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TNF-alpha Genes Polymorphisms As A Markers In Non-Alcoholic Fatty Liver Disease Patients In Sohag Governorate

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Abstract

Background: Tumor necrosis factor-α (TNF-α) polymorphism has been suggested to play an important role in the pathogenesis of non alcoholic fatty liver disease (NFALD), and known to be a mediator of insulin resistance. **Aim of the work**: to investigate the influence of G 308 A (allele TNFA) & G 238 A (allele TNF2) TNF -α polymorphisms and its relationship with insulin resistance. **Patient and Method:** Sixty patient with NAFLD AND 30 controls were tested by homeostatic metabolic assessment insulin resistance (HOMA-IR),serum level of Human Insulin and TNF -α assay by ELISA kit and TNF -α polymorphism by polymerase chain reaction (PCR) analysis **Result:** Insulin resistance was detected in all patient and was highly significant in NAFLD in comparison with control (4.32±1.96 vs 1.75±0.48) respectively,there is significant value In TNF -α 238 allels polymorphism in NAFLD in comparison with control (P=0.048)but not in 308 gene , and Multivariate regression analysis showed that the only independent risk factors for development of NAFLD included higher body mass index (BMI) and higher HOMA-IR index. **Conclusions:** we conclude that TNF -α polymorphism could represent a susceptibility genotype for insulin resistance and NAFLD.

Keyword: Insulin resistance (IR), tumor necrosis factor-alpha(TNF -α) ,gene polymorphisms, Non alcoholic fatty liver disease(NAFLD),polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP),body mass index (BMI),) non-alcoholic steatohepatitis (NASH).

Introduction

Non-alcoholic fatty liver disease (NAFLD) has emerged as the most common form of chronic liver disease. The spectrum of NAFLD ranges from simple steatosis through non-alcoholic steatohepatitis (NASH) to advanced fibrosis and cirrhosis, and the minority of patients progress to end-stage liver disease requiring liver transplantation or develop hepatocellular carcinoma (1). However, the vast majority of patients only have simple steatosis with a benign long-term prognosis.

It has been observed that even when considering patients with similar environmental and metabolic NAFLD risk factors (diet, exercise, obesity and insulin resistance being the most important factors), they still differ largely in terms of disease phenotype and degree of progression (2).

There is a slight or no production of cytokines in the liver, under physiological conditions. However, pathological stimuli, such as lipid gathering, trigger the production of inflammatory molecules by hepatic cells. The active role that cytokines might play lies in the evolution of NAFLD by stimulating hepatic inflammation, cell necrosis and apoptosis, as well as by inducing fibrosis (3).

Tumor necrosis factor alpha (TNF- α) is an adipokine produced by multiple types of inflammatory cells, such as monocyte /macrophages, neutrophils and T - cells, as well as by several types of tissue, like the endothelium, adipose tissue or neuronal tissue. TNF-α is produced either directly, by hepatocytes and Kupffer cells, or indirectly, by abdominal fat, in the liver (4). There have been reports that TNF-α is highly implicated in the evolution of NAFLD and NASH in both humans and animals. The connection between TNF-α expression and insulin resistance in NASH was first reported in a study conducted by Hotamisligil et al. (5).

TNF-α polymorphisms have been reported to influence the susceptibility to different hepatic diseases, including alcoholic steatohepatitis (6) and increasing evidence suggests that TNF-α is involved in the pathogenesis and progression of liver diseases of different etiology (7,8) Data from experimental and clinical studies indicate that this cytokine plays a role not only in the early stages of fatty liver disease but also in the transition to more advanced stages of liver damage (9).Two polymorphisms in the TNF-α promoter have been identified: one at position 308 (TNF2 allele) and another at position 238 (TNFA allele) (10). Studies with the TNF-α promoter have shown that the TNF2 allele leads to increased constitutive and inducible expression compared with the wild type (TNF-1)(11,12); conflicting data have been reported on the TNFA allele, (6,13) but most investigators believe that TNFA allele increases the release of this cytokine.

Aim of the work:

Determine gene polymorphism in TNF-α and its levels in diagnosis of non alcoholic fatty liver disease patients to prospect a new strategy of treatment

Patients and Methods :

Our study was conducted on 60 patients (21 males, 39 females) with NAFLD. Their ages ranged from 25-55 years. All patients were referred to the Departments of Tropical Medicine and Gastroenterology, Sohag University Hospital (in the period from June 2011 to June 2014). All patients were Egyptian, residing in sohag and unrelated to each other. The control group was include 30 healthy subjects of the same geographical

Origin, This group was formed by enrolling volunteers from hospital staff, medical students, and acquired relatives of the patients. The mean age of the

control group was 25-56 years (14 males, 16 females). None of the subjects referred alcohol abuse or had a BMI above 25.

Before inclusion in the study, all participants gave informed consents and the study was approved by the Local Ethics Committee.

- ***Exclusion criteria:***

Presence of hepatitis B Virus (HBV), hepatitis C Virus (HCV) infection and cirrhotic changes.

- ***History taking:***

Complete medical history including presence or absence of fever, fatigue, flatulent dyspepsia, abdominal pain, yellowish discoloration of the sclera, change in the color of urine and feces, swelling of legs or abdomen and bleeding from nose or gums or alimentary tract were recorded. Past history of any liver diseases. Family history of liver disease was also reported.

- ***Clinical examination:***

Full clinical examination including calculation of BMI, looking for signs of liver diseases other than NAFLD as fever, jaundice, pigmentation, purpura, finger clubbing, white nails, vascular spiders, palmer erythema, gynecomastia, testicular atrophy, distribution of body hair and peripheral edema was performed. Abdominal examination for enlarged liver and spleen, ascites and abdominal wall veins was also done.

Methods:

Blood samples were collected by venipuncture in the fasting state with adequate precautions. From each participant, 10 mL of fasting blood was divided into two portions, the whole blood was collected in sterile EDTA containing tubes for DNA extraction, and the rest was left for 30-60 minutes for spontaneous

clotting at room temperature then centrifuged at 3000 rpm for 10 minutes. Serum samples were separated into another set of tubes and kept frozen at -80°C until assay.

Laboratory investigation:

1-Routin examination: Hepatitis markers: hepatitis B surface antigen (HBs Ag) and hepatitis C virus antibody (HCV Ab).Liver function tests**:** serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, and prothrombin time and concentration and Fasting serum glucose**.**

2-Fasting insulin level: was measured by Enzyme-Linked Immunosorbent Assay (ELISA) as Quantitative Determination of Insulin Concentrations in Human Serum (Immunospec No. E29-072, Immunospec corporation, ELISA kit,7018 Owensmouth Ave. Suite 103, Canoga Park, CA, 91303, USA), From which HOMA-IR was calculated. HOMA-IR= fasting blood glucose [mmol/L] X fasting blood insulin [mIU/L] / 22.5. (IR was defined as HOMA-IR > 2)

3-Serum TNF-α:was measured by ELISA kit designed for detection of TNF-***α*** in human plasma (Assaypro LLC No. ET2010-1)

4-Genetic polymorphisms

Genomic DNA was extracted from whole heparinized blood samples, using (CinnaPure DNA Cat No. PR881612. provided by SinaClon,Tehran). TNF-α genotyping was conducted using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). PCR amplification was carried out in a total volume o of 50 μ L. The reaction mixture contained 1.25 pmol of each primer for the polymorphism of TNF- promoter at position -238 we used the following primers: sense primer 5'-AGACCCCCCTCGGAACC-3' and antisense primer 5'-ATCTGGAGGAAGCGGTAGTG-3'while for the -308 TNF- polymorphism we

used the sense primer 5'-AGGCAATAGGTTTTGAGGGCCAT(forward) and ACACTCCCCATCCTCCCTGCT(reverse)-3 in a final volume 250 μM dNTP, 2.5 mM MgCl2, 10× buffer, 0.15 unit Taq polymerase, and the genomic DNA. The PCR cycling conditions were denaturation at 94oC for 30 s, annealing at 65°C for 30 s, and extension at 72oC for 30 s. For genotyping of TNF-α promoter polymorphisms, a reaction mixture was prepared containing the PCR products, RFLP technique was accomplished to detect TNF-α polymorphism using a restriction endonuclease enzyme Mspl and Ncol (provided by JenoBioscience) were used to detect the polymorphisms -238and -308 respectively, which recognizes a short specific DNA sequence to cleave double stranded DNA at specific site within the recognition sequence.. Restricted products were electrophoresed on 3% agarose gel stained with ethidium bromide at 100v for 45 min and visualized under UV.

Statistical analysis:

Statistical package for social sciences (IBM-SPSS), version 19 IBM-Chicago, USA was used for statistical data analysis. Data expressed as mean, standard deviation (SD), number and percentage. Mean and standard deviation were used as descriptive value for quantitative data.Student t test was used to compare the means between two groups, and one-way analysis of variance (ANOVA) test was used to compare means of more than two groups.Number and percentages were used to describe qualitative data. Chi square was used to compare percentages between cases and controls.For all these tests, the level of significance (P-value) is considered significant if $p<0.05$.Multivariate and univariate regression analysis were used to predict the risk factors for NAFLD.

Results

Demographic and clinical characteristics data of 60 patients with and 30 controls as healthy subjects are shown in table (1).

Table (1): Demographic and clinical characteristics of NAFLD and controls.

characteristics	NAFLD	Controls	P Value
Age(year)	42.10±8.72	41.63±8.41	0.809(NS)
Sex **Female** **male**	 39 21	 16 14	0.285(NS)
BMI(kg/m^2)	30.70±31.0	26.46±26	P<0.001***
ALT(Iu/L)	46.25±25.25	44.500±25.96	0.761(NS)
AST(Iu/L)	40.23±17.73	45.30±22.62	0.248 (NS)
Total Cholesterol(mg/dl)	200.58±49.22	164.86±30.74	P<0.001***
Fasting blood glucose(mmol/L)	6.10±1.41	4.70±0.90	P<0.001***
Fasting Insulin(mIU/mL)	15.83±5.85	8.32±1.28	P<0.001***
HOMA-IR%	4.32±1.96	1.75±0.48	P<0.001***
TNF α(pg/mL))	21.50±5.09	16.25±4.8	P<0.001***

*** =Highly significant NS= Non significant

Table 2 it represents that there is no differences were observed in the TNF- α polymorphisms at the -308 locus between the Controls, but there is a significant difference in TNF- α polymorphisms at the -238 locus.

Table (2): shows the frequencies of TNF-α polymorphisms at -308 and -238 in NAFLD and controls

TNFα polymorphism			Group: NAFLD cases	Group: Controls	Total	Chi square	C.I.	P value
- 308 locus	GG genotype	Count	29	14	43	0.024	0.902-1.000	0.988 (NS)
		%	48.3%	46.7%	47.8%			
	GA genotype	Count	27	14	41			
		%	45.0%	46.7%	45.6%			
	AA genotype	Count	4	2	6			
		%	6.7%	6.7%	6.7%			
	G allele frequency		70.8%	70%	70.6%			
	A allele frequency		29.2%	30%	29.4%			
- 238 locus	GG genotype	Count	15	14	29	6.073	0.045-0.054	0.048 (S)
		%	25%	46.7%	32.2%			
	GA genotype	Count	35	15	50			
		%	58.33%	50%	55.6%			
	AA genotype	Count	10	1	11			
		%	16.7%	3.3%	12.2%			
	G allele frequency		54.2%	71.7%	60%			
	A allele frequency		45.8%	28.3%	40%			
Total		Count	60	30	90			
		%	100.0%	100.0%	100.0%			

Regression analysis is used to detect the factors which can be used to "predict" certain outcome. Here, we used univariate regression analysis to detect the predictive factors for cases (patients with NAFLD) from controls (healthy non NAFLD subjetcs). All possible variables were introduced into a univariate binary logistic regression analysis. The factors which showed non significant effect were excluded from the subsequent analysis. Factors which showed significant effect by univariate regression were then introduced into multivariate regression analysis (table 3).

Univariate regression analysis showed that the possible risk factors for the prediction of NAFLD development included high BMI, high serum cholesterol, high serum fasting blood sugar, higher fasting serum insulin, higher HOMA-IR index. Multivariate regression analysis showed that the only independent risk factors for development of NAFLD included higher BMI and higher HOMA-IR index (table 4).

Table (3):Univariate analysis of factors associated with significant in NAFLD

	Sig.	Odd's ratio	95% C.I.for Odd's	
			Lower	Upper
Age	0.807	0.994	0.944	1.046
Female sex	0.286	1.625	0.666	3.966
BMI	**<0.001**	1.562	1.277	1.911
ALT	0.758	1.003	0.985	1.021
AST	0.249	0.987	0.966	1.009
Cholesterol	**0.001**	1.020	1.008	1.032
FBS	**<0.001**	1.057	1.028	1.087
Insulin	**<0.001**	2.604	1.644	4.124
HOMA-IR	**<0.001**	11.619	3.785	35.670
TNF-α level	0.322	1.049	0.954	1.153

Table 4. Multivariate Binary Logistic Regression Analysis

	Sig.	Odd's ratio	95% C.I.for Odd's	
			Lower	Upper
BMI	**0.007**	1.559	1.130	2.151
Cholesterol	0.058	1.026	0.999	1.053
FBS	0.388	0.883	0.666	1.171
Insulin	0.784	0.668	0.037	11.934
HOMA-IR	**0.031**	4.457	0.002	9.727

Discussion

Worldwide, the presumed prevalence of nonalcoholic fatty liver disease (NAFLD) in the general population is 6-33%, with a median of 20%; sonographically the prevalence rate varied between 17% and 46%,1 thereby possibly being the most common cause of chronic liver disease (14).

Insulin resistance is crucial in the pathogenesis of the metabolic syndrome, of which NAFLD is considered as the hepatic component. Insulin resistance appears to have a crucial role in the pathogenesis of NAFLD and NASH (15). Besides the suggested role of insulin resistance in the development of steatosis, hepatic insulin resistance could promote hepatocyte injury and inflammation (16). Gut flora and gut derived endotoxemia are considered main factors in developing insulin resistance. The key link is represented by the lipopolysaccharide–toll like receptor 4 (TLR4)– monocyte differentiation antigen CD14 system (17,18,19,20).

IR determined by the homeostasis model assessment (HOMA) is widely used in clinical and epidemiological studies, particut al.,larly with large patient populations (21,22,23). We observed significant increase of fasting blood glucose and fasting serum insulin level in NAFLD patients in comparison with control (Table 1).

Insulin usually controls fuel homeostasis through the glucose uptake into peripheral tissues and by suppressing the release of stored lipids from adipose tissues (24). Insulin activates lipoprotein lipase that promotes the catabolism of triglyceride-rich lipoproteins, such as chylomicrons and VLDL, and the clearance of LDL. Insulin also affect HDL metabolism through LCAT activation (25). In agreement with our result that ther is significant elevated cholesterol in NAFLD patients compared to the normal subjects in this study, strengthening the association between overweight and the appearance of NAFLD (26,27).

TNF-α is a pro-inflammatory cytokine and is associated with Steatohepatitis(28,29,30) The association may be causal or secondary. On one hand, patients with a particular genetic make-up may have unfavorable adipokine profiles and are more prone to develop NAFLD. On the other hand, the adipokine profile may be just a global manifestation of insulin resistance without any Causal role.

TNF-α also increases with increasing fatty mass, because, when adipose tissue is expanding, it is infiltrated by more macrophages; subsequently macrophage-induced increased TNF-α enhances IR, fibrogenesis and apoptosis. Nevertheless, increased TNF-α simultaneously induces cell-protective mechanisms in hepatocytes by enhancing anti-apoptotic and regenerative mechanisms (31). It seems that various adipokines are in balance, when fatty mass is limited, but if fatty mass exceeds a limit, this balance tends to be lost. Compensatory adipokine alterations may be initially efficient to preserve insulin sensitivity and deter steatosis or the progression to NASH; however, by further expanding of adipose tissue, adipokine alterations may be more detrimental than beneficial for NAFLD (14).

Pro-inflammatory cytokines TNF-α level were elevated significantly in NAFLD patients with severity of the disease compared to the normal subjects in this study. TNF-α is known to activate intracellular signaling molecules, including stress related kinases such as Jun N-terminal kinase and inhibitor kappa beta kinase beta, that make cells resistant to the actions of insulin (32).

Evidence suggests that the cellular mechanisms for insulin resistance also activate TNF-α production (33). Raised serum TNF-α level has also been demonstrated in several studies of fatty liver disease (34). TNF-α activates harmful proatherogenic pathways partially through the reduction of HDLcholesterol, elevated expression of cholesterogenic genes, accompanied by an increase in potentially harmful precholesterol metabolites, and suppression of cholesterol elimination(35). TNF-α can induce both hepatocyte cell death and hepatocyte proliferation (31), and is critically involved in the pathogenesis of liver fibrosis in NASH model (36).

TNF-α gene is located in chromosome 6p21.3 and is polymorphic at several positions including -G308A, -G238A, -T1031C, -C863A, -C851T, -G419C, and -G376A (37). Among these single nucleotide polymorphisms (SNPs), -G308A and -G238A lie within the promoter, and previous studies have shown that the -308, and possibly the -238, alleles are associated with the increased production of TNF-α in various inflammatory diseases (38). Since TNF-α polymorphism was first suggested to be associated with the Pathogenesis of alcoholic liver disease (6) several studies have been published supporting the hypothesis that TNF-α polymorphism might also be associated with the pathogenesis and progression of NAFLD (39,40).

G to A polymorphic sequence at position -308 (TNF1 to TNF2 allele) and -238 (TNFG to TNFA allele) in TNF-α gene promoter region has been shown to influence TNF-α gene expression and may induce IR (12) . Moreover, these polymorphisms have been involved in the pathogenesis and progression of NASH (29). Valenti et al. (38) reported that italian NAFLD patients have a high frequency of G-238A, but not of G-308A TNF-α polymorphisms in comparison with controls and this is agree with our result as shown in table 2. In addition, these polymorphisms were associated with IR. Another report by Tokushige et al. (39) found that there were no significant differences in the genotype frequency of any of the five TNF-α polymorphisms (T-1031C, C-863A, C-857T, G-308A and G-238A) analyzed among Japanese NAFLD patients compared with healthy controls; however, the polymorphisms T-1031C and C-863A were associated with IR.

In conclusion, our results indicate that insulin resistance is present in NAFLD, and the prevalence of the 238 TNF-α polymorphisms (TNFA allele) is higher in patients of NAFLD than controls. Also the TNFα polymorphisms are associated with insulin resistance.

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