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Is C771G Polymorphism of MLX interacting protein-like (MLXIPL) Gene a Novel Genetic Risk Factor for Non-alcoholic Fatty Liver Disease?

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Abstract

In a recent study, a genome-wide scan has identified C771G (His241Gln) polymorphism of MLX interacting protein like (MLXIPL) gene that is associated with the level of plasma triglycerides. Since, no study has been reported on the association between MLXIPL gene and non-alcoholic fatty liver disease (NAFLD), we aimed to identify a connection between this genetic variation and NAFLD. Two hundred and thirteen patients with NAFLD and 252 healthy controls were entered into this study. MLXIPL genotypes were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Our study showed that the single nucleotide polymorphism (SNP) of MLXIPL is significantly associated with NAFLD. Significant differences between cases and controls were observed for MLXIPL genotype frequencies ($p<0.002$). The frequency of C allele of MLXIPL in patient group was higher than the control group (68.30% vs. 51.59%, respectively; $p<0.05$). C771G polymorphism in the MLXIPL gene potentially plays a significant role in pathophysiology of non-alcoholic fatty liver disease. Further research is needed to confirm this finding.

Key words: MLX interacting protein-like, non-alcoholic fatty liver disease, polymerase chain reaction, polymorphism.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease. The spectrum of NAFLD ranges from simple steatosis through steatohepatitis (NASH) to advanced fibrosis and cirrhosis. A minority of patients with NAFLD progress to end-stage liver disease, which requires liver transplantation or develop hepatocellular carcinoma (1). The observed inter-individual variability in susceptibility to NAFLD and progressive NASH is possibly explained by heritability and genetics (2,3). Studies investigating the ethnic differences in the prevalence of NAFLD, vigorously propose that susceptibility to NAFLD (rather than to its risk factors) may have a genetic component (4,5). It has been shown that mutations in genes related to insulin resistance, lipid metabolism, oxidative stress, inflammation, and telomerase activity may all rise susceptibility to NAFLD development (6). Recent studies have revealed that single nucleotide polymorphisms (SNPs) in patatin-like phospholipase 3 (PNPLA3) (7), microsomal triglyceride transfer protein (MTTP) (8), leptin receptor (9), adiponectin (10), and hepatic Lipase (11) are playing fundamental role in development of NAFLD. Findings from the previous studies have revealed that the SNP localized within the MLXIPL (MLX interacting protein like; ChREBP, carbohydrate response element binding protein) loci is associated with plasma triglycerides (12,13). The most significant association was described for the rs3812316 SNP (C771G, His241Gln); the CC genotype was associated with elevated plasma triglycerides (TGs). The identified SNP is located at the evolutionary conserved domain responsible for glucose dependent activation of MLXIPL. After activation and binding to the MLX, the complex increases the transcription of genes involved, among others, in lipogenesis, and triglyceride synthesis. As elevated TGs are a risk factor for NAFLD development, it is supposed that MLXIPL is a novel genetic risk factor for NAFLD. The present study is the first to demonstrate the association between genetic variation in C771G polymorphism of MLXIPL gene and NAFLD in an Iranian population. Further studies are needed to identify more candidate genes which will undoubtedly be informative, not only for revealing the pathogenesis and prognosis of the disease, but also for suggesting novel treatment targets.

Materials and methods

Study Population

Two hundred and thirteen consecutive unrelated patients (102 males and 111 females) with NAFLD were recruited from Digestive Disease Research Institute (DDRI) of Shariati Hospital of Tehran University of Medical Sciences (TUMS) between 2009 and 2012. Two hundred and fifty-two healthy individuals (122

males and 130 females) with the same demographic background who underwent the annual health check up during the same study period were included in the study as the control group. All healthy controls were subjected to ultrasonographic (USG) examination of abdomen. None of the individuals in the control group had any evidence of fatty change, biochemical abnormalities or features indicative of metabolic syndrome. The inclusion criteria was the age over 19 years with the diagnosis of fatty liver disease. Exclusion criteria were positive viral markers (such as hepatitis B and C), history of alcohol use, diagnosis of autoimmune hepatitis, liver disease associated with drug use, primary biliary cirrhosis, and metabolic liver diseases (such as hemochromatosis and Wilson disease). Written consent was obtained from all the participants in accordance with the procedures approved by the Ethics Committee of Digestive Disease Research Institute (DDRI) of Shariati Hospital of Tehran University of Medical Sciences (TUMS), which was in compliance with the Helsinki declaration.

Liver Biopsies

The diagnosis of NAFLD was confirmed by percutaneous liver biopsy performed in all subjects. Liver biopsy specimens were routinely fixed in 40 g/L formaldehyde (pH=7.4), embedded in paraffin and stained with hematoxylin and eosin, Masson trichrome and silver impregnation for reticular fibers. For the evaluation of the disease severity, we followed the classification of Brunt *et al*. (14). Brunt *et al*. scored a total of 10 findings to develop a grading and staging system for NASH. These included hepatic macrovesicular steatosis, hepatocellular ballooning, intra-acinar inflammation, portal tract inflammation, Mallory's hyaline, acidophil bodies, glycogen nuclei, lipogranulomas, and hepatocellular iron. Each of these was scored separately. Three parameters of hepatic fibrosis were scored: perisinusoidal fibrosis, portal fibrosis and bridging fibrosis. Based on these, the necroinflammatory activity was graded as mild (grade 1, 10%-33% of hepatocytes affected), moderate (grade 2, 34%-66% of hepatocytes affected) and severe (grade 3, >66% of hepatocytes affected). Brunt classification of fibrosis assessment includes five stages: stage 0: no fibrosis; stage 1: zone 3 perisinusoidal or pericellular fibrosis, focally or extensively present; stage 2: zone 3 perisinusoidal or pericellular fibrosis with focal or extensive periportal fibrosis: stage 3: zone 3 perisinusoidal or pericellular fibrosis and portal fibrosis with focal or extensive bridging fibrosis; stage 4: cirrhosis.

Laboratory measurement

Laboratory investigations were performed in the morning, following a not less than 12-h fasting period. Blood samples were collected as follows: fasting clotted samples for lipid profile and liver functions and EDTA blood for DNA analysis. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2) and was used as the index for relative weight. Additionally, waist and hip circumferences were also assessed. Serum insulin, total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), TGs, plasma glucose, and liver function enzymatic tests were measured by standard clinical laboratory techniques. Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) and the homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values.

Gene Polymorphism

Genomic DNA was extracted from peripheral blood leucocytes. MLXIPL C771G polymorphism was detected by polymerase chain reaction (PCR). The template

Table 1. Demographic, Anthropometric, and Clinical Features of Patients with NAFLD and Control Subjects.

Variable	Control subjects	Patient group	P-value
Sex (M/F)	122/130	102/111	>0.05
Age (years)	49.14±4.63	51.22±5.12	>0.05
BMI (kg/m2)	26.39± 0.42	27.11 ± 2.32	>0.05
Total cholesterol (mg/dL)	167±29.62	170±32.41	>0.05
HDL-C(mg/dL)	43.32±9.32	40.52±6.29	>0.05
TGs (mg/dL)	164.14±62.45	272.11±60.29	0.02
LDL-C (mg/dL)	100.71±23.63	103.23±20.81	>0.05
Uric acid (mg/dL)	5.23±1.71	5.34±1.04	>0.05
Glucose (mg/dL)	91.54±9.38	103.18±8.76	>0.05
Homa-R	4.21±1.53	4.32±1.72	>0.05
ALT (IU/mL)	30.13±5.23	100.24±12.67	0.01
AST (IU/L)	29.34±5.32	92.56±9.81	0.02
GGT (IU/L)	36.51 ± 7.2	48.32 ± 6.45	>0.05
ALP (IU/L)	210.17 ± 14.42	218.50 ± 25.67	>0.05
Adiponectin (ng/ml)	37.54±11.36	35.13±13.4	>0.05
Resistin (ng/ml)	3.10±1.43	2.9±1.39	>0.05
Leptin (ng/ml)	38.32± 26.61	35.13± 31.43	>0.05

All data were indicated the mean±SD. $p<0.05$ was considered to be statistically significant.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL-C, high density lipoprotein-cholesterol; HOMA-R, index of insulin resistance; LDL-C, low density lipoprotein-cholesterol; TGs, triglycerides

DNA was amplified by following primers: (forward) 5'ATCCTCAGGCGGCAGCTGCAGGGGA3', and (reverse) 5'AATGGTGCAAACAGCTCTTCTCCA 3'. These primers (0.5 µmol/l of each) were added to a mixture containing 5 µl of 10× reaction buffer (pH8.3), 200 µmol/l (dATP, dCTP, dGTP, dTTP) and 1.0 units of Taq DNA polymerase. 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C and primer extension for 1 minute at 72°C were applied for amplification. PCR products of MLXIPL gene locus were examined by 3% agarose gel electrophoresis and visualized at room temperature under UV after ethidium bromide staining.

Statistical analysis

Student's t-test was used for comparison of age, sex, BMI, and lipid profile in control and patient groups. Genotype frequencies were compared by the Chi-square test. Allele frequencies were determined by the gene counting method, and Hardy-Weinberg equilibrium was tested by the χ^2 test. The statistical analysis was performed using SPSS v 19.0.

Results

The clinical and laboratory measurement of the data in the study groups were shown in Table 1. As it can be seen from the table, significant differences were observed in the parameters of TGs, Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (AST). But, the other factors did not change between patients and control subjects. Table 2 represents the distribution of histopathological findings of biopsy samples of patients. Approximately 50% of patients had the mild forms of Necroinflammatory and Fibrosis, 51.17% and 54%, and the minority of patients had the acute form of the disorders, 15.5% and 5.16%, respectively.

Table 2. The distribution of histopathological findings of biopsy samples of patients.

Necroinflammatory grade	n (%)
Grade 1 (mild)	109 (51.17%)
Grade 2 (moderate)	71 (33.33%)
Grade 3 (severe)	33 (15.5%)
Fibrosis (Stage)	
Stage 0	115 (54%)
Stage 1	45 (21.12%)
Stage 2	30 (14.1%)
Stage 3	12 (5.62%)
Stage 4	11 (5.16%)

Table 3 shows the genotype distribution of the MLXIPL polymorphism in individuals with high and normal TGs. In those with high TGs, the frequency of CC genotype is higher than GG genotype ($p<0.01$) (nearly four times) and such difference was not seen in individuals with normal TGs, in whom the distribution of the genotypes were similar to each other ($p>0.05$). This table shows an odds ratio of 2.20 for C allele in individuals with high TGs. Table 4 indicates the frequency of homozygous and heterozygous genotypes for MLXIPL polymorphism in patients and control subjects. The genotype distributions satisfied the Hardy-Weinberg equilibrium. Significant differences were observed in the frequency of MLXIPL genotypes between controls and NAFLD groups. Patients with NAFLD had a higher frequency of the CC genotype than the control group (49.2% vs. 26.19%, respectively; $p<0.002$). According to the genotype data, the frequency of C allele in the patients was significantly higher than the control groups (68.30% vs. 51.59%, respectively; $p<0.05$) with an odds ratio of 2.02. The distribution of the genotypes of the MLXIPL polymorphism in histopathological findings of patients has been shown in Table 5. No significant differences were observed between distribution of MLXIPL genotypes and the histopathological findings of patients ($p>0.05$).

Discussion

In the present study we examined the distribution of C771G Polymorphism of MLXIPL Gene in NAFLD patients and control subjects and our result revealed that C771G Polymorphism of MLXIPL could be considered as a genetic risk factor for the patients with NAFLD. NAFLD can lead to major complications such as cirrhosis and hepatocellular carcinoma, which are associated with a high rate of mortality unless managed by liver transplantation. Therefore, identification and understanding of novel factors leading to the progression of the disease are important. The pathogenesis and progress of NASH remain unclear, however, the most advocated theory is the "two-hit hypothesis" (15). Briefly, the first hit is the deposition of fatty acid in hepatocytes triggered by different factors, whereas the second hit is the concomitant liver damage induced by oxidative stress and lipid peroxidation. The fact that NASH is observed only in a fraction of patients with NAFLD, suggests genetic predisposition to this disease. In this study, there was a statistical difference between MLXIPL genotypes

Table 3. Genotypes distribution of the MLXIPL rs3812316 (C771G, His241Gln) polymorphism in persons with high and normal TGs.

Triglyceride levels	N	CC n(%)	CG n(%)	GG n(%)	P	Odds ratio (95% confidence interval)
High (>200 mg/dl)	231	117 (50.65%)	83 (35.93%)	31 (13.42%)	<0.01	2.20 (1.69-2.88)
Normal (60-200mg/dl)	234	72 (30.77%)	89 (38.03%)	73 (31.20%)	>0.05	

Table 4. The genotypes and allele distributions of the MLXIPL polymorphisms among controls and patients.

Genotype	Patient group n=213	Control group n=252	P	Odds ratio (95% confidence interval)
CC	105 (49.2%)	66 (26.19%)	p<0.002	
CG	81 (38%)	128 (50.80%)	>0.05	
GG	27 (12.6%)	58 (23.01%)	0.001	
C	291 (68.30%)	260 (51.59%)	<0.05	2.02 (1.55-2.65)
G	135 (31.70%)	244 (48.41%)	>0.05	

Table 5. Genotypes distribution of the MLXIPL rs3812316 (C771G, His241Gln) polymorphism in histopathological findings of patients.

Necroinflammatory grade	CC	CG	GG	p
Grade 1 (mild)	37	34	38	>0.05
Grade 2 (moderate)	23	25	23	>0.05
Grade 3 (severe)	12	10	11	>0.05
Fibrosis (Stage)				
Stage 0	40	37	38	>0.05
Stage 1	14	17	14	>0.05
Stage 2	10	9	11	>0.05
Stage 3	4	3	5	>0.05
Stage 4	4	3	4	>0.05

and TGs level of case and control groups. The distribution of MLXIPL genotypes by TGs levels were similar to frequencies obtained in the previous studies (16-17). However, in a study by Vrablik *et al*., no significant difference was observed between people with high TG and controls, and no links between the MLXIPL variant and plasma TG levels was identified among the controls. (18). Thus, the failure to replicate the previously reported effect of the MLXIPL variant on TG may be partly due to the modest number of subjects with very high TG levels. Plasma levels of TGs are influenced by dietary composition, smoking, body weight, and genetic factors. Similar to the other risk factors, it is estimated that the contribution of genetic and environmental factors on plasma levels of TG is roughly the same. The genetic predisposition to a high level of plasma TG levels has been intensively analyzed. There are polymorphisms in different genes that could have some effect on plasma TG levels (19, 20) and genome-wide scan identifies variation in MLXIPL associated with plasma TGs (12). Increased glucose metabolism has been shown to trigger an independent signaling pathway that transcriptionally activates lipogenic genes, in many cases synergistically with insulin (20, 21). Genes that respond to glucose contain a specific regulatory site, the carbohydrate response element (ChoRE), in their promoter regions. To date, ChoREs have been mapped within the promoter regions of the liver-type pyruvate kinase (PK), S14, fatty acid synthase (FAS), acetyl-CoA carboxylase 1 (ACC), and thioredoxin- interacting protein genes (21, 22). ChREBP is most abundantly expressed in tissues where lipogenesis is highly active, such as the liver. Mice with a disruption of the ChREBP gene or hepatocytes treated with siRNA to reduce ChREBP expression cannot induce lipogenic gene expression in response to carbohydrate (21, 22). In hepatocytes prepared from ChREBP null mice, the induction can be restored by the addition of a ChREBP expression vector (21). Thus, ChREBP is essential for regulating lipogenic gene expression.

Our study showed that MLXIPL variant is associated with NAFLD. A number of studies over the years have implicated genetic predisposition in NAFLD. For example, it was noted that NAFLD appears to have a familial component (2, 23). As data have accumulated, it is obvious that ethnic differences play a role in susceptibility to NAFLD, especially progressive NAFLD, which cannot be explained simply on the basis of diet or socioeconomic differences. Other groups have examined SNP variants in candidate genes chosen for their known implication in regulation of lipid metabolism or relationship with risk factors for NAFLD (24). To our knowledge, this is the first study that examines the relationship between C771G polymorphism in the MLXIPL gene and NAFLD. Since it is assumed that MLXIPL is essential for regulating lipogenic gene expression and we found a difference not only between MLXIPL genetic variation and TG levels, but also between this gene and NAFLD. Therefore, it seems reasonable that this gene might be a novel genetic risk factor for NAFLD. The present study indicates that the C771G polymorphism in the MLXIPL gene is not associated with histopathological findings in NAFLD. The etiology of NAFLD and its progression are complex and remains incompletely understood. Many cases are related to a "Western lifestyle," i.e., abundance of nutrients coupled with a sedentary lifestyle; however, it is likely that the

genetic predisposition plays an important, if not decisive, in determining which individuals are at increased risk for development of NAFLD and for its progression. In this study, we assessed only one factor in the pathogenesis of this disease i.e. the polymorphism of a single related gene that is one of the several factors involved in this complex disease. As there are different underlying factors leading to pathogenesis of this disease as well as its severity, we suggest that MLXIPL CC genotype is not the only molecular change associated with NAFLD. Probably, other more important factors, rather than polymorphism, are involved in determining the severity of the disease. Thus, further studies are needed to confirm our results and also identify the other risk factors involved in pathogenesis and severity of NAFLD.

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