ASSOCIATION OF *PNPLA3* (rs738409) AND *GCKR* (rs1260326) GENE POLYMORPHISMS WITH THE DEVELOPMENT OF NON-ALCOHOLIC FATTY LIVER DISEASE IN OBESE EGYPTIAN CHILDREN

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Non-alcoholic fatty liver disease (NAFLD) is known as a major cause of chronic liver disease in pediatric population. The disease forms vary from simple steatosis, and non-alcoholic steatohepatitis (NASH), fibrosis to hepatocellular carcinoma (Li *et al.*, 2016; Sookoian and Pirola, 2017). NAFLD is known as the metabolic syndrome, as its main components are abdominal obesity, insulin resistance, atherogenic dyslipidemia and hypertension (Li *et al.*, 2016; Sun *et al.*, 2016). Its incidence is 9.6% of children in general and up to 38% of obese children (Manti *et al.*, 2014; Molleston *et al.*, 2014). Genetics as well as environmental factors effect on NAFLD and its development (Hotta *el al.*, 2010).

Several gene variants have been identified by genome wide association studies (GWAS) as associated with fatty liver disease. The strongest gene variants associated with fatty liver are the petanin-like phosphate 3 (*PNPLA3*) and the glucokinase regulatory protein (*GCKR*) (Marzuillo *et al.*, 2014). Single nucleotide polymorphism (SNP) rs738409 in *PNPLA3* gene is characterized by a mutation for C base to G base, which causes a change in an amino acid from isoleucine (I) to methionine (M) at the position 148 of the obtained protein (1148 M). This protein is associated with elevated hepatocyte fat content (Santoro *et al.*, 2012). SNP rs1260326 in the *GCKR* gene is a C to T substitution which leads to change in amino acid proline (Pro) to leucine (L) at the position 446 in the obtained protein (Santoro *et al.*, 2012). The effective protein of this gene is a regulatory protein that inhibits glucokinase in liver and pancreatic islet cells by binding non-covalently to form an inactive complex with the enzyme (Santoro *et al.*, 2012).

Although, the association of the *PNPLA3* and *GCKR* genes in the NAFLD around the world has been recognized, few studies on the Egyptian patients were reported. Therefore, this study aimed to investigate the role of *PNPLA3* rs738409 and the *GCKR* rs1260326 SNPs in the development of NAFLD in a sample of obese Egyptian children.

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PATIENTS AND METHODS

This study was carried out with the consent of all patients' and controls' parents to participate in the study as well as acceptance of the ethics committee of Ain Shams University. The work has been carried out in accordance with the code of Ethics of the World Medical association (Declaration of Helsinki) for experiments involving humans.

Subjects

The present study included 80 Egyptian obese children diagnosed with NAFLD aged from 5 to 13 years with mean age of 8.5 ± 4.4 and 80 healthy children as a control aged from 4 to 13 years with mean age of 8.7 ± 4.7. The patients were divided into 2 groups; group1 were 40 patients with NAFL, which included obese children with fatty liver without evidence of hepatic engine (Simple steosis). Group 2 were 40 patients with NASH, which include obese children with fatty liver with evidence of hepatic engine (with hepatomegaly and elevated liver enzymes).

Medical records for the patients were reviewed for full medical history included age, gender, weight, height, body mass index (BMI), hip waist circumferences. Blood analysis for all the patients was performed include CBC, ALT, AST, Cholesterol, LDL and TG (Table 1).

DNA extraction and genotyping

Genomic DNA was extracted from whole blood using spin column method of Gene-JETTM Genomic DNA purification kit (Fermentas Life Sciences, Finland). The eluted DNA was stored at -20°C until further use.

Genotyping for the rs738409 SNP in the *PNPLA3* gene and the rs1260326 polymorphisms in the GCKR gene was performed by PCR based RFLP analysis. The following primers were used: rs738409 SNP: F: 5'-TGG GCC TGA AGT CCG AGG GT-3' and R: 5'-CCG ACA CCA GTG CCC TGC AG-3' (Alyavi *et al.*, 2014). For the rs1260326 SNP: F: 5'-TGC AGA CTA TAG TGG AGC CG-3' and R: 5'-CAT CAC ATG GCC ACT GCT TT-3' (Santoro *et al.*, 2012). A total of 20 ng of genomic DNA was amplified by polymerase chain reaction (PCR) containing 0.2 mM of forward primer and 0.2 mM of reverse primer, 10x PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, and 1 unit of Taq DNA Polymerase (Promega, UK) in a 25 ml volume. Biometra PCR (Germany) was used for the amplification. PCR conditions were 95°C for 4 minutes, followed by 35 cycles of: 95°C for 30 seconds (s), 58°C for 30 s for rs738409/ 60°C for 30 s for rs1260326, 72°C for 30 s. and final extension 72°C for 8 minutes. The obtained amplicon size was 333 bp for rs738409 and 231 bp for rs1260326. The PCR products were digested using *BstF5 I* (Fermentas thermo scientific) for rs738409 SNP and *HpaII* (Fermentas

thermo scientific) for rs1260326 SNP. The digestion products were visualized on 8% polyacrylamide gel electrophoresis. The digested PCR product for rs738409 SNP resulted in 200 and 133 bp for the wild type genotype (GG), 333, 200 and 133 bp for the heterozygous (GC) genotype and 333 bp for the mutant genotype. Whereas, for the rs1260326 SNP, the digestion revealed 150, 63, 18 bp for the wild type (CC) genotype, 213, 150, 63 and 18 bp for the heterozygous (TC) genotype and 213 and 18 bp for the mutant (TT) genotype (Table 2).

Statistical analysis

Statistical analyses were performed using statistical package of social sciences (SPSS) software Program for IBM SPSS statistics (Version 21.0). Data were expressed as median and percentages for quantitative nonparametric measures in addition to number and percentage for categorized qualitative variables. Quantitative data were presented as mean ± SD for normally distributed data. Chi-square (X^2) test was used to compare between groups. P value < 0.05 was considered as significant and P< 0.01 as highly significant.

RESULTS AND DISCUSSION

NAFLD is a common public health problem. *GCRR* and *PNPLA3* genes are known to be associated with the NFLD worldwide. To our knowledge, in Egypt, only one studies showed the effect of genetic polymorphism in the promoter of microsomal triglyceride transfer protein (MTP) in NAFLD (El-Koofy *et al.*, 2011). But No previous study illustrated the effect of *GCKR* and *PNPLA3* variants on NAFLD. Increasing body mass index (BMI) and obesity are known as risk factor for NAFLD (Aragones *et al.*, 2016).

In order to achieve our goal, 80 patients diagnosed with NAFLD and 80 healthy children were recruited to study the association between *GCKR* rs1260326 and *PNPLA3* rs738409 polymorphisms with NAFLD Egyptian obese patients as well as to understand the role of these polymorphisms in the development of NAFLD. Forty-six (57.5%) out of the patients group were males and 34 (42.5%) were females. The patients' ages ranged from 5 to 13 years with mean of (8.5 years ± 4.4). There were no significant differences in age and gender between NAFLD patients and their control. NAFLD patients divided into two groups; NAFL (group 1) which include patients with simple steosis, and NASH (group 2) which includes patients suffered from hepatomegaly and elevated liver enzyme. There were highly significant differences between the two groups in weight, height, BMI, hip and waist circumferences, cholesterol, TG, LDL, ALT and AST (Table 1).

Genotyping for *GCKR* rs1260326 and *PNPLA3* rs738409 polymorphisms was carried out using PCR based RFLP. Figure (1) shows the differences between the three genotypes of both polymorphisms visualized on polyacrylamide gel.

Regarding the *PNPLA3* rs738409 polymorphisms, there were 17 individuals (21.25%) homozygous for the risk allele (GG). Whereas, 39 individuals (48.75%) were heterozygous (GC). Moreover, 24 individuals (30%) were homozygous with the wild allele (CC). There were significant differences between the tree genotypes in patients compared to their control. The risk allele (G) frequency was 45.6% in patients and 36.3% in control, with highly significant differences between them (Table 3).

As for the *GCKR* rs1260326 polymorphisms, among all the patients, 15 individuals (18.75%) were homozygous for the risk allele (TT) with no significant difference between them and their control. Whereas, 48 individuals (60%) were heterozygous (TC). Moreover, 17 individuals (21.25%) were homozygous with the wild allele (CC). There were highly significant differences between the heterozygous and wild homozygous (CC) between patients and their control. The risk allele (T) frequency was 48.8% in the patients and 33.1% in the control, with highly significant differences between them (Table 4).

Many studies showed that the allele frequency of *PNPLA3* and *GCKR* varied in different ethnic groups (Palmer *et al.*, 2013; Lin *et al.*, 2014; Zhang *et al.*, 2015). The frequency of the risk allele (G) in *PNPLA3* rs738409 polymorphism was 0.266 in Caucasian, 0.170 in African American, 0.417 in the Hispanics (Santoro *et al.*, 2012), 0.448 in Asians (Lin *et al.*, 2014). While the risk allele frequency (T) in the *GCKR* rs1260326 variant was 0.446 in Caucasian, 0.129 in African American and 0.355 in Hispanics (Santoro *et al.*, 2012).

In the present study, Table (5) illustrates a comparison between NAFL (group 1) and NASH (group 2) in genotypes and allele frequency for *PNPLA3* rs738409 polymorphism. It is evident that there were seven individuals (17.5%) and 10 individuals (25%) in NAFL and NASH group, respectively, who carried the homozygous genotype (GG). There were significant differences between the three genotypes in the two groups. Moreover, there were significant differences between the two groups in the frequency of the risk allele (G), this mean that the *PNPLA3* rs738409 polymorphism may play a role in the progressive of NAAFLD in the Egyptian obese children

On the other hand, group 1 (NAFL) and group 2 (NASH) in the *GCKR* rs1260326 polymorphisms revealed that there were five individuals (12.5%) and 11 individuals (27.5%) in NAFL and NASH group, respectively, who carry the homozygous genotype (TT). Moreover, the risk allele frequencies (T) were 41.3% and 57.5% in group 1 and group 2, respectively (Table 6). The results indicated that (T) allele is more strongly associated with NASH. Therefore, it is expected that this polymorphism may have an important role in the development of NAFLD in the Egyptian obese children.

Studies on obese children who suffer develop NAFLD indicated that genetic and/or environmental factor may influence the susceptibility of the patient (Lin *et al.*, 2014). Hotta *et al.* (2010) reported that (G) allele of rs738409 may be involved in the development of NALD in Japanese patients. Moreover, Santoro *et al.* (2012) suggested that both of *PNPLA3* and *GCKR* act to confer susceptibility to NAFLD. It is noteworthy; that Aragones *et al.* (2016) illustrated that *PNPLA3* could be related to the progression of simple steatosis in obese women. Our results showed partial agreement with these findings.

Final conclusion**,** our findings suggest that *GCKR* rs1260326 and *PNPLA3* rs738409 with obesity may play an important role in the development of NAFLD in obese Egyptian children, with later seem to have greater impact in this respect.

SUMMARY

Non-Alcoholic fatty liver disease (NAFLD) is known as a common public health problem worldwide. Genetic variant as well as environmental factor interact to develop NAFLD. The allele frequency of *PNPLA3* and *GCKR* variant varied in different ethnic groups. Our study aimed to recognize the association of the *PNPLA3* rs738409 and *GCKR* rs1260326 with the occurrence and progression of NAFLD in obese Egyptian children.

PNPLA3 rs738409 and *GCKR* rs1260326 were genotyped using RFLP-PCR in 80 patients with NAFLD (40 with NAFL and 40 with non-alcoholic steatohepatitis [NASH]) and 80 control subjects. Comparison was made between NAFLD and control as well as between NAFL and NASH. The risk allele (G) allele frequency of *PNPLA3* rs738409 was 0.456 in patients and 0.363 in control. Whereas, the risk allele (T) allele frequency of *GCKR* rs1260326 was 0.488 in patients and 0.331 in control. These two alleles show highly association with NAFLD (P = 0.00). Comparison between NAFL and NASH revealed that the frequencies of the (G) allele in *PNPLA3* rs738409 were 0.3875, 0.5 in NAFL and NASH, respectively. Moreover, the allele frequencies of the (T) allele in *GCKR* rs1260326 were 0.413 and 0.575 in NAFL and NASH, respectively. Our findings demonstrate that both SNPs were highly associated with NASH. It is concluded that the *PNPLA3* rs738409 and *GCKR* rs1260326 may have an important role in the development of NAFLD in obese Egyptian Children.

REFERENCES

Alyavi, A. L., G. N. Sobirova and M. M. Karimov (2014). Association of rs738409 polymorphism in the *PNPLA3* gene with nonalcoholic fatty liver disease. International Journal of BioMedicine, 4: (Suppl 1) S8-S11.

Aragones, G., T. Auguet, S. Armengol, A. Berlanga, E. Guiu-Jurado, C. Aguilar, S. Martínez, F. Sabench, J. A. Porras, M. D. Ruiz, M. Hernández, J. J. Sirvent, D. Del Castillo and C. Richart (2016). *PNPLA3* expression is related to liver steatosis in morbidly obese women with non-alcoholic fatty liver disease. Int. J. Mol. Sci., 17: pii: E630.

El-Koofy, N. M., H. M. El-Karaksy, I. M. Mandour, G. M. Anwar, M. S. El-Raziky and A. M. El-Hennawy (2011). Genetic polymorphisms in non-alcoholic fatty liver disease in obese Egyptian children. Saudi J. Gastroenterol, 17: 265-270.

Hotta, K., M. Yoneda, H. Hyogo, H. Ochi, S. Mizusawa, T. Ueno, K. Chayama, A. Nakajima, K. Nakao and A. Sekine (2010). Association of the rs738409 polymorphism in *PNPLA3* with liver damage and the development of nonalcoholic fatty liver disease. BMC Medical Genetics, 11: 172.

Li, X. L., J. Q. Sui, L. L. Lu, N. N. Zhang, X. Xu, Q. Y. Dong, Y. N. Xin and S. Y. Xuan (2016). Gene polymorphisms associated with non-alcoholic fatty liver disease and coronary artery disease: a concise review. Lipids in Health and Disease, 15: 53.

Lin, Y. C., P. F. Chang, M. H. Chang and Y. H. Ni (2014). Genetic variants in *GCKR* and *PNPLA3* confer susceptibility to nonalcoholic fatty liver disease in obese individuals. Am. J. Clin. Nutr., 99: 869-874.

Manti, S., C. Romano, V. Chirico, M. Filippelli, C. Cuppari, I. Loddo, C. Salpietro and T. Arrigo (2014). Nonalcoholic fatty liver disease/non-alcoholic steato-hepatitis in childhood: Endocrine-Metabolic "Mal-Programming". Hepat., 14: el17641.

Marzuillo, P., E. Del Giudice and N. Santoro (2014). Pediatric fatty liver disease: role of ethnicity and genetics. World J. Gastroenterol, 20: 7347-7355.

Molleston, J. P., J. B. Schwimmer, K. P. Yates, K. F. Murray, O. W. Cummings, J. E. Lavine, E. M. Brunt, A. O. Scheimann, A. Unalp-Arida and NASH Clinical Research Network. (2014). Histological abnormalities in children with nonalcoholic fatty liver disease and normal or mildly elevated alanine aminotransferase levels. J. Pediatr., 164: 707-713e3.

Palmer, N. D., S. K. Musani, L. M. Yerges-Armstrong, M. F. Feitosa, L. F. Bielak, R. Hernaez, B. Kahali, J. J. Carr, T. B. Harris, M. A. Jhun, S. L. R. Kardia, C. D. Langefeld, T. H. Mosley, J. M.

Norris, A. V. Smith, H. A. Taylor, L. E. Wagenknecht, J. Liu, I. B. Borecki, P. Peyser and E. K. Speliotes (2013). Characterization of European ancestry nonalcoholic fatty liver disease-associated variants in individuals of African and hispanic descent. Hepatology, 58: 966-975.

Santoro, N., C. K. Zhang, H. Zhao, A. J. Pakstis, G. Kim, R. Kursawe, D. J. Dykas, A. E. Bale, C. Giannini, B. Pierpont, M. M. Shaw, L. Groop and S. Caprio (2012). Variant in the glucokinase regulatory protein (*GCKR*) gene is associated with fatty liver in obese children and adolescents. Hepatology, 55: 781-789.

Sookoian, S. and C. J. Pirola (2017). Genetic predisposition in nonalcoholic fatty liver disease. Clin. Mol. Hepatology, 23: 1-12.

Sun, M. Y., L. Zhang, S. L. Shi and J. N. Lin (2016). Associations between methylenetetrahydrofolate reductase (MTHFR) polymorphisms and non-alcoholic fatty liver disease (NAFLD) risk: a meta-analysis. PLoS ONE, 11: e0154337.

Zhang, L., W. You, H. Zhang, R. Peng, Q. Zhu, A. Yao, X. Li, Y. Zhou, X. Wang, L. Pu and J. Wu (2015). *PNPLA3* polymorphisms (rs738409) and non-alcoholic fatty liver disease risk and related phenotypes: a meta-analysis. J. Gastroenterol Hepatology, 30: 821-829.

Table (1): Comparison between NAFL (group 1) and NASH (group 2) in clinical characteristics and laboratory evaluation.

Parameter	NAFL (group 1)	NASH (group2)	P value
Weight	49.81 ± 4.90	85.23 ± 2.85	< 0.01**
Height	122.39 ± 14.07	123.60 ± 2.03	> 0.05
BMI	32.02 ± 2.04	54.95 ± 2.76	< 0.01**
Hip circumference	90.28 ± 2.27	112.10 ± 2.71	< 0.01**
Waist circumference	94.12 ± 2.39	111.58 ± 2.52	< 0.01**
Cholesterol	175.10 ± 18.79	224.10 ± 13.10	< 0.01**
TG	122.90 ± 10.12	166.50 ± 18.90	< 0.01**
LDL	129.80 ± 15.70	165.90 ± 10.30	< 0.01**
ALT	12.57 ± 8.25	26.20 ± 8.30	< 0.01**
AST	12.60 ± 7.50	22.50 ± 6.10	< 0.01**

* Significant difference ** Highly significant difference

Table (2): The DNA oligonucleotide primers, reaction conditions for PCR and RFLP analysis of *PNPLA3* rs738409/*GCKR* rs1260326.

Gene	SNP N	Primer sequence	TM	Amplicon Size	Restriction Enzyme	RFLP analysis
PNPLA3	rs738409	F: 5'-TGG GCC TGA AGT CCG AGG GT-3' R: 5'-CCG ACA CCA GTG CCC TGC AG-3'	58°C	333 bp	*BstF5I*	GG: 333 bp GC: 333, 200, 133 bp CC: 200, 133 bp
GCKR	rs1260326	F: 5'-TGC AGA CTA TAG TGG AGC CG-3' R: 5'-CAT CAC ATG GCC ACT GCT TT-3'	60°C	231 bp	*HpaII*	TT: 213, 18 bp TC: 213, 150, 63, 18 bp CC: 150, 63, 18 bp

Table (3): Genotypes and alleles frequency and percentage of the *PNPLA3* rs738409 polymorphism for patients and control.

Genotype/allele	Patients		Control		P value
	No.	Percent%	No.	Percent%	
GG	17	21.25	10	12.5	< 0.05*
GC	39	48.75	38	47.5	> 0.05
CC	24	30.00	32	40.5	< 0.05*
Total	80	100.00	80	100.0	
Frequency of T allele	0.456	45.60	0.363	36.3	< 0.01**

* Significant difference ** Highly significant difference

Table (4): Genotypes and alleles frequency and percentage of the *GCKR* rs1260326 polymorphism for patients and control.

Genotype/allele	Patients		Control		P value
	No.	Percent%	No.	Percent%	
TT	15	18.75	12	15.00	> 0.05
CT	48	60.00	29	36.25	< 0.01**
CC	17	21.25	39	48.75	< 0.01**
Total	80	100.00	80	100.00	
Frequency of T allele	0.488	48.80	0.331	33.10	< 0.01**

* Significant difference ** Highly significant difference

Table (5): Comparison between Group 1 and Group 2 in genotypes and allele frequency for *PNPLA3* rs738409 polymorphism.

Genotype/ allele	Group 1		Group 2		P value
	No.	Percent%	No.	Percent%	
GG	7	17.50	10	25.00	<0.05*
GC	17	42.50	20	50.00	< 0.01**
CC	16	40.00	10	25.00	<0.05*
Total	40	100.00	40	100.00	
Frequency of T allele	0.3875	38.75	0.5	50.00	<0.05*

* Significant difference ** Highly significant difference

Table (6): Comparison between Group 1 and Group 2 in genotypes and allele frequency for *GCKR* rs1260326 polymorphism.

Genotype/ allele	Group 1		Group 2		P value
	No.	Percent%	No.	Percent%	
TT	5	12.5	11	27.5	< 0.01**
CT	23	57.5	24	60.0	> 0.05
CC	12	30.0	5	12.5	< 0.01**
Total	40	100.0	40	100.0	
Frequency of T allele	0.413	41.30	0.575	57.5	< 0.01**

* Significant difference ** Highly significant difference

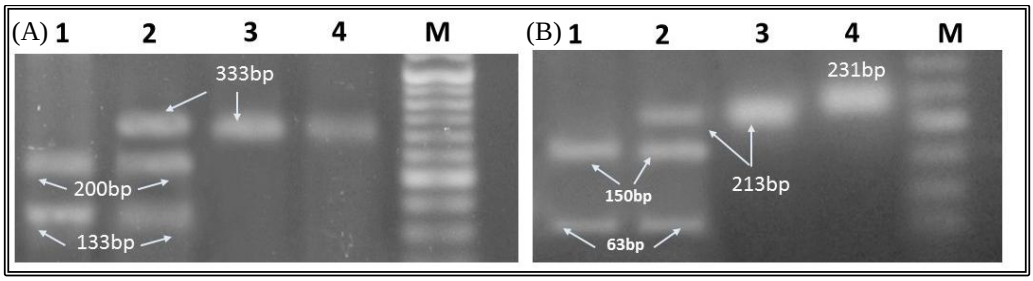

Fig. (1): The PCR product and the RFLP for the *PNPLA3* rs7383409 and *GCKR* rs1260326 polymorphisms.

(A): Lane 1: show the normal homozygous genotype (CC) for the *PNPLA3* rs7383409 Polymorphism. Lane 2: show the heterozygous genotype (GC) for the *PNPLA3* rs7383409 Polymorphism. Lane 3: show the mutant homozygous genotype (GG) for the *PNPLA3* rs7383409 Polymorphism. Lane 4: Native PCR product for the *PNPLA3* rs7383409 SNP. M: Marker (50 bp Ladder).

(B): Lane 1: show the normal homozygous genotype (CC) for the *GCKR* rs1260326 Polymorphism. Lane 2: show the heterozygous genotype (TC) for the *GCKR* rs1260326 Polymorphism. Lane 3: show the mutant homozygous genotype (TT) for the *GCKR* rs1260326 Polymorphism. Lane 4: Native PCR product for the *GCKR* rs1260326 SNP. M: Marker (50 bp Ladder).