin mean values of AA, GG and AG subgroups were found different in Group 3 where obese and diabetic people existed. While the highest insulin values were seen in AA (homozygote 'wild' type) genotype, lower insulin values were found in GG (homozygote mutant type) and AG (heterozygote) genotypes. This difference can be caused by different body mass index in the subgroups. With this idea, the comparison between subgroups was analyzed regarding BMI. No significant difference was found in this analysis. As a result, it was thought that the difference between insulin values in Group 3 can be related with genotype. However, to be able to test this, a larger group and a new plan with a different methodology are needed.

Human AQP7 gene mutation was reported in searching 160 Japanese subjects (27). In that study, three types of missense mutation were identified; R12C (a C→T substitution at nucleotide 206 in exon 3 led to substitution of arginine with cysteine at position 12, which resides in the N-terminal cytoplasmic domain): 1 subject, V59L (a G→C substitution at nucleotide 347 in exon 4 caused substitution of valine with leucine at position 59, which resides in the 1st bilayer-spanning domain): 13 subjects, G264V (a G→T substitution at nucleotide 963 in exon 8 led to substitution of glycine with valine at position 264, which resides in the 6th bilayer-spanning domain): 6 subjects.

AQP7-knockout (AQP7-KO) mice models developed obesity and insulin resistance, had impaired plasma glycerol permeability (altered uptake or secretion rates) compared with wild-type mice, and exhibited accumulated excess glycerol and triglyceride concentrations in adipocytes (25). Hibuse et al. (29) have shown that AQP7-KO mice exhibit, even at young age, diet-induced obesity associated with severe insulin resistance and adult-onset obesity.

Because of the limited budget and the high cost of kits and probes, 49 people in Group 1, 60 people in Group 2 and 89 people in Group 3 were able to participate in this study. According to the results, A953G and V59L

gene polymorphisms of AQP7 gene and T279A and C43T polymorphisms of AQP9 gene were not found different in obese, obese+diabetic and non-obese+non+diabetic groups. Further studies are going to be conducted with larger groups with different polymorphisms. By doing this, it is aimed that we are going to be able to explain the effect of polymorphism differences in AQP7 and AQP9 liquid tracts on the development of obesity and DM diseases. These results indicate that polymorphisms belonging to A953G and V59L genotypes of AQP7 gene and T279A and C43T genotypes of AQP9 gene do not have different distribution in obese, obese+T2DM and non-obese+ non-T2DM groups. More mechanistic studies are needed to further explore the implication and response of AQP7 and AQP9 in physiologic and pathologic glucose homeostasis.

ACKNOWLEDGEMENT

This study is funded by Trakya University, The Faculty of Medicine, Scientific Research Projects Unit (TÜBAP 2011-115).

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