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Assessment of the rs2645424 C/T single nucleotide polymorphisms in the *FDFT1* gene, hepatic expression, and serum concentration of the *FDFT* in patients with nonalcoholic fatty liver disease

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ABSTRACT

Despite being the most common chronic liver disease, the pathogenesis of nonalcoholic fatty liver disease (NAFLD) still remains unclear. According to the genome-wide association studies (GWAS) alternative alleles of the farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*) gene involved in cholesterol biosynthetic pathway are known to affect hepatic squalene synthase (*SQS* or *FDFT*) expression. Recent studies have shown that the *FDFT1* gene is associated with the clinical and histopathological characteristics of patients with NAFLD and thus is a candidate gene for NAFLD susceptibility. Our aim was to investigate the effect of rs2645424 C/T single nucleotide polymorphisms (SNPs) in NAFLD patients in the Turkish population. For this purpose, 64 Turkish NAFLD patients who underwent liver biopsy and 60 Turkish healthy control subjects were included in the study. We have evaluated the rs2645424 C/T SNPs genotypes (CC, wild type; CT, heterozygous; TT, mutant type) and the hepatic expression of the *FDFT1* gene with real-time PCR and serum concentration of *FDFT* with ELISA method. The frequencies of the *FDFT1* gene rs2645424, TT, CC and TC genotypes were the similar between patients with NAFLD and controls. Additionally, there was no significant correlation between serum *FDFT1* mRNA expression and histological parameters in patients with NAFLD while it was significantly higher in patients with NAFLD in comparison to the healthy controls. The expression and variants of *FDFT1* gene should be investigated in larger populations and different ethnic groups in order to clarify their impact on NAFLD pathogenesis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of metabolic syndrome and is the most common cause of chronic liver disease in many developed countries worldwide. The histological spectrum of NAFLD ranges from simple steatosis (steatosis without hepatocellular injury), to nonalcoholic steatohepatitis (steatosis with inflammation), advanced fibrosis, cirrhosis, and eventually to hepatocellular carcinoma. The prevalence of NAFLD is dramatically rising and is predicted to become the most frequent indication for liver transplantation by 2030 in Western countries (Armstrong et al., 2012; Shaker et al., 2014). Recent findings indicate that, NAFLD is a precursor

Abbreviations: ALT, Alanine Aminotransferase; AST, Aspartate aminotransferase; BMI, Body Mass Index; CI, Confidence Intervals; ELISA, Enzyme-Linked Immunosorbent Assay; FDFT1, Farnesyl-Diphosphate Farnesyltransferase 1; FDFT, Squalene Synthase; GWAS, Genome Wide Association Studies; HbA1c, Hemoglobin A1C; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; NAFLD, Nonalcoholic Fatty Liver Disease; NAS, NAFLD Activity Score; NASH, Nonalcoholic steatohepatitis; OR, Odds ratios; ROC, Receiver Operating Characteristic; RT-PCR, Real-Time Polymerase Chain Reaction; SNPs, Single Nucleotide Polymorphisms; SQS, Squalene Synthase; VLDL, Very-Low-Density Lipoprotein; WC, Waist Circumference

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for the development of insulin resistance (Ballestri et al., 2016).

The farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*) gene encodes squalene synthase (*SQS* or *FDFT*) which is an enzyme localized to the membrane of the endoplasmic reticulum in humans, animals, plants, and yeasts. *FDFT1* gene is a key regulator enzyme of the cholesterol biosynthesis pathway. It catalyzes the formation of squalene, the first cyclic structure and the first step in the sterol biosynthesis pathway. Recent studies have shown that chemical inhibition of *FDFT* is effective in reducing plasma levels of total and low-density lipoprotein cholesterol (LDL-c) (Kourounakis et al., 2011). In addition to that, a genome-wide association studies (GWAS) has identified a novel association between the rs2645424 C/T single nucleotide polymorphisms (SNPs) in the *FDFT1* gene on chromosome 8 in patients with NAFLD (Chalasani et al., 2010). The present study was designed to investigate the relationship between rs2645424 C/T SNPs in the *FDFT1* gene, hepatic *FDFT1* gene expression and serum levels of *FDFT*, as well as their association with clinical and histopathological parameters in patients with NAFLD.

2. Methodology

2.1. Study subjects

Our local ethics committee approved the study protocol (Istanbul University School of Medicine Ethic committee approval number: 2010/789-244, date: 26th October 2010) and a written informed consent was obtained from all volunteers. Sixty-four Turkish patients with NAFLD (32 male, and 32 female; mean age 45,3 ± 11,1 years) and 60 Turkish healthy control subjects (27 male, and 33 female; mean age, 47 ± 13,4 years) were recruited into the study. All of the 64 NAFLD patients underwent ultrasound (US) guided liver biopsy. A detailed clinical history was obtained and a full physical examination was performed on the NAFLD patients and healthy controls. All of them had transaminases elevations for at least 6 months. The criteria for exclusion from participation in the study were as follows: ingestion of hepatotoxic drugs or herbal medications, using medications which cause hepatosteatosis, such as amiodarone, aspirin, diltiazem, tamoxifen, corticosteroids, consumption of > 20 g/day of alcohol, history of any liver disease, such as chronic hepatitis B, C, D or serum positive for hepatitis B surface antigen, hepatitis B DNA, hepatitis C RNA, history of autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, alpha-1 antitrypsin deficiency, hemochromatosis, Wilson's disease, and history of malignancy. Patients with elevated levels of transaminases persisting longer than sixth months underwent an US-guided liver biopsy and those diagnosed with NAFLD were enrolled in the study. Clinicopathological characteristics of the NAFLD group can be in Table 1. The healthy control group had no illness, no history of previous liver disease, no history of drugs abuse, herbal medications or alcohol consumption and was negative for viral hepatitis serology tests and with normal liver US and transaminases levels.

2.2. Clinical assessment

All patients and healthy control subjects underwent a complete physical examination. Anthropometric assessment of weight, height, and waist circumference (WC) were measured, and body mass index (BMI) (kg/m^2) was calculated. All ultrasonography examinations and US-guided percutaneous liver biopsies were performed by the same radiologist. Blood pressure was measured after ten minutes of quiet rest. Blood cell counts and biochemistry analysis were performed using standard methods. The serum samples were centrifuged at 2500 ×*g* for 10 min and samples were recovered and stored at −80 °C until analysis. Homeostatic Model Assessment - Insulin Resistance (HOMA-IR) index [fasting insulin (μU/ml) × fasting glucose (mg/dL)/405.23] was used for determining insulin resistance. The American Diabetes Association (ACE/ADA Task Force on Inpatient Diabetes, 2006) criteria were used for diabetes mellitus and Adult Treatment Panel III (Grundy et al., 2004) criteria were used for metabolic syndrome diagnosis.

Table 1
Clinicopathological characteristics of the NAFLD group.

Patient characteristics	Status	n (%)
Steatosis	I	21 (36.8)
	II	24 (42.1)
	III	12 (21.1)
	Mild (I)	21 (36.8)
	Moderate - Severe (II,III)	36 (63.2
Lobular inflammation	0	11 (19.3)
	I	34 (59.6)
	II	10 (17.5)
	III	2 (3.5)
Lobular inflammation	Yes	46 (80.7)
	No	11 (19.3)
Ballooning	0	10 (17.5)
	I	33 (57.9)
	II	14 (24.6)
Ballooning	Yes	47 (82.5)
	No	10 (17.5)
Portal inflammation	0	31 (54.4)
	I	22 (38.6)
	II	4 (7.0)
Portal inflammation	Yes	26 (45.6)
	No	31 (54.4)
Fibrosis	0	24 (38.1)
	I	25 (39.7)
	II	3 (4.8)
	III	5 (7.9)
	IV	6 (9.5)
Fibrosis	Yes	39 (61.9)
	No	24 (38.1)
IHC	Mild - Moderate (I,II)	71,8
	Severe (III)	28.2
Type 2 diabetes (%)		22.8
Hypertension (%)		26.3
Hyperlipidemia (%)		29.8

The results are shown as "%". NAFLD, Nonalcoholic Fatty Liver Disease; IHC: Immunohistochemistry.

Serum *FDFT* levels were measured duplicately using a commercial enzyme-linked immunosorbent assay (ELISA) (NovaTeinBio, Analyte ELISA Kit, Cat.No: NB-E100001) kit according to the manufacturer's instructions. The minimum detectable concentration was 0.031 pg/ml. The intra and inter-assay coefficients of variation for the *FDFT1* gene were < 10%. All ELISA tests were performed in a blind manner.

2.3. Genetic analyses (DNA extraction and genotyping)

While selecting the SNPs, both the results of population genetics on the *FDFT1* gene, found in the ENSEMBL genome database, and the literature support were taken into consideration. SNPs (rs2645424 C/T) was analyzed in the *FDFT1* gene [ACCTGCCATCCCTTTCCCCTTCC TGC[C/T]GCAGAATTCTTTCTTTGGGGGAAAT], by using quantitative real-time PCR (RT-PCR, LightCycler; Roche Diagnostics GmbH, Mannheim Germany). Peripheral venous blood samples were collected from each subject into tubes containing the anticoagulant EDTA for DNA isolation. Genomic DNA was isolated from whole blood with a spin column kit (Roche Diagnostics GmbH, Mannheim Germany) according to the manufacturer's instructions. Samples were stored in aliquots at −20 °C until they were analyzed. Also, polymerase chain reaction (PCR) and reverse hybridization methods were used to genotypes samples for SNPs analyses. Quantitative RT-PCR for genotyping was performed on the LightCycler 1.5 system using 1 μl of hybridization probe pair (Light Cycler Fast Start DNA Master HybProbe) labeled with 3′- fluorescein and -5′ LightCycler Red-labeled pair of oligonucleotide probes (TIB MOLBIOL GmbH, Berlin, Germany). Genotyping was performed in a 20 μl volume containing 2.0 μl of LightCycler FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany), 1.0 μl

Reagent Mix, 3.0 mM $MgCl_2$, and 50 ng of genomic DNA. The following protocol was used for amplification; initial denaturation at 95 °C for 10 min, followed by 40 cycles with denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. An additional melting curve analysis was performed at 95 °C for 30 s, 40 °C for 2 min and 75 °C for 0 s in order to assign any non-specific amplification.

2.4. Histological analysis

All of the patients in this study underwent US-guided percutaneous liver biopsy. The obtained liver biopsy specimens are considered as adequate if the tissue was > 2 cm and/or having more than six portal areas in histological examination. The liver biopsy specimens were stained with hematoxylin-eosin, reticulin, and Masson's trichrome stains and all specimens were evaluated by an experienced pathologist blinded to the clinical data and laboratory results. NAFLD was classified according to histopathological features of such as steatosis, lobular inflammation, ballooning, fibrosis, by NASH Clinical Research Network (Kleiner et al., 2005). Briefly, hepatic steatosis was ranked from 0 to 3 (score 0, steatosis ratio lower than 5%; score 1, steatosis ratio 5-33%; score 2, 33% to 66%; and score 3, > 66%), lobular inflammation was graded from 0 to 3 (score 0, no foci; score 1, < 2 foci per x200 field; score 2, 2-4 foci per x 200 field; score 3, > 4 foci per x 200 field), ballooning scoring was ranked from 0 to 2 (score 0, no ballooning; score 1, few ballooning; score 2, numerous ballooning of hepatocytes), and fibrosis was graded from 0 to 4 (stage 0, no liver fibrosis; stage 1, perisinusoidal or periportal fibrosis; stage 2, perisinusoidal and portal/periportal fibrosis; stage 3, bridging fibrosis and stage 4, cirrhosis). Based on this scoring system, the total NASH score 0-2 as simple steatosis, 3-4 as borderline NASH, 5 or greater were diagnosed as definitive NASH (Kleiner et al., 2005).

2.5. Statistical analysis

The statistical analyses were performed using SPSS 21.0 software package (SPSS Inc., Chicago, IL, USA). Continuous variables with normal and skewed distributions were presented by the mean ± standard deviation, while categorical variables are presented as frequencies. Mean values were compared between patients and controls by using a Student's *t*-test or Mann-Whitney *U* test and differences in the distribution of genotypes and alleles between study groups were tested using the chi-square, depending on the normality of the variable distribution. Data were further analyzed for significant differences between the three genotypes using one-way Anova test and post hoc Bonferroni test where appropriate. Allele frequencies were calculated by gene counting methods and the chi-square test was used to test the departure from the Hardy-Weinberg equilibrium. Whenever an expected cell value of < 5 was obtained, Fisher's exact-test was used. The odds ratios (OR) and the confidence intervals (CI) were calculated to estimate the relative risk. Multivariate analysis was performed with linear logistic regression. This analysis was used to determine the association of NAFLD disease with several independent factors. In the logistic regression model, presence of NAFLD was used as the dependent variable. Model included TC genotype ≥5.18 mmol/l, BMI ≥30 kg/m^2, serum *FDFT* Level ≥ 15 and serum insulin level ≥ 5 μU/ml levels as independent variables. Receiver operating characteristic (ROC) curve analysis was used to assess the sensitivity and specificity of the *FDFT1* gene to distinguish between NAFLD and healthy controls. P values < 0.05 were considered statistically significant.

3. Results

The main clinical and physical characteristics and laboratory findings of the patients and controls are described in Table 2. Gender and age distribution and smoking rates were similar between patients with NAFLD and controls. As expected, WC, BMI, systolic and diastolic blood pressure, C-reactive protein, white blood cells, HOMA-IR index, lipid profile, transaminases, uric acid levels, ferritin, and the *FDFT* serum levels in patients with NAFLD were significantly higher from those of the healthy controls (Table 2). Ten (16%) patients had simple steatosis, 54 (84%) patients had NASH in the NAFLD group. Metabolic syndrome was found in 43 (67%) of the patients with NAFLD.

Table 2
Clinical, physical, and biochemical characteristics of the NAFLD patients and healthy controls.

	NAFLD group (n = 64)	Healthy controls (n = 60)	p value
Gender (males/females)	32/32	27/33	NS
Age (years)	45,3 ± 11,1	47 ± 13,4	NS
BMI (kg/m2)	32,3 ± 5,5	22,6 ± 3,7	< 0.001
Smoking (%)	31	33	NS
Waist circumference (WC)	104(98-108)	76,5(69-89)	< 0.001
Systolic blood pressure (mmHg)	123,3 ± 17,5	105,9 ± 12,6	< 0.001
Diastolic blood pressure (mmHg)	81,7 ± 10,3	68,6 ± 8,3	< 0.001
Sedimentation (mm/h)	15,6 ± 9,5	14,1 ± 9,1	NS
C-reactive protein (mg/l)	4,5(3-8)	2,8(3-9)	0.01
White blood cells (x109/l)	6,3(5,4-7,6)	5,7(5,4-6,3)	< 0.001
Hemoglobin (g/l)	137 ± 20	134 ± 19	NS
Platelets (x 109/l)	240(163-310)	250(213 − 302)	NS
Hemoglobin A1c (%)	5,9 ± 0,6	5,3 ± 0,1	< 0.001
HOMA-IR	2,8(2-3,5)	0,8(0,5-1,2)	< 0.001
Fasting Insulin (iU/ml)	4.53 ± 1.93	13.64 ± 7.77	< 0.001
Total cholesterol (mmol/l)	5,3 ± 1,3	4,65 ± 0,85	0.006
Triglycerides (mmol/l)	1,7(1,3-1,7)	1(0,7-1,4)	< 0.001
LDL cholesterol (mmol/l)	3,6 ± 0,9	2,8 ± 0,7	0.03
HDL cholesterol (mmol/l)	1,1(1-1,3)	1,4 ± 0,3	0.008
AST (U/l)	33(26-45)	19(15-22)	< 0.001
ALT (U/l)	45(34-67)	15(8-21)	< 0.001
Uric acid (ìmol/l)	327,7 ± 59,5	297,4 ± 59,5	< 0.001
Ferritin (pmol/l)	90(37,5-202)	21,7(10-37)	< 0.001
FDFT (U/l)	19,5 ± 4	14,3 ± 3	< 0.001
Metabolic syndrome (%)	68%	0	–

Data are shown as the mean ± standard deviation (for normally distributed continuous variables); median and interquartile ranges (for skewed continuous variables), with statistical analysis using a Student-*t*-test for means and a Pearson χ^2 test for numbers.
Normal values in laboratory tests: Sedimentation (0-20 mm/h); C-reactive protein (< 8 mg/l); white blood cell count (4-10 × 10^9/l); hemoglobin (130-180 g/l in males and 110-160 g/l in females); platelet (150-400 × 10^9/l); HbA1c (4.3-5.8 proportion of total hemoglobin); total cholesterol (2.6-5.2 mmol/l); LDL cholesterol (1-3.37 mg/dl); HDL cholesterol (> 0.9 mmol/l); triglyceride (0.7-1.7 mmol/l); AST (5-32 U/l); ALT (5-38 U/l); ferritin (54-755 μg/l in males and 25-755 μg/l in females); BMI (body mass index) (18-25 kg/m^2); Homeostatic Model Assessment-Insulin Resistance (HOMA-IR); Farnesyl-diphosphate farnesyltransferase (*FDFT*) and metabolic syndrome are described in the text.

3.1. Serum levels of the FDFT

Serum levels of *FDFT* in patients with NAFLD were significantly higher than those of the controls (p < 0.001) (Table 1). Levels of the *FDFT* in the study groups are reported in Fig. 1. As assessed by one-way ANOVA, serum *FDFT* levels were significantly different across the three study groups (p < 0.001). Specifically, the Bonferroni multiple comparison post-hoc tests showed that levels of *FDFT* were significantly higher in patients with simple steatosis (19.1 ± 3.5 U/l, p = 0.007) and NASH (19.5 ± 4 U/l, p < 0.001) compared with controls (14.5 ± 3.3 U/l).

In correlation analyses for the entire study cohort, *FDFT* was significantly and positively associated with BMI (r = 0.52, p < 0.001), WC (r = 0.503, p < 0.001), ALT (r = 0.501, p < 0.001), AST (r = 0.524, p < 0.001), total cholesterol (r = 0.384, p < 0.001), and

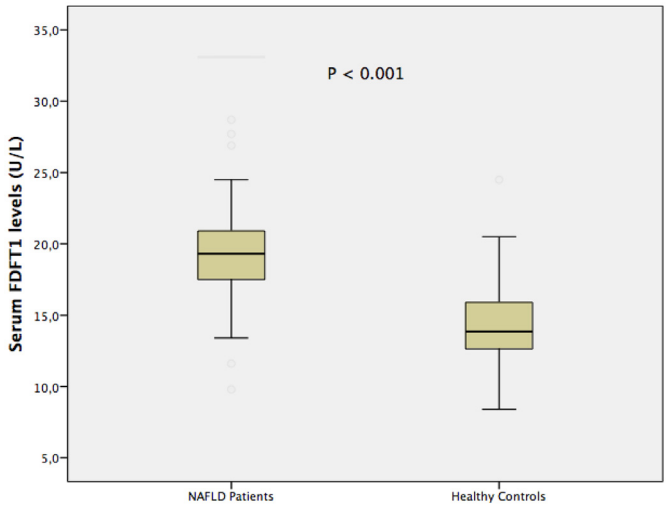

Fig. 1. Serum farnesyl-diphosphate farnesyltransferase (*FDFT*) levels in the study groups. Nonalcoholic Fatty Liver Disease (NAFLD) and healthy controls.

LDL-c ($r = 0.468$, $p < 0.001$). In multiple linear regression analyses, LDL-c level was a strongly associated to *FDFT* ($\beta = 0.668$, $t = 2.397$, $p = 0.019$). There was not significantly a correlation between serum *FDFT* levels and histological parameters in patients with NAFLD.

The ROC analysis to determine the discriminative power of *FDFT* for discrimination of NAFLD and healthy controls yielded an AUC of 0.852 (Fig. 2). Using a cut-off of 16.65 U/l for *FDFT* the sensitivity and specificity values were calculated as 80.1 and 81.5%, respectively. The positive and negative predictive values for the serum *FDFT* level of 16.65 U/l were 82.2% and 79%, respectively.

3.2. The rs2645424 C/T single nucleotide polymorphisms and allel distribution in the FDFT1 gene

The allelic and genotypic frequencies of the *FDFT1* gene, rs2645424 C/T, SNPs were not significantly different between the study groups

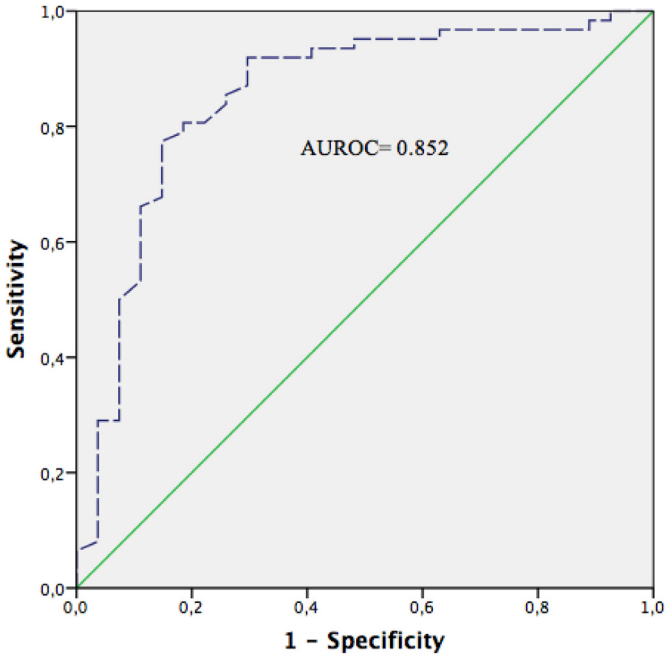

Fig. 2. Receiver-operating characteristics (ROC) curve for distinguishing between nonalcoholic fatty liver disease (NAFLD) and healthy controls using serum farnesyl-diphosphate farnesyltransferase (*FDFT*) levels.

Table 3
The distribution of the *FDFT1* gene, rs2645424 C/T genotypes and alleles in the study groups.

FDFT1 genotypes	Groups	
	Controls (n = 60)	NAFLD (n = 64)
TT	12 (20.0%)	17 (26.6%)
TC	34 (56.7%)	27 (42.2%)
CC	14 (23.3%)	20 (31.3%)
HWE	p = 0.297	p = 0.216
Alleles		
T	58 (48.33%)	61 (47.66%)
C	62 (51.67%)	67 (52.34%)

HWE: Hardy-Weinberg Equilibrium.

($p > 0.05$) (Table 3). The frequency of all alleles and genotypes in the study groups were distributed according to Hardy-Weinberg equilibrium ($p > 0.05$).

The frequencies of the *FDFT1* gene rs2645424, (CC, wild type; CT, heterozygous; TT, mutant type), TT, CC and TC genotypes among the NAFLD patients were 0.266, 0.313 and 0.422, respectively and among the control subjects, they were 0.200, 0.233 and 0.567, respectively ($p > 0.05$).

3.3. Association of the FDFT1 gene, rs2645424 C/T, SNPs with lipid and metabolic parameters in the study groups

To assess whether *FDFT1* gene, rs2645424 C/T, SNPs had any effect on lipid and metabolic parameters, we compared the baseline characteristics among the genotype groups in the control and patients with NAFLD (Tables 4, and 5). The serum *FDFT* levels were significantly higher in rare CC genotype carriers in control subjects in comparison to T-allele carriers ($p = 0.009$). In contrast, the control subject with CC genotype showed a lower BMI ($p = 0.007$), WC ($p = 0.03$), hip circumference ($p = 0.03$), LDH-c level ($p = 0.038$) and LDL-c ($p = 0.018$) than those with the common T-allele (TT and TC genotypes) (Table 4).

In the NAFLD group, subjects with the *FDFT1* gene rare CC genotype showed lower creatinine (0.020), HbA1c ($p = 0.003$), and HDL-c ($p = 0.001$) concentrations than with the T-allele. Hovewer, creatinine level and waist circumference were higher in the CC genotype carrying NAFLD subjects than those with the T-allele with statistical significance ($p = 0.020$, and $p = 0.036$, respectively) (Table 5). In accordance with this in the NAFLD group, in subjects with the rs2645424 rare CC genotype creatinin levels were significantly higher in comparison to TT and TC carriers, while HDL-c and HbA1c levels were significantly lower. Subjects with rs2645424 TC genotype have shown a significant increase in waist and hip circumference in comparison to TT genotype carriers, while TT genotype carriers were observed to have higher levels of microalbumin when compared to subjects with rs2645424 TC genotype.

3.4. Association of the FDFT1 gene rs2645424 C/T SNPs with clinical characteristics in the NAFLD group

Subjects in the NAFLD group with TC or CC genotypes were more likely to have moderate or severe type of steatosis in comparison to subjects with TT genotype (83.7%, $p = 0.007$). There were not any significant associations between rs2645424 C/T genotypes and clinical characteristics (Table 6).

3.5. Multivariate analysis

In the multivariate logistic regression analysis, the dependent variable, NAFLD was significantly associated with total cholesterol ≥5.18 mmol/l ($p = 0.003$), BMI ≥ 30 kg/m^2 ($p < 0.001$), serum *FDFT*

Table 4
Comparison of biochemical and clinical characteristics among the different genotypes of the *FDFT1* gene, rs2645424 C/T SNPs in the control group.

	FDFT1 rs2645424 genotypes			p values		*FDFT1* rs2645424 Alleles		p values	
	FDFT1 TT	FDFT1 CC	FDFT1 TC	CC vs TT	CC vs TC	FDFT1 T Allel	FDFT1 C Allel	CC vs T	TT vs C
FDFT1	13.78 ± 0.06	17.03 ± 3.73	12.84 ± 2.31	0.063	0.002	13.06 ± 2.04	14.66 ± 3.62	0.009	0.259
BMI	26.14 ± 2.25	22.59 ± 3.15	25.35 ± 3.69	0.017	0.014	25.52 ± 3.42	24.51 ± 3.73	0.007	0.211
Waist circumference	85.00 ± 11.31	74.30 ± 10.37	86.21 ± 14.24	0.05 <	0.036	86.06 ± 13.84	81.25 ± 14.05	0.030	0.718
Hip circumference	95.50 ± 2.12	91.60 ± 5.52	99.43 ± 9.52	0.05 <	0.026	98.94 ± 8.98	96.17 ± 8.87	0.030	0.918
SBP	120.00 ± 14.14	107.60 ± 12.52	106.15 ± 13.25	0.05 <	0.05 <	108.00 ± 13.73	106.52 ± 12.65	0.855	0.164
DBP	75.00 ± 7.07	69.00 ± 8.76	69.23 ± 8.62	0.05 <	0.05 <	70.00 ± 8.45	69.13 ± 8.48	0.778	0.354
Fasting glucose	91.88 ± 14.09	81.00 ± 16.10	91.57 ± 24.52	0.05 <	0.05 <	91.64 ± 22.24	88.05 ± 22.44	0.113	0.645
Urea	25.63 ± 11.33	25.93 ± 12.13	29.36 ± 9.99	0.05 <	0.05 <	28.53 ± 10.25	28.21 ± 10.73	0.449	0.538
Creatinine	0.78 ± 0.20	0.84 ± 0.30	0.81 ± 0.17	0.05 <	0.05 <	0.80 ± 0.17	0.82 ± 0.22	0.557	0.691
ALT	22.13 ± 10.62	20.62 ± 14.09	17.86 ± 7.81	0.05 <	0.05 <	18.81 ± 8.53	18.73 ± 10.12	0.588	0.393
AST	23.00 ± 8.07	21.38 ± 7.40	20.79 ± 3.97	0.05 <	0.05 <	21.28 ± 5.10	20.98 ± 5.21	0.955	0.365
ALP	70.83 ± 24.66	64.15 ± 16.48	72.13 ± 19.23	0.05 <	0.05 <	71.87 ± 19.96	69.32 ± 18.48	0.229	0.860
GGT	34.50 ± 30.61	20.21 ± 13.46	25.61 ± 15.77	0.05 <	0.05 <	27.58 ± 19.83	23.81 ± 15.10	0.208	0.364
LDH	188.25 ± 30.30	167.86 ± 52.16	194.64 ± 31.05	0.05 <	0.036	193.22 ± 30.57	185.71 ± 40.75	0.038	0.868
Total bilirubin	0.78 ± 0.17	0.76 ± 0.54	0.77 ± 0.73	0.05 <	0.05 <	0.77 ± 0.69	0.76 ± 0.65	0.965	0.972
Direct bilirubin	0.14 ± 0.04	0.23 ± 0.18	0.18 ± 0.13	0.05 <	0.05 <	0.18 ± 0.12	0.20 ± 0.15	0.421	0.563
Total protein	7.42 ± 0.40	7.01 ± 2.07	7.45 ± 0.39	0.05 <	0.05 <	7.44 ± 0.39	7.30 ± 1.23	0.447	0.790
Albumin	4.51 ± 0.30	4.29 ± 1.28	4.51 ± 0.25	0.05 <	0.05 <	4.51 ± 0.26	4.43 ± 0.75	0.322	0.779
Total cholesterol	4.85 ± 0.71	4.45 ± 0.78	4.72 ± 0.79	0.05 <	0.05 <	4.75 ± 0.77	4.64 ± 0.79	0.222	0.473
Triglyceride	1.54 ± 0.78	1.20 ± 0.74	1.27 ± 0.55	0.05 <	0.05 <	1.31 ± 0.59	1.25 ± 0.61	0.571	0.285
HDL-c	1.54 ± 0.46	1.42 ± 0.38	1.28 ± 0.28	0.05 <	0.05 <	1.31 ± 0.30	1.34 ± 0.32	0.417	0.405
LDL-c	3.58 ± 0.83	2.75 ± 0.80	3.31 ± 0.83	0.022	0.040	3.37 ± 0.83	3.14 ± 0.85	0.018	0.154
VLDL-c	0.71 ± 0.36	0.55 ± 0.34	0.58 ± 0.25	0.05 <	0.05 <	0.60 ± 0.27	0.57 ± 0.28	0.571	0.285

The parametric results are shown as mean ± SD or %. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; VLDL-c, very low-density lipoprotein-cholesterol. n, number of individuals. Bold values of p indicate statistical significance. Differences in *FDFT1* genotypes and alleles were assessed by one-way ANOVA and unpaired Student's t-test, respectively.

Table 5
Comparison of biochemical and clinical characteristics among the different genotypes of the *FDFT1* gene, rs2645424 C/T SNPs in the patients with NAFLD.

	FDFT1 gene rs2645424 C/T genotypes			p values			*FDFT1* gene rs2645424 C/T Alleles		p values	
	FDFT1 TT	*FDFT1* CC	*FDFT1* TC	CC vs TT	CC vs TC	TT vs TC	*FDFT1* TAllele	*FDFT1* C Allele	CC vs T	TT vs C
FDFT1	20.06 ± 4.71	18.70 ± 3.73	19.68 ± 3.48	0.05 <	0.05 <	0.05 <	19.83 ± 3.96	19.26 ± 3.58	0.295	0.477
BMI	31.62 ± 4.47	31.52 ± 3.81	32.80 ± 6.99	0.05 <	0.05 <	0.05 <	32.03 ± 6.07	32.25 ± 5.80	0.635	0.724
Waist Circumference	98.86 ± 9.00	103.68 ± 10.00	106.39 ± 9.45	0.05 <	0.05 <	0.024	103.54 ± 9.88	105.17 ± 9.68	0.959	0.036
Hip Circumference	108.36 ± 8.12	111.11 ± 9.85	114.96 ± 13.05	0.05 <	0.05 <	0.05 <	112.46 ± 11.77	113.21 ± 11.74	0.669	0.157
SBP	121.43 ± 16.46	122.63 ± 17.90	123.48 ± 17.99	0.05 <	0.05 <	0.05 <	122.70 ± 17.22	12.31 ± 17.74	0.989	0.758
DBP	78.57 ± 8.64	81.32 ± 9.98	82.17 ± 12.04	0.05 <	0.05 <	0.05 <	80.81 ± 10.90	81.79 ± 11.03	0.867	0.326
HbA1c	6.14 ± 0.76	5.57 ± 0.40	6.00 ± 0.78	0.020	0.039	0.05 <	6.05 ± 0.76	5.81 ± 0.67	0.003	0.133
Insulin	13.99 ± 10.30	15.57 ± 6.18	11.91 ± 7.06	0.05 <	0.05 <	0.05 <	12.70 ± 8.35	13.52 ± 6.86	0.202	0.845
Fasting glucose	99.86 ± 16.69	96.16 ± 12.77	102.42 ± 32.18	0.05 <	0.05 <	0.05 <	101.47 ± 27.26	99.65 ± 25.43	0.425	0.978
Urea	26.07 ± 7.33	29.63 ± 8.04	29.04 ± 6.53	0.05 <	0.05 <	0.05 <	27.95 ± 6.89	29.30 ± 7.15	0.414	0.150
Creatinine	0.89 ± 0.13	0.99 ± 0.14	0.91 ± 0.15	0.0043	0.046	0.05 <	0.90 ± 0.14	0.95 ± 0.15	0.020	0.236
Uric acid	5.31 ± 1.22	5.65 ± 2.13	5.52 ± 1.67	0.05 <	0.05 <	0.05 <	5.44 ± 1.51	5.58 ± 1.87	0.679	0.626
ALT	61.00 ± 44.22	56.89 ± 29.52	50.75 ± 22.50	0.05 <	0.05 <	0.05 <	54.53 ± 32.04	53.47 ± 25.69	0.788	0.434
AST	45.93 ± 33.81	38.84 ± 14.89	38.03 ± 13.47	0.05 <	0.05 <	0.05 <	40.97 ± 22.99	38.42 ± 13.94	0.715	0.432
ALP	79.21 ± 29.05	84.37 ± 30.62	85.21 ± 20.91	0.05 <	0.05 <	0.05 <	83.00 ± 24.02	84.84 ± 25.33	0.854	0.489
GGT	70.57 ± 73.83	45.16 ± 20.65	55.33 ± 44.03	0.05 <	0.05 <	0.05 <	60.95 ± 56.35	50.84 ± 35.64	0.244	0.183
LDH	205.07 ± 56.53	219.21 ± 49.17	206.63 ± 31.34	0.05 <	0.05 <	0.05 <	206.05 ± 41.64	212.19 ± 40.17	0.294	0.606
Total bilirubin	0.65 ± 0.28	0.81 ± 0.45	0.69 ± 0.23	0.05 <	0.05 <	0.05 <	0.67 ± 0.25	0.74 ± 0.35	0.160	0.395
Direct bilirubin	0.11 ± 0.06	0.17 ± 0.12	0.13 ± 0.04	0.038	0.05 <	0.05 <	0.12 ± 0.05	0.15 ± 0.09	0.101	0.160
Total protein	8.10 ± 0.41	8.00 ± 0.33	7.93 ± 0.32	0.05 <	0.05 <	0.05 <	7.99 ± 0.36	7.96 ± 0.03	0.938	0.206
Albumin	4.65 ± 0.27	4.81 ± 0.32	4.64 ± 0.32	0.05 <	0.05 <	0.05 <	4.65 ± 0.30	4.72 ± 0.32	0.064	0.508
HOMA-IR	3.78 ± 3.37	3.55 ± 1.45	3.07 ± 3.32	0.05 <	0.05 <	0.05 <	3.35 ± 3.31	3.29 ± 2.63	0.811	0.586
Microalbumin (mg/day)	14.94 ± 12.32	7.84 ± 10.36	5.82 ± 6.52	0.05 <	0.05 <	0.040	8.72 ± 9.52	6.72 ± 8.33	0.805	0.137
Total cholesterol	5.61 ± 1.79	4.96 ± 1.08	5.25 ± 1.09	0.05 <	0.05 <	0.05 <	5.38 ± 1.38	5.12 ± 1.08	0.247	0.218
Triglyceride	2.49 ± 1.97	2.30 ± 1.64	2.29 ± 2.39	0.05 <	0.05 <	0.05 <	2.37 ± 2.22	2.29 ± 2.07	0.909	0.754
HDL-c	1.24 ± 0.23	1.04 ± 0.18	1.22 ± 0.17	0.004	0.004	0.05 <	1.22 ± 0.19	1.14 ± 0.19	0.001	0.104
LDL-c	3.76 ± 1.22	3.44 ± 0.88	3.54 ± 0.66	0.05 <	0.05 <	0.05 <	3.62 ± 0.90	3.49 ± 0.76	0.470	0.330
VLDL-c	0.82 ± 0.39	0.91 ± 0.40	0.85 ± 0.46	0.05 <	0.05 <	0.05 <	0.84 ± 0.43	0.87 ± 0.43	0.573	0.703

The parametric results are shown as mean ± SD or %. NAFLD, Nonalcoholic Fatty Liver Disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; VLDL-c, very low-density lipoprotein-cholesterol. n, number of individuals. Bold values of p indicate statistical significance. Differences in *FDFT1* genotypes and alleles were assessed by one-way ANOVA and unpaired Student's t-test, respectively.

Table 6
Comparison of clinical characteristics among the different genotypes of the *FDFT1* gene, rs2645424 C/T SNPs in the NAFLD group.

Clinical characteristics		*FDFT1* gene rs2645424 C/T genotypes					
		TT	TC + CC	p value	CC	TC + TT	p value
Steatosis	Mild (I)	8 (38.1)	13 (61.9)	0.07	4 (19.0)	17 (81.0)	0.144
	Moderate-severe (II, III)	6 (16.7)	30 (83.3)		15 (41.7)	21 (58.3)	
Lobular inflammation	(−)	1 (9.1)	10 (90.9)	0.261	3 (27.3)	8 (72.7)	0.735
	(+)	13 (28.3)	33 (71.7)		16 (34.8)	30 (65.2)	
Lobular inflammation	Mild (I)	9 (20.0)	36 (80.0)	0.121	17 (37.8)	28 (62.2)	0.301
	Moderate-severe (II, III)	5 (41.7)	7 (58.3)		2 (16.7)	10 (83.3)	
Ballooning	(−)	3 (30.0)	7 (70.0)	0.694	1 (10.0)	9 (90.0)	0.140
	(+)	11 (23.4)	36 (76.6)		18 (38.3)	29 (61.7)	
Portal inflammation	(−)	8 (25.8)	23 (74.2)	0.812	9 (29.0)	22 (71.0)	0.452
	(+)	6 (23.1)	20 (76.9)		10 (38.5)	16 (61.5)	
Fibrosis	(−)	4 (16.7)	20 (83.3)	0.242	10 (41.7)	14 (58.3)	0.185
	(+)	13 (33.3)	26 (66.7)		10 (25.6)	29 (74.4)	
Simple steatosis/NASH	Simple steatosis	4 (23.5)	13 (76.5)	1.000	4 (23.5)	13 (76.5)	0.370
	NASH	10 (25.0)	30 (75.0)		15 (37.5)	25 (62.5)	
IHC	Mild- Moderate (I, II)	6 (21.4)	22 (78.69	1.000	9 (32.1)	19 (67.9)	1.000
	Severe (III)	2 (18.2)	9 (81.89)		4 (36.4)	7 (63.6)	

The results are shown as "%". NAFLD, Nonalcoholic Fatty Liver Disease; NASH, Nonalcoholic steatohepatitis; IHC: Immunohistochemistry.

level ≥ 15 U/l (p = 0.000), and serum insulin level ≥ 5 μU/ml (p = 0.000), in study group.

This analysis revealed that the NAFLD was associated with *FDFT* levels ≥15 U/l (p = 0.003), fasting insulin levels ≥5μU/ml (p = 0.022), and BMI ≥ 30 kg/m^2 (p = 0.022) (Table 7). The logistic regression analysis confirmed that the *FDFT* levels was associated with NAFLD in this study.

4. Discussion

NAFLD is the most common cause of chronic liver disease in developed countries. Insulin resistance and metabolic syndrome are the risk groups of NAFLD. It is characterized by simple steatosis, lobular inflammation, hepatic injury, hepatocyte ballooning, and NASH (Edelman et al., 2015). The lack of a preventive treatment makes the significance of genetic susceptibility more evident.

NASH is a subset of NAFLD. Hepatic cholesterol accumulation in association with steatosis, inflammatory cell infiltration and hepatocytes ballooning, with fibrosis are hallmarks of NASH (Farrell and Van Rooyen, 2012). In the present study, 16% of patients had simple steatosis while 84% of them had NASH. The *FDFT1* mRNA levels were significantly higher in patients with simple steatosis (p = 0.007) and NASH (p < 0.001) in comparison to controls. In addition to that, *FDFT1* was also found to be significantly correlated with BMI, WC, ALT, and AST levels, total and LDL-c. In the control group, *FDFT* levels increased in individuals with CC genotype, while *FDFT1* gene variations in NAFLD had no significant effect on *FDFT* levels.

The existence of dyslipidemia with NAFLD results in hypertriglyceridemia and leads to an increase in VLDL-c levels and a decrease in HDL-c (Sun et al., 2016). Such alterations are usually followed by an increase in LDL-c concentrations, which is associated with NAFLD and NASH (Barb et al., 2016). Since there is no data regarding the association of normal LDL-c levels with NAFLD, it is still unclear if elevated LDL-c levels are risk factor for NAFLD (Sun et al., 2016). Yet our findings indicate a strong association between *FDFT* and LDL-c levels.

Table 7
Multivariate logistic regression analysis[a].

Independent variables	B	Beta	P value	95% CI for B
FDFT serum levels ≥ 15 U/l	0.289	0.289	0.003	0.105–0.473
Body mass index ≥ 30 kg/m^2	0.173	0.199	0.022	0.026–0.319
Fasting insulin ≥ 5μU/ml	0.289	0.289	0.022	0.243–0.649
Total-cholesterol ≥ 5.18 mmol/l	0.121	0.139	0.093	−0.021–0.262

[a] All NAFLD patients are included (n = 64). Dependent variable: Group (NAFLD).

In the control group, BMI, WC, hip circumference and LDH, LDL-c levels were significantly higher than those carrying the C-allele vs T-allele carriers. In contrast, in the NAFLD group, WC, hip circumference, creatinine, and bilirubin levels were found significantly higher in the C-allele carriers. In NAFLD, T-allele responsible for high levels of HDL-c, HbA1c and microalbumin levels were detected.

In this case, we may suggest C-allele for elevation of WC, hip circumference, and creatinine, bilirubine plasma levels, which could predict NAFLD, rather than C-allele which is responsible for the *FDFT* levels elevation in the control group. However, this equation disrupts HDL-c, HbA1c and microalbumin levels, which are significantly associated with the T-allele in the NAFLD group. In addition to, it is important to remember the relationship between of BMI, WC, hip circumference, and LDH-c levels in the T-allele carriers of the control group.

The *FDFT1* gene, also known as *SQS* is localized to the endoplasmic reticulum membrane and catalyzes the synthesis of squalene from farnesyl pyrophosphate as the first step of cholesterol synthesis (Liu et al., 2014). Recent clinical studies reported that high levels of plasma LDL-c was directly proportional to the *FDFT* serum levels (Do et al., 2009).

In regard to cholesterol metabolism, visceral obesity which is a risk factor for NAFLD, is associated with increased cholesterol synthesis and low cholesterol absorption (Bedogni et al., 2005; Klop and Elte, 2013). This association can be explained by the stimulation of cholesterol synthesis as a result of fatty acid release by visceral fat into the portal vein.

Visceral fat is composed of adipose depots, where adipose tissue itself produces and stores high amounts of squalene (Peltola et al., 2006). In connection with this, many studies reported a correlation between WC and visceral fat (Pouliot et al., 1994; Janssen et al., 2002; Camhi et al., 2011). In corroboration we observed, *FDFT* enzyme levels were significantly associated with WC and BMI. Previous studies reported that *FDFT* serum levels may be associated with visceral obesity by effecting WC (Peltola et al., 2006).

The rs2645424 C/T is an intronic SNPs on chromosome 8 in the *FDFT1* gene (Fukuma et al., 2012). According to our results the allelic and genotypic frequencies of rs2645424 were not significantly different between the study groups. CC genotype was associated with low BMI, WC, hip circumstance, LDL-c in controls. Patients with CC genotype had lower HDL-c than patients with T-allele (CT + TT). The *FDFT1* genotypes were associated neither with triglyceride levels nor with total cholesterol levels.

In another study, the *FDFT1* gene genotype frequencies have shown a significant difference in the prevalence of fibrosis when classified as none/mild (stages 0 - 1) versus moderate/severe (Ballestri et al., 2011).

The *FDFT1* gene protein levels are inversely correlated to cholesterol. The decrease in the *FDFT1* gene activity may result in metabolic with key roles in inflammatory pathways (Tansey and Shechter, 2001). A GWAS performed in a cohort of adult women indicated an association between rs2645424 C/T and NAFLD activity score (NAS) (Chalasani et al., 2010).

Since the rs2645424 C/T is an intronic variant, it does not have an obvious effect on the enzyme activity. It is possible that rs2645424 C/T is in linkage disequilibrium with a promoter variant resulting enhanced expression leading to increased squalene and cholesterol accumulation.

In this study, the fact that the *SQS* enzyme level is higher in the NAFLD group than in the control group supports the studies in the literature. However, it is still unclear how effective *FDFT1* gene variants are at high enzyme levels. This translates our focus into the possible effects of other genes on the *SQS* enzyme level.

In accordance with this previous animal studies have shown that *FDFT1* mRNA overexpression results in increased LDL-c synthesis and higher total cholesterol levels (Marzuillo et al., 2014). Recent studies corroborated this and have shown that intrahepatic cholesterol accumulation plays a significant role in NASH pathogenesis (Santoro et al., 2013).

According to our findings rs2645424 T-allele was associated with low *FDFT* serum levels and high LDL-c in controls. The T-allele may be contributing to the LDL-mediated suppression of squalene synthase (Trapani et al., 2012; Honda et al., 1998). In conclusion, we did not observe an association between the *FDFT1* gene rs2645424 C/T genotypes and NAFLD, but we observed an association between the rs2645424 C/T genotypes and severity of steatosis. Due to the small sample size of this study, our findings require confirmation by studies in different ethnicities with larger sample sizes.

Disclosure statement

The authors declare no conflict of interest.

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