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PNPLA3 and GCKR Gene Polymorphisms influence genetic Susceptibility to NAFLD in Obese Egyptians

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ABSTRACT

Environmental and genetic factors have a crucial role in the development and progression of non-alcoholic fatty liver disease (NAFLD). The aim of the present study is to investigate the association between single nucleotide polymorphism (SNP) rs738409 in PNPLA3 gene and rs1260326 in GCKR gene and the development of NAFLD in obese Egyptian population. Forty obese subjects (20 with NAFLD and 20 without NAFLD) and 20 control subjects were enrolled in this study. All subjects were genotyped for (rs738409) PNPLA3 and (rs1260326) GCKR gene polymorphisms using the TaqMan assay. Results revealed that the homozygous mutant GG genotype of the PNPLA3 was the most frequent among patients with NAFLD (15%) as compared to controls (0%) *(p=0.036)*. Regarding the GCKR rs1260326; the homozygous mutant TT genotype was most frequent among patients with NAFLD (40%) as compared to controls (20%) with trend significance *(p=0.083)* and as compared to obese non-FL group (10%) (*p*=0.014). The frequency of the T allele was found to be significantly higher in NAFLD patients (62.5%) compared to obese non-FL group (42.5%) *(p=0.037).* In conclusion our study confirmed the association between PNPLA3 (rs738409) and GCKR (rs1260326) polymorphisms and susceptibility to NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a frequent and growing cause of chronic liver diseases, affecting about 20%– 30% of the general population worldwide. Patients with NAFLD and especially those with non-alcoholic steatohepatitis (NASH), are at risk of progression to cirrhosis and its complications, presenting also a high rate of cancer and cardiovascular events compared to subjects without fatty liver [1].

NAFLD encompasses a broad clinicopathologic spectrum ranging from simple steatosis to NASH. Simple steatosis is relatively benign. It is characterized by hepatic steatosis without inflammation or fibrosis. The NASH progression is characterized by hepatocyte ballooning, cellular necrosis and inflammatory infiltration that may eventually result in complications such as hepatic cirrhosis, liver failure and hepatocellular carcinoma [2].

Obesity is one of the risk factors for NAFLD, but not all obese persons are affected. Familial clustering for the disease has been recognized, suggesting that NAFLD may be influenced by genetic variants [3]. Recently, genome-wide association studies (GWAS) have given insights into the possible molecular pathway associating gene polymorphism to NAFLD. Single nucleotide polymorphisms (SNPs) of genes encoding proteins in the pathways of lipogenesis have been linked with NAFLD [4].

Adiponutrin/PNPLA3 which is a patatin-like phospholipase domain containing-3 belongs to the family of patatin-like phospholipase. PNPLA3 gene is expressed in the adipose tissue and liver and it possesses acyl hydrolase activity [5]. Expression of adiponutrin is increased by carbohydrate feeding and a diet of Western-type. It has activity of acylglycerol transacylase and lipase activity against triglycerides, thus being likely implicated in mobilization of energy and storage in the form of lipid droplets [6].

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Recently, it has been mentioned that the I148M (The 148 amino acid substitution of isoleucine to methionine) allele of adiponutrin is a loss-of function variant which predisposes an individual to steatosis by reducing hydrolysis of triglyceride in hepatocytes [7].

The glucokinase regulatory protein (GCKRP) which is a GCKR gene product, binds allosterically to GCK and thus regulates its activity [8]. Glucokinase (GCK) is a phosphorylating enzyme that activates hepatic lipogenesis and regulates glucose metabolism in liver [9]. GCKR seems to interfere with homeostasis of glucose and lipid by regulating the glucose storage/disposal and by providing de novo lipogenesis substrates through inhibition of glucokinase [10].

The present study aimed to investigate the association between single nucleotide polymorphism (SNP) rs738409 in PNPLA3 gene and rs1260326 in GCKR gene and the development of NAFLD in obese Egyptian population.

2. Subjects and Methods

This study was conducted on 40 obese adult subjects aged between 18-65 years, selected from the outpatient clinic attending Gastro-intestinal unit of Nasser Institute hospital, Cairo, Egypt. The subjects were 20 Egyptian obese patients with NAFLD and another 20 obese participants without NAFLD. Patients with the following diseases were excluded: Chronic viral hepatitis B or C, autoimmune disorders, Wilson's disease, hemochromatosis, drug-induced hepatitis, alcoholic hepatitis or those with any clinical evidence of hepatic decompensation or a contraindication for liver biopsy. In addition, 20 age-matched healthy subjects were included and served as a normal control group.

The study protocol conformed to ethical guidelines of the 1975 declaration of Helesinki, was approved by the Institutional Review Board (IRB) for Human Subject Research at National Hepatology & Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt (serial no.: 11-2016). After obtaining a written informed consent, all patients were subjected to medical history for risk factors of NAFLD (diabetes, hypertension, dyslipidemia, bypass surgeries), anthropometric measures (Body Mass Index (BMI)). All included subjects were of the same socioeconomic standard and dietary habits.

All control subjects were confirmed to have no risk of fatty liver (FL), determined from the following parameters: BMI <25 kg/m2; FPG <100 mg/dL, serum TGs <150 mg/dL, serum high density lipoprotein-cholesterol (HDL-C) > 40 mg/dL; systolic blood pressure < 130 mmHg; and diastolic blood pressure < 85 mmHg (Data not shown). Blood sample was collected from each participant for genetic analysis of PNPLA3 & GCKR SNPs.

2.1 Analysis of PNPLA3 & GCKR SNPs

DNA was extracted from all patients and control groups using: Genomic whole blood extraction kit (Puregene, USA) according to the instructions of the manufacturer. The DNA was dissolved in TE buffer and the DNA purity and concentration were done to all samples using spectrophotometry or Nano drop [11]. All extracted DNA samples were stored at -20 ◦C and used as templates for Real-time PCR.

Real time PCR was applied to detect single nucleotide polymorphisms in PNPLA3 gene (rs738409) [12] and GCKR gene (rs1260326) [13]. The allelic discrimination of the PNPLA3 and GCKR polymorphisms were assessed using ABI Step One TM Real- Time PCR System (Applied Biosystems, Singapore) and analyzed using SDS v3.0 software (Applied Biosystems), using the TaqMan assay.

The final volume for each reaction was 20µL, containing 10µL TaqMan Genotyping Master Mix, 0.5µLTaqMan probes mix, and 1 µL genomic DNA was then completed with distilled water up to 20ul. Real-time PCR reaction included an initial denaturation step at 95 ˚C for10 min, followed by 40 cycles, each consisting of 95 ˚C for 15 second (denaturation) and 60 ˚C for 1 min (annealing/extension).

2.2 Statistical analysis

Data were statistically analyzed by the computerized program “Statistical Package for Social Sciences (SPSS)” software, version “20” for Windows. Genotype and allele frequencies were compared between the different study groups using chi-square test. Values were considered significant at $P \leq 0.05$. Student t test was used for comparison between 2 groups for BMI and results are shown as mean±SD

3. Result and Discussion

The study included 40 (32 females & 8 males) subjects, median age 44.5 years (20-57). The BMI was found to be significantly higher in NAFLD cases (40.86 ± 5.45 kg/m^2) than in normal control participants (22.07 ± 2.10 kg/m^2) (*p<0.001).* BMI was significantly lower in obese non-FL group (35.83 ± 5.94 kg/m^2) compared to (40.86 ± 5.45 kg/m^2) in obese FL group *(p = 0.003).* This finding agrees with the results obtained by other authors who reported that NAFLD has been detected as a complication for the majority (>95%) of patients having severe obesity (BMI=35 or more) [14,15,16]. Other previous studies concluded that NAFLD can occur in non-obese subjects who are physically inactive “Metabolic obesity” [17,18].

Table (1) shows that the rs738409 PNPLA3 C allele was higher in normal controls (70%) compared to (55%) in obese FL group (p = 0.083), while the rs738409 PNPLA3 G allele was higher in obese FL group (45%) compared to (30%) in normal controls (p = 0.083). The (wild type) CC genotype was higher in normal controls (40%) compared to (25%) in obese FL group while homozygous mutant GG genotype was (15%) in obese FL group compared to (0%) in normal controls *(P = 0.036).* GG+CG genotypes were detected in 15 cases (75%) and in 12 controls (60%); GG+CG were higher in obese FL group than control.

Table (1): Comparison of genotype and allele frequencies of PNPLA3 rs738409 SNP in obese FL and control group.

		Group				P value
		Obese FL group (N=20)		Control group (N=20)		
		Count	%	Count	%	
PNPLA3	CC	5	25	8	40	0.156
	CG	12	60	12	60	0.5
	GG	3	15	0	0	0.036*
	GG+CG	15	75	12	60	0.156
	Allele C	22	55	28	70	0.083
	Allele G	18	45	12	30	0.083

* significant

Table (2): Comparison of genotype and allele frequencies of PNPLA3 rs738409 SNP in obese non FL and control group.

		Group				P value
		Obese non FL group (N=20)		Control group (N=20)		
		Count	%	Count	%	
PNPLA3	CC	10	50	8	40	0.525
	CG	8	40	12	60	0.206
	GG	2	10	0	0	0.074
	GG+CG	10	50	12	60	0.525
	Allele C	28	70	28	70	0.5
	Allele G	12	30	12	30	0.5

Table (3): Comparison of genotype and allele frequencies of PNPLA3 rs738409 SNP in obese FL and obese non FL groups.

		Group				P value
		Obese FL group (N=20)		Obese non FL group (N=20)		
		Count	%	Count	%	
PNPLA3	CC	5	25	10	50	0.051*
	CG	12	60	8	40	0.206
	GG	3	15	2	10	0.633
	GG+CG	15	75	10	50	0.051*
	Allele C	22	55	28	70	0.083
	Allele G	18	45	12	30	0.083

* significant

These results agree with Wood et al., [19] who reported that individuals harbouring the G-allele of rs738409 PNPLA3 gene were susceptible to NAFLD. Also, the obtained results agree with Hegazy et al., [20] who found that there is a strong association between the PNPLA3 rs738409 G allele and the susceptibility to NAFLD and its progression in Egyptian patients. Moreover, Medhat et al., [21] confirmed the association of the PNPLA3 rs738409 G allele with the susceptibility to NAFLD and its progression to NASH in Egyptian patients.

The obtained data from the present study (Tables 2 & 3) shows that the C allele in normal controls and in obese non-FL group were equal (70%) and the G allele in normal controls and in obese non-FL group were equal (30%). Also, the data revealed that rs738409 PNPLA3 CC genotype (wild type) was detected in 40%, 25% and 50% for normal control participants, obese FL group and obese non-FL group, respectively, thus it was significantly higher in obese non-FL group compared to obese FL group with (*P = 0.051*). However, rs738409 PNPLA3 GG mutant genotype was detected in 15%, 0%, 10% for obese FL group, normal control and obese non-FL group, respectively, thus it was higher in obese non-FL group compared to control group with trend significance *(P = 0.074).* On the other hand, rs738409 PNPLA3 CG heterozygote genotype occurred with equal percentage in NAFLD patients and normal control participants (12/20, 60%) respectively. While the rs738409 PNPLA3 CG heterozygote genotype was detected in (8/20, 40%) in obese non-FL group which was lower than the two groups (NAFLD patient and normal control participants) but still statistically non-significant (*p = 0.206*). GG+CG is higher in obese FL group than control and obese non-FL group: GG+CG genotypes were detected in up to (15/20, 75%) of the NAFLD patients compared to (12/20, 60 %) of the normal control participants and (10/20, 50%) of obese non-FL group and it was higher in obese FL group than in obese non-FL group *(P = 0.051).* These results agreed with those of Trépo et al. [22] who found that mutant GG polymorphism in the Rs738409 PNPLA3 gene was strongly associated with development and progression of non-alcoholic fatty liver disease. Also, Baclig et al. [23] reported that there was a dose effect of the PNPLA3 I148M genotype, in that CG heterozygotes had a risk percent of NAFLD between that of CC and GG homozygotes.

Table (4): Comparison of genotype and allele frequencies of GCKR rs1260326 SNP in obese FL and control group.

		Group				P value
		Obese FL group (N=20)		Control (N=20)		
		Count	%	Count	%	
GCKR	CC	3	15	3	15	0.5
	CT	9	45	13	65	0.169
	TT	8	40	4	20	0.084
	TT+CT	17	85	17	85	0.5
	Allele C	15	37.5	19	47.5	0.183
	Allele T	25	62.5	21	52.5	0.183

Table (5): Comparison of genotype and allele frequencies of GCKR rs1260326 SNP in obese non FL and control group.

		Group				P value
		Obese non FL group (N=20)		Control (N=20)		
		Count	%	Count	%	
GCKR	CC	5	25	3	15	0.215
	CT	13	65	13	65	0.5
	TT	2	10	4	20	0.188
	TT+CT	15	75	17	85	0.215
	Allele C	23	57.5	19	47.5	0.185
	Allele T	17	42.5	21	52.5	0.185

Table (6): Comparison of genotype and allele frequencies of GCKR rs1260326 SNP in obese FL and obese non FL group.

		Group				P value
		Obese FL group (N=20)		Obese non FL group (N=20)		
		Count	%	Count	%	
GCKR	CC	3	15	5	25	0.350
	CT	9	45	13	65	0.102
	TT	8	40	2	10	0.014*
	TT+CT	17	85	15	75	0.350
	Allele C	15	37.5	23	57.5	0.037*
	Allele T	25	62.5	17	42.5	0.037*

* Significant

On the other hand, results obtained for the other gene rs1260326 GCKR SNP (Tables 4, 5 & 6) revealed that C allele was higher in the normal control participants (19/40, 47.5%) compared to obese FL group (15/40, 37.5%), while the C allele recorded a lower value in the normal control participants when compared to obese non-FL group (23/40, 57.5%). It is worth mentioning that the difference between the values obtained for the C allele was significant between the obese FL group and obese non-FL group (*P = 0.037*). The rs1260326 GCKR T allele recorded a higher value in obese FL group (25/40, 62.5%) compared to the normal control participants (21/40, 52.5%), while it was lower in obese non-FL group (17/40, 42.5%) compared to the normal control group. The mean value recorded for the T allele of the obese FL group was significantly higher compared to its corresponding value of the obese non-FL group (*P = 0.037*). The results of the present study agree with Wood et al. [19] who reported that the variant T allele frequencies of the GCKR rs1260326 was found to be more prevalent in patients with both NAFLD and NASH when compared with control and also agree with Tan et al. [24] who stated that GCKR rs1260326 allele T was associated with susceptibility to NAFLD. Moreover, Sliz et al. [25] confirmed the association of the GCKR rs1260326 T allele with the susceptibility to NAFLD. Rs1260326 GCKR CC (wild type) genotype occurred in the same percentage (3/20, 15%) in NAFLD patients and normal control participants, while wild type was detected in (5/20, 25%) of obese non-FL group. Rs1260326 GCKR TT mutant genotype was detected in 40% (8/20) of obese FL group compared to 20% (4/20) for normal control with trend significant difference (p = 0.084). TT mutant genotype was found to be 10% (2/20) of obese non-FL group with significant difference when compared to NAFLD patient which was 40% (8/20) (p = 0.014) but there was no significant difference when compared to normal control participants. Rs1260326 GCKR CT heterozygote genotype occurred in equal percentages (13/20, 65%) in obese non-FL group and normal control participants, while was detected in (9/20, 45%) of obese FL group. TT + CT were higher in obese FL group than obese non-FL group. TT + CT genotypes occurred in equal percentages (17/20, 85%) in NAFLD patients and normal control participants, while TT + CT genotypes was detected in (15/20, 75%) of obese non-FL group. The results of the present study agree with El-Nady and Abo El-Fath [26] who stated that TT polymorphism in the rs1260326 GCKR gene was strongly associated with development of NAFLD in the Egyptian obese children.

4. Conclusion

This study suggests that SNPs at rs1260326 allele of GCKR gene and at rs738409 allele of PNPLA3 gene may be useful tools for predicting the susceptibility to non-alcoholic fatty liver disease (NAFLD) related to obese patients and should be applied in the clinical mangment of these patients.

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