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Title: Adipokine levels and perilipin gene polymorphisms in obese Turkish adolescents with non-alcoholic fatty liver disease

Running Head: PLIN polimorphisms and adipokins in NAFLD

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ABSTRACT

Objective: The aim of the present study was to evaluate the relationship between adipokines and perilipin (PLIN) polymorphisms with non-alcoholic fatty liver disease (NAFLD).

Methods: Obese Turkish adolescents were assessed in the study. The patients were divided into two groups: obese (NAFLD and non-NAFLD) and non-obese. Serum leptin, adiponectin, resistin, and ghrelin levels and PLIN gene analysis (PLIN 1, 4, and 6) were studied in all patients and healthy control group. Data obtained were compared with those of healthy control group.

Results: Overall, 83 obese adolescents with NAFLD, 123 obese adolescents with normal liver, and 102 healthy non-obese adolescents as the control group were evaluated. No significant difference was observed in terms of serum adipokine (leptin, adiponectin, resistin, and ghrelin) levels in patients with NAFLD and non-NAFLD obese adolescents. The incidence of major alleles of PLIN 6 genotype in obese adolescents without NAFLD was slightly higher than that in the control group (p=0.06). PLIN 6 minor allele incidence was significantly lower (p=0.01), and PLIN 4 major allele frequency in patients with NAFLD was slightly lower than those in the control group (p=0.05).

Conclusion: These findings suggested that no adipokine role was determined in the development of fatty liver in obese adolescents. The low rate in PLIN 6 minor allele can be a risk factor for NAFLD in adolescents.

Keywords: Adolescent, non-alcoholic fatty liver disease, perilip in polymorphism, adipokines, obesity

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects more than 30% of the adult population and 10% of the children population in developed countries (1). NAFLD is a reflection of an underlying metabolic function disturbance that is thought to be greatly related to insulin resistance, dyslipidemia, cardiovascular disease, and, above all, metabolic risk factor such as obesity (2, 3). To understand fatty liver development in humans, it is important to comprehend lipid pathophysiology. Fatty tissue is not just a storage organ, but it also plays a key role in the organization of lipid metabolism. A series of proteins plays an important role in the organization of lipid metabolism. Perilipin (PLIN) is the primary protein (4, 5). PLINs are phosphorylated proteins present on the surface of lipid droplets in the adipocytes, steroid producing cells, liver, heart, and muscle cells (6). These proteins have central roles in the mobilization and storage of triglycerides (TGs) in the adipose tissue (7). In the fasting state, catecholamines bind their cell surface receptors and initiate signals that activate cyclic adenosine monophosphate-dependent protein kinase A (PKA), PKA stimulates the phosphorylation of PLIN A, and finally, activated PLIN A facilitates lipolysis in the adipocytes (8). Five proteins of the PLIN family (PLINs 1–5) are associated with lipid droplet formation in the liver (9). PLIN 1 expression is synchronized with TG storage in the liver, and it has been shown that it has an important role in the NAFLD physiopathology (10, 11). A variety of polymorphisms associated with genes that code PLINs have been identified, and they have been linked with several clinical conditions (e.g., obesity, metabolic syndrome, insulin resistance, and glucose) (12). In a study that we conducted in obese adolescents followed by our clinic, it was shown that PLIN 6, one of the most commonly encountered PLIN single nucleotide polymorphisms (SNPs), has a relationship with obesity (13). To our knowledge,

there is no study that analyzes the relationship of PLIN genetic alterations with NAFLD development in human studies. We aimed to search the role of PLINs in the development of NAFLD.

MATERIALS and METHODS

Study population

The study was conducted between 2011 and 2012. Patients aged between 10 and 18 years who were referred to the Dokuz Eylül University Department of Pediatric Gastroenterology, Hepatology and Nutrition with complaint of excess weight and diagnosed with obesity according to anthropometric measurements composed the obese group. Patients with genetic diseases (Prader-Willi syndrome and Down syndrome), chronic diseases (Cushing syndrome, growth hormone deficiency, diabetes mellitus, and hypothyroidism), and who use drugs (corticosteroids and anti-diabetics) were excluded in the study.

A complete physical examination and anthropometric measurements were performed to all patients. Height was measured in stocking feet to the nearest 0.5 cm using a stadiometer. Body weight was measured using calibrated scales in light clothing to the nearest 0.1 kg. Height and body weight were measured twice, and the mean of two readings was calculated. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters squared). Patients whose lean BMI was higher than the 95th percentile for patient age and sex were diagnosed as obese (14).

Diagnosis of NAFLD was performed based on increased echogenicity via ultrasonography (USG) compatible with fatty infiltration of the liver with or without elevated alanine aminotransferase (ALT) levels. NAFLD grading by USG was measured according to a previous study (15). According to liver

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brightness at USG, obese patients were divided into two groups: patients with NAFLD and patients without NAFLD.

The control group consisted of asymptomatic healthy children and adolescents who were admitted to the well child outpatient clinic of our hospital for medical screening (e.g., hepatitis, thyroid functions, lipid profile, celiac disease, or anemia). All of these screening test results were normal. Anthropometric values and abdominal USG of the controls were also in the normal ranges. Serum glucose levels, lipid profiles, and liver function tests of the control group were measured and found to be in normal ranges.

The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from all obese and control adolescents.

Biochemical Analysis

Venous blood collection was obtained between 08:00 AM and 10:00 AM after fasting for 12h overnight. Fasting serum glucose, insulin, ALT, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and TG levels were measured. Low-density lipoprotein cholesterol (LDL-C) level was calculated using the Fried Ewald formula (LDL-C = TC − HDL-C − TG/5) (16). Infectious (hepatotropic viruses), metabolic (Wilson disease and alpha-1 antitrypsin deficiency), and autoimmune (autoimmune hepatitis and celiac disease) disorders of the liver were screened if the patients' ALT levels were above the accepted values.

The estimate of insulin resistance was calculated by a homeostasis model assessment of insulin resistance (HOMA-IR) index (fasting insulin × fasting glucose/22.5) (17). A cut-off HOMA level of>3.16 was used to identify an insulin resistance status for adolescents (18).

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Venous blood samples were centrifuged at 3000*g*at 4 °C for 15 min. Serum was frozen at −20 °C until enzyme-linked immunosorbent assay (ELISA) analysis. Leptin serum concentration was measured using a human leptin ELISA kit (catalog no. EK0437, Boster Biological Technology Co., Ltd.). The samples were read on a Synergy HT Multi-Detection Microplate Reader (Bio-Tek) measuring the absorbance at a wavelength of 450 nm. ELISAs were performed according to the manufacturer's instructions, and the intra- and inter-assay coefficients of variation were <10%. All assays were conducted twice on the same occasion, and the average value obtained and conducted within the same laboratory under the same conditions.

Genetic Analysis

A 1-milliliter blood sample was collected in a tube withK_2-EDTA from all of the patients for genetic investigation. Samples were stored at −20 °C. Genomic DNA was isolated from these blood samples according to the manufacturer's instructions (Zymo Research). After DNA was isolated, single gene polymorphisms at the locus ofPLIN1gene (Entrez Gene ID:5346) 15q26 were evaluated by real-time polymerase chain reaction (RT-PCR) using specific fluorochrome-labeled probes in accordance with the manufacturer's instructions (Roche). SNPs of rs894160, rs2289487, and rs1052700 in thePLIN1 gene locus were investigated by RT-PCR using the model for end-stage liver disease (MELD) analysis method from DNA specimens isolated from blood. Florescence-labeled hybridization probes were used for detection of mutations. Hybridization probes have been used for detection of mutations by MELD curve analysis using fluorescence resonance energy transfer FRET technology. Primers and probes have been designed to detect SNPs. Reaction conditions were prepared as the maximum volume of specimen would be 20 µL. A 0.5 µM sample was obtained from "forward" and "reverse" primers and 0.2 µM from anchor and sensor probes. SNPs were detected by RT-PCR using the

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LightCycler® 480 device (Roche). SNPs containing parts of DNA were amplified by the LightCycler® FastStart DNA Master HybProbe (Roche) using forward and reverse primers for each SNP. Amplification signals were detected by florescence-labeled probes. Mutations were detected by the MELD analysis after PCR processing.

Statistical Analysis

Statistical analysis was performed using the SPSS software 15.0. Data were analyzed as mean ± standard deviation, odds ratio, and 95% confidence interval. The chi-square test was used to assess whether the genotypes were in Hardy–Weinberg equilibrium and to compare the genotype and allele frequencies between case and control subjects. Differences in mean values between the groups were analyzed by Student'st-test or Mann–Whitney U test, when sample size is <30. Pearson's correlation analysis was performed to determine the correlation between the two groups. All p-values are two-tailed, and group differences or correlations with a $p<0.05$ were accepted as significant.

Ethical Approval

The study protocol was designed in compliance with the Declaration of Helsinki. Written informed consent was obtained from both the children and/or their parents on enrollment in the study. The study was approved by the ethics committee of the Dokuz Eylül University Faculty of Medicine (25/18/2009).

RESULTS

A total of 206 obese adolescents including 83 obese + NAFLD, 123 obese + normal liver, and 102 healthy control group were evaluated. Age distribution was found to be similar between the group

with NAFLD and non-NAFLD. Body weight and height, BMI and BMI standard deviation score, aspartate aminotransferase, ALT, fasting insulin, and HOMA-IRin the NAFLD group were significantly higher than those in the non-NAFLD group. Blood sugar averages and fasting lipid levels were determined as similar between both groups. Serum leptin, adiponectin, resistin, and ghrelin levels were also identified to be similar between two groups (Table 1).

The frequencies for the less common allele of the PLIN1 (rs2289487), PLIN4 (rs894160), and PLIN6 (rs1052700) polymorphisms were 0.13, 0.20, and 0.31, respectively. Genotype distributions did not deviate from Hardy–Weinberg expectations ($p>0.05$).

We analyzed major and minor allele distributions to understand the role of PLIN variations in NAFLD development. The incidence of major alleles of PLIN 6 genotype in obese adolescents without NAFLD was slightly higher than that in the control group ($p=0.06$). PLIN 6 minor allele incidence was significantly lower, and PLIN 4 major allele frequency in the group with NAFLD was lightly lower than those in the control group ($p=0.01$ and $p=0.05$, respectively) (Table 2).

DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) is a complex metabolic condition in which lifestyle and genetic factors play significant roles. It generally develops in obesity and plays a key role in the development of insulin resistance fatty liver (19). Numerous mechanisms are blamed in the tendency of people to be obese. The primary factors of these are adipokines, PLINs, and gene variations that code them (20). In the present study, we evaluated the role of adipokines in obese children in the development of NAFLD. In previous studies, adiponectin serum levels in patients with NAFLD were

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reported to be lower than those in the control group (21, 22); leptin, ghrelin, and resistin plasma levels were found to be high (23, 24). In the present study, we found leptin, adiponectin, resistin, and ghrelin averages to be high similar in both groups. Adipokines in obese adolescents were identified as an effective factor in the development of NAFLD.

PLIN protein family (PLIN), another factor blamed for the tendency of obesity, plays a vital role in fat cell metabolism. Nonetheless, the expression and functions of these proteins can be different in distinctive cells (25). Caldwell et al. (10) and Straub et al. (11) showed that there is a PLIN 1 expression in the liver tissue in NAFLD. PLIN 1 generally covers large fat cells and is seen as the same in hepatocyte cells. It is thought to have contributions to the development of large fat cells by combining small fat cells and stabilizing these cells. It has been indicated to have a contribution in alcoholic liver deposition and NAFLD. In both conditions, PLIN 1 protein family was displayed to be upregulated in the tissue (26, 27). To our knowledge, there is no study examining the relationship with PLIN SNPs.

In our study, we found that PLIN 6 major allele frequency in the group without NAFLD was slightly higher than that in healthy controls. PLIN 4 major allele frequency in the group with NAFLD was lightly lower than that in the control group. However, these results were not statistically significant in both cases. PLIN 1, 4, and 6 are the most frequently investigated polymorphisms in obesity. However, to our knowledge, there is no research about the relationship between PLIN SNPs and NAFLD. Studies investigating the relationship between PLIN SNPs and obesity have found very different results.

Qi et al. (28) demonstrated that PLIN 1 and PLIN 4 gene variations are associated with sex, and minor alleles of PLIN 1 and PLIN 4 are also related to low BMI in Spanish women. Mottagui-Tabar et al. (29) stated that the presence of PLIN 4 minor allele has been shown to cause decrement in PLIN levels

and increment in lipolytic activity. Perez-Martinez et al. (30) had demonstrated that the presence of minor C and A alleles in PLIN 1 and PLIN 4 results in low postprandial and atherosclerotic response. Sone et al. (31) had shown that PLIN 4 polymorphism is associated with body weight in middle-aged Japanese men. The association of PLIN 4 minor allele with metabolic syndrome had been demonstrated in a study conducted in 234 obese children and adolescents (32). On the contrary, in a study performed in France, PLIN 4 had been indicated not to be linked to anthropometric measurements and plasma leptin, glucose, and insulin concentrations (33). Similar results had been obtained in China. They had investigated the association between PLIN 1, 4, 5, and 6 and obesity and could not show any correlation (34).

We determined that PLIN 6 minor allele frequency in the NAFLD group was significantly lower than that in the control group. Qi et al. (28) had demonstrated PLIN 6 to be associated with high body fat mass and waist circumference in women. In another study conducted by Qi et al. (34), PLIN 6 SNP had been shown to be associated with obesity in Malaysian and Indian patients but not in Chinese patients. Soenen et al. (35) had detected low body weight in patients with homozygote PLIN 6 T-T allele. In these studies, several connections of PLIN 6 with obesity were identified. We think as a result of data obtained in the present study that in addition to this, there may be a role of PLIN 6 allele in the development of fatty liver. The relationship was described for the first time; however, there are also limitations in our study. One of them is that NAFLD diagnosis can only be made by USG; the other one is that there are a relatively limited number of patients diagnosed with NAFLD. On the other hand, it should be kept in mind that PLIN variations can vary according to ethnicity and gender.

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Consequently, of the PLIN variations known to have an association with obesity and metabolic syndrome, the inferiority in the minor allele rate of PLIN 6 can be a risk factor in the development of NAFLD. Advanced studies with more comprehensive and a large series of cases are required on this issue.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Dokuz Eylul University Faculty of Medicine (25/18/2009).

Informed Consent: Informed consent was obtained from both children and their parents.

Peer-review: Externally peer-reviewed.

Author Contributions: Conceived and designed the experiments or case: YT, NA, CBE. Performed the experiments or case: YT, NA, CBE, ST. Analyzed the data: SA, TK, OS, EE. Wrote the paper: YT, CBE, ST, NA. All authors have read and approved the final manuscript.

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Table 1. Clinicaland laboratory features of obese adolescents with and without NAFLD.

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	NAFLD		p
	Yes	No	
Gender			
Male	54	59	**0.02**
Female	29	64	
Age (year)	13.0 ± 2.1	12.5 ± 2.0	0.69
Body length (cm)	160.1 ± 11.3	154.6 ± 10.5	**0.001**
Body weight (kg)	82.3 ± 22.6	66.6 ± 14.5	**0.0001**
BMI (kg/m^2)	31.7 ± 6.3	27.5 ± 3.5	**0.0001**
BMI (SDS)	2.1 ± 0.36	1.8 ± 0.4	**0.0001**
AST (IU/L)	31.3±15.3	22.5±6.8	**0.000**
ALT (IU/L)	40.9±31.6	19.7±9.8	**0.000**
Glucose (mg/dL)	86.5±7.9	87.0±7.5	0.68
Insulin (µU/mL)	14.8±13.1	9.9±7.7	**0.03**
HOMA-IR	3.2±3.0	2.1±1.7	**0.02**
LDL (mg/dL)	103.8±25.7	104.9±33.0	0.40
TG (mg/dL)	129.5±73.3	121.5±73.2	0.46

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Total cholesterol (mg/dL)	172.3±31.6	173.4±35.1	0.83
Leptin (pg/mL)	11.3±2.4	11.4±2.2	0.74
Adiponectin (ng/mL)	6.0±3.4	5.9±3.6	0.13
Resistin (pg/mL)	18.0±7.2	16±6.7	0.34
Ghrelin (ng/mL)	2.4±0.69	2.4±0.70	0.89

ALT: alanine aminotransferase, AST: aspartate aminotransferase, BMI: body mass index, HOMA-IR: homeostasis model assessment of insulin resistance, LDL: low-density lipoprotein, NAFLD: non-alcoholic fatty liver disease, SDS: standard deviation score, TG: triglyceride.

Values given in bold denote statistically significant results ($p<0.05$).

Table 2. PLIN SNPs in obese adolescents with and without NAFLD and controls.

PLIN SNPs	Alleles	NAFLD+ (n:83)	NAFLD− (n:123)	Controls (n:102)	p-value NAFLD+ vs NAFLD−	p-value NAFLD+ vs controls	p-value NAFLD− vs controls
rs2289487 (PLIN 1)	G-G	56	83	69	0.70	0.57	0.90
	A-A + G-A	27	40	33			
rs894160 (PLIN 4)	G-G	32	56	54	0.44	0.05	0.23
	A-A + G-A	51	67	48			

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rs1052700 (PLIN 6)	T-T	68	97	68	0.29	**0.01**	0.06
	A-A + T-A	15	26	34			

Values given in bold denote statistically significant results (p<0.05).

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