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Influence of ala54thr polymorphism of fatty acid-binding protein 2 on histological alterations and insulin resistance of non alcoholic fatty liver disease

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Abstract. – A transition G to A at codon 54 of fatty acid binding protein type 2 (FABP2) produces an amino acid substitution (Ala 54 to Thr 54). This amino acid substitution was associated with modifications of insulin resistance, adipokines and insulin concentrations. The aim of this study was to evaluate the influence of Ala54Thr polymorphism in the FABP2 gene on the histological alterations of non-alcoholic fatty liver disease (NAFLD) and insulin resistance.

Thirty subjects with the presence of biopsy-proven NAFLD were enrolled for this study. Glucose, Insulin, Insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, resistin, leptin, adiponectin, interleukin-6 and TNF-alfa serum levels were measured at basal time. A tetrapolar bioimpedance, BMI, waist circumference, waist to hip ratio, blood pressure and a prospective serial assessment of nutritional intake with 3 days written food records were examined. Genotype of Ala54Thr FABP2 gene polymorphism was studied.

The mean age was 41.6±11 years and the mean BMI 29.2±6.6 with 24 males (80%) and 6 females (20%). Fifteen patients (50%) had the genotype Ala54/Ala54 (wild type group) and 15 (50%) patients Ala54/Thr54 (13 patients) or Thr54/Thr54 (2 patients) (mutant type group). Both genotype groups have the similar anthropometric parameters. Serum aspartate aminotransferase and alcaline phosfatase were higher in wild type group than mutant type group, with an unclear explanation. Dietary intake was similar in both groups. A non-statistical significant low levels of adiponectin in mutant group was observed. No differences were detected among other adipokines. There were no differences between genotypes in histological results of inflammation (portal or lobular inflammation) or grade of steatosis or fibrosis.

In conclusion, the present study demonstrates that the polymorphism Ala54Thr of FABP in patients with NAFLD doesn't predict liver histological changes, nor both insulin resistance and serum adipokines variations.

Key Words:

Adipokines, Histology, FABP, NAFLD.

Introduction

Non-alcoholic fatty liver disease (NAFLD) includes a wide spectrum of hepatic alterations of metabolic origin, significantly associated with the metabolic syndrome (MS) and its individual features[1]. The clinical-histological spectrum of NAFLD includes non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH)[2]. The progression of steatosis to steatohepatitis is associated with increasing oxidative stress within hepatocytes. NASH can progress to cirrhosis in 15-20% of subjects[3]. NAFLD is emerging as a common cause of chronic liver disease in Western countries. Steatohepatitis is present in 18.5% of markedly obese patients and 2.7% of lean patients[4].

Hyperinsulinemia and hyperglycaemia, related with adipose tissue metabolism, promote de novo lipogenesis, and in turn both hepatic triglyceride accumulation and high circulating free fatty acid (FFA) levels contribute to hepatic and peripheral insulin resistance[5]. Hepatic lipid accumulation is not the sole factor responsible for hepatocellular

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injury. Increase hepatic FFA oxidation can generate oxygen radicals promoting lipid peroxidation, cytokine secretion and mitochondrial dysfunction. Hepatocytes handle the increase FFA load by increasing FFA beta-oxidation, thus contributing to generation of reactive oxygen species with subsequent cytokine induction (i.e. TNF-alfa) that eventually leads to mitochondrial dysfunction[6].

Adipose tissue secretes several bioactive proteins, or adipokines, that regulate hepatic and peripheral glucose and lipid metabolism. These adipokines include leptin, tumor necrosis factor alfa (TNF-alfa), resistin, and adiponectin. Recently, Hui et al[6] suggested that raised serum leptin levels in NASH may be a reflection of the failure of leptin to stimulate hepatic lipid turnover that, is hepatic leptin resistance. Reduced adiponectin level is associated with more extensive liver necroinflammation and may contribute to the development of necroinflammatory forms of NAFLD[6].

Some proteins have been related with fat liver store, such as fatty binding protein (FABP). FABP regulates murine hepatic fatty acid trafficking in response to fasting. FABP may function as a metabolic sensor in regulating lipid homeostasis[7]. These Authors consider that knock-out FABP mice are protected against Western diet-induced obesity and hepatic steatosis through a series of adaptations in both hepatic and extra hepatic energy substrate use[7]. In 1995, Baier et al[8] reported a new G/A mutation in FABP. A transition G to A at codon 54 of FABP2 results in an amino acid substitution (Ala 54 to Thr 54). This polymorphism is common, with a Thr54 allelic frequency of 30% in most populations. This amino acid substitution was associated with high insulin resistance, and fasting insulin concentrations[8,9].

The aim of this study was to evaluate the influence of Ala54Thr polymorphism in the FABP2 gene on the histological alterations of NAFLD and its relationship with insulin resistance and serum adipokines.

Patients and Methods

Study Population

Consecutive 30 patients seen in the Clinic Hospital at the Valladolid, Spain, between 2004 and 2007, were enrolled for this study. The inclusion criterion was the presence of liver biopsy-proven NAFLD and a history of either no alcohol consumption or consumption of <20 g/day on the average. Exclusion criteria were hepatitis B and C, hemochromatosis (iron panel and gene test for those with an iron saturation >50%), Wilson disease, autoimmune hepatitis, and alfa-1-antitrypsin deficiency.

Liver Biopsies

The diagnosis of NAFLD was confirmed by percutaneous liver biopsy performed in all subjects with a 1.6-mm Menghini needle. Liver samples were routinely processed, sectioned, and stained with haematoxylin-eosin and Manson's trichrome. All biopsies were examined by the same liver pathologist (T.A.G.) using the Brunt classification[10]. Steatosis was graded as follows: mild-moderate (<66% of hepatocytes affected); severe (≥66% of hepatocytes affected). The Brunt system also includes as grading: portal inflammation, ballooning, lobular inflammation and staging fibrosis: stage 1: zone 3 perivenular perisinusoidal/pericellular fibrosis, focal or extensive; stage 2: as above with focal or extensive periportal fibrosis; stage 3: bridging fibrosis, focal or extensive; stage 4: cirrhosis. In our study, stage was divided as absent or presence of fibrosis.

Procedures

Glucose, C-reactive protein (CRP), insulin, Insulin resistance (HOMA), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, resistin, leptin, adiponectin, interleukin-6 and TNF-alfa serum samples were measured at basal time. A tetrapolar bioimpedance, weight, BMI, blood pressure and a prospective serial assessment of nutritional intake with 3 days written food records were realized. Genotype of Ala54Thr FABP2 gene polymorphism was studied.

Genotyping of FABP2 Gene Polymorphism

Oligonucleotide primers and probes were designed with the Beacon Designer 4.0 (Premier Biosoft International®, Los Angeles, CA, USA). The polymerase chain reaction (PCR) was carried out with 50 ng of genomic DNA, 0.5 μL of each oligonucleotide primer (primer forward: 5'-CAG TTC CGT CTG CTA GAT TGT-3'; primer reverse: 5'-GCT GAC AAT TAC ACA AGA AGG AA-3'), and 0.25 μL of each probes

(wild probe: 5'-Fam-CAA AGA ATC AAG CAC TTT TCG AAA CA-BHQ-1-3') and (mutant probe: 5'-Hex-AGA ATC AAG CGC TTT TCG AAA CA-BHQ-1-3') in a 25 µL final volume (Termociclador iCycler IQ (Bio-Rad®), Hercules, CA). DNA was denaturated at 95ºC for 3 min; this was followed by 50 cycles of denaturation at 95ºC for 15 s, and annealing at 59.3º for 45 s). The PCR were run in a 25 µL final volume containing 12.5 µL of IQTM Supermix (Bio-Rad®, Hercules, CA) with hot start Taq DNA polymerase. Hardy Weimberger equilibrium was assessed.

Assays

Serum total cholesterol and triglyceride concentrations were determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y.), while HDL cholesterol was determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulphate-magnesium. LDL cholesterol was calculated using Friedewald formula. Serum Alanine amino transferase and aspartate aminotransferase activity were determined by enzymatic colorimetric assay Hitachi 917 (Roche Diagnostics, Geneve, Switzerland).

Plasma glucose levels were determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, CA). Insulin was measured by RIA (RIA Diagnostic Corporation, Los Angeles, CA) with a sensitivity of 0.5 mUI/L (normal range 0.5-30 mUI/L)[11]. The homeostasis model assessment for insulin sensitivity (HOMA) was calculated using these values[12].

Adipocytokines

Resistin was measured by ELISA (Biovendor Laboratory, Inc., Brno, Czech Republic) with a sensitivity of 0.2 ng/ml with a normal range of 4-12 ng/ml[13]. Leptin was measured by ELISA (Diagnostic Systems Laboratories, Inc., TX) with a sensitivity of 0.05 ng/ml and a normal range of 10-100 ng/ml[14]. Adiponectin was measured by ELISA (R&D systems, Inc., Minneapolis, MN) with a sensitivity of 0.246 ng/ml and a normal range of 8.65-21.43 ng/ml[15]. Interleukin 6 and TNF alpha were measured by ELISA (R&D systems, Inc., Minneapolis, MN) with a sensitivity of 0.7 pg/ml and 0.5 pg/ml, respectively. Normal values of IL6 was (1.12-12.5 pg/ml) and TNF-alpha (0.5-15.6 pg/ml)[16,17].

Anthropometric Measurements

Body weight was measured to an accuracy of 0.5 Kg and BMI computed as body weight/(height^2). Waist (narrowest diameter between xiphoid process and iliac crest) and hip (widest diameter over greater trochanters) circumferences to derive waist-to hip ratio (WHR) were measured, too. Tetrapolar body electrical bioimpedance was used to determine body composition[18]. An electric current of 0.8 mA and 50 kHz was produced by a calibrated signal generator (Biodynamics Model 310e, Seattle, WA) and applied to the skin using adhesive electrodes placed on right-side limbs. Resistance and reactance were used to calculate total body water, fat and fat-free mass.

Blood pressure was measured twice after a 10 minutes rest with a random zero mercury sphygmomanometer, and averaged.

Dietary Intake and Habits

To control the influence of dietary intake patients received prospective serial assessment of nutritional intake with 3 days written food records. All enrolled subjects received instruction to record their daily dietary intake for three days including a weekend day. Handling of the dietary data was by means of a personal computer equipped with personal software, incorporating use of food scales and models to enhance portion size accuracy. Records were reviewed by a registered dietitian and analyzed with a computer-based data evaluation system. National composition food tables were used as reference[19].

Statistical Analyses

Sample size was calculated to detect differences over 5% of frequencies in histological categories of liver disease (n=30). The results were expressed as average ± SD. The distribution of variables was analyzed with Kolmogorov-Smirnov test. Quantitative variables with normal distribution were analyzed with a two-tailed Student's-t test. Non-parametric variables were analyzed with the U-Mann-Whitney test. Qualitative variables were analyzed with the chi-square test, with Yates correction as necessary, and Fisher's test. The statistical analysis was performed for the combined Ala54/Thr54 and Thr54/Thr54 as a mutant group and wild type Ala54/Ala54 as second group. A p-value under 0.05 was considered statistically significant.

Table I. Anthropometric variables (mean ± SD).

Characteristics	Ala54/Ala54	(Ala54/Thr54 or Thr54/Thr54)	
	(n = 15)	(n = 15)	p
BMI	30 ± 3.2	29.3 ± 5.6	NS
Weight (kg)	84.8 ± 10.2	84.3 ± 12.5	NS
Fat mass (kg)	25.2 ± 7.7	24.1 ± 9.6	NS
WC (cm)	97.4 ± 9.2	97.2 ± 10.7	NS
Waist to hip ratio	0.91 ± 0.1	0.9 ± 0.04	NS

WC: Waist circumference.
(*) $p<0.05$, in each group with basal values.

Results

Thirthy patients gave informed consent and were enrolled in the study. The mean age was 41.6±11 yrs and the mean BMI 29.2±6.6 with 24 males (80%) and 6 females (20%). Fifteen patients (50%) had the genotype *Ala54/Ala54* (wild type group) and 15 (50%) patients *Ala54/Thr54* (13 patients) or *Thr54/Thr54* (2 patients) (mutant type group). Age was similar in both groups (43.2±13.8 vs 42.7±6.1:ns). Sex distribution was similar in both groups, males (11 wild type group vs 13 mutant type group) and females (4 wild type group and 2 mutant type group).

Table I shows the anthropometric variables. No differences were detected among these variables.

As show in Table II, serum alkaline phosfatase and serum aspartate aminotransferase were higher in wild type group than mutant type group. No differences were detected in other biochemical parameters between groups.

Table III shows nutritional intake with 3 days written food records. No statistical differences were detected in caloric, carbohydrate, fat, and protein intakes.

Table IV shows levels of serum adipokines. In mutant group serum, adiponectin levels were lower than wild group, without statistical differences. No differences were detected among other adipokines.

Table V shows frequencies of histological changes in liver biopsy without differences between genotypes. There were no differences in serum adipokines levels and insulin resistance among histological categories of Table V (data not shown).

Table II. Basal serum biochemical parameters of the studied patients (mean ± SD).

Characteristics	Ala54/Ala54	(Ala54/Thr54 or Thr54/Thr54
	(n = 15)	(n = 15)
Glucose (mg/dl)	102.7 ± 20.2	101.4 ± 26
Total Ch. (mg/dl)	218.2 ± 53	222.3.5 ± 38
LDL-Ch. (mg/dl)	141.1 ± 55	139.6 ± 29
HDL-Ch. (mg/dl)	64.2 ± 40	51.6 ± 14.3
TG (mg/dl)	142.9 ± 61.9	128.5 ± 58
Insulin (mUI/L)	13.6 ± 5.9	13.3 ± 8.9
HOMA	3.65± 2.2	3.4 ± 2.5
ALT (IU/L)	96.2 ± 44.5	87.2 ± 46.1
AST (IU/L)	60.7 ± 36.3	44.3 ± 14*
GGT (IU/L)	78.5 ± 60.5	74.2 ± 54.3
AP (IU/L)	72.3 ± 10.8	103.3 ± 53.4*

Ch: Cholesterol. TG: Triglycerides. HOMA: Homeostasis model assessment.
ALT: alanine aminotransferase. AST: aspartate aminotransferase. GGT: gamma glutamyl transferase. AP: alcaline phosphatase
(*) $p<0.05$.

Table III. Dietary intake (mean ± SD).

Characteristics	Ala54/Ala54 (n = 15)	(Ala54/Thr54 or Thr54/Thr54) (n = 15)
Energy (kcal/day)	2127 ± 740	2261 ± 756
CH (g/day)	228.8 ± 82	248 ± 88
Fat (g/day)	89.7 ± 48	92.2 ± 40
Protein (g/day)	95.2 ± 22	98.7 ± 27
Dietary fiber	15.3 ± 7.8	18.4 ± 6.8

CH: Carbohydrate. No statistical differences.

Discussion

The necroinflammatory component of NASH appears to be modulated by interactions among various factors (for example, cytokines, hormones, neurotransmitters) that regulate the biological activity of TNF-alpha and other pro-inflammatory (th-1) cytokines[20]. In our study, lack of association of biochemical markers, dietary intake, adipocitokines, anthropometric variables and single nucleotide polymorphism (SNP) of FABP (Ala54Thr) with histological variables of NAFLD were detected.

Increasing evidence suggests that non-sex-linked genetic factors play a role in determining both susceptibility to, and progression of liver fibrosis. The elucidation of these factors will have many potential benefits in the management of patients with chronic liver disease. A variety of approaches can be used to look for genetic factors playing a role in liver fibrosis. Genes encoding proteins involved in fibrogenesis in the liver are clearly candidates for a role in NAFLD-related fibrosis[21]. The only relevant study thus far in this regard in NAFLD is a recent report that obese patients possessing both the high TGFbeta-1 and angiotensinogen producing SNP may be more susceptible to advanced fibrosis[22].

Carriers of the Thr54 allele have a 2-fold greater affinity for the long-chain fatty acids than those with the Ala 54, which supports the role of the FABP2 Ala54Thr polymorphism in the etiology of metabolic disorders. Baier et al([8]) concluded that threonine-containing protein may increase absorption and/or processing dietary fatty acids by the intestine and therefore increase fat oxidation, which has been shown to reduce insulin action, with high insulin, LDL-cholesterol, BMI and triglycerides levels.

Perhaps, these different results could be explained by inclusion criteria of subjects in previously studies of the literature. For example, Carlsson et al[23] have detected higher concentrations of triglyceride and cholesterol in the Thr54 allele patients, in a population of obese patients with parenteral history of cardiovascular disease. In a population of type 2 diabetes mellitus[24], a linear relationship of mean fasting plasma triglyceride levels was found and after fat ingestion, in homozygous for the Thr54 allele than in wild type patients. In type 1 dia-

Table IV. Circulating serum adypocitokines (mean ± SD).

Characteristics	Ala54/Ala54 (n = 15)	(Ala54/Thr54 or Thr54/Thr54) (n = 15)
IL-6 (pg/ml)	3.1 ± 3.9	2.1 ± 2.8
TNF-alfa (pg/ml)	5.4 ± 4.4	4.4 ± 2.6
Adiponectin (ng/ml)	55.5 ± 26.8	31.4 ± 28
Resistin (ng/ml)	2.7 ± 0.8	2.5 ± 0.5
Leptin (ng/ml)	43 ± 28	34.9 ± 41

IL-6: interleukin-6.

Table V. Circulating serum adypocitokines (mean ± SD).

Characteristics	Ala54/Ala54	(Ala54/Thr54 or Thr54/Thr54)	
	(n = 15)	(n = 15)	P
Portal inflammation (%)	14.3	9.1	Ns
Lobular inflammation (%)	78.6	81	Ns
Low-moderate steatosis (%)	42.9	36	Ns
Severe steatosis (%)	57.1	63	Ns
Fibrosis (%)	33.3	33.3	Ns

betes mellitus patients[25] do not interact with the codon 54 polymorphism of the FABP2 gene to cause dyslipemia. The population association studies with insulin resistance a type 2 diabetes mellitus[26,27] were also essentially negative.

The lack of association with cholesterol and triglyceride levels is clear in our study. Other Authors have shown[23,24] higher levels of cholesterol and triglycerides in patients with Thr allele. Nevertheless, Duarte et al[28] have shown a lower total and LDL cholesterol levels in patients with Thr allele. These previous works would require composition analysis of the diet to determine whether dietary components could be responsible for the lipid profile modifications. In our study dietary intake didn't show statistical differences between groups. In this way our data have controlled by dietary intake and previous discrepancies could be explain by this uncontrolled factor (dietary intake).

Body mass index of the patients could be other factor to explain these discrepancies. First, the average BMI was higher in bariatric surgery researches[29] than our study. Second, some studies evaluated weight loss and the influence of this polymorphism in metabolic parameters (cohort studies) and our research is a non interventional study[9,30]. A type II error of our study due to a small sample size could be other hypothesis to explain the lack of association of our design. Interaction with other polymorphisms could be other confounding factor. For example, Valenti et al[31] have demonstrated that TNF-alpha polymorphisms (238 and 308) could represent a susceptibility genotype for insulin resistance, steatohepatitis and nonalcoholic fatty liver. Regardless of the mechanisms involved for these discordant findings, further researches are needed to elucidate potential metabolic pathways explaining the lack of Ala54Thr FABP polymorphism-related effect on metabolic parameters and histological changes in patients with NAFLD. This topic area has different ways to explain the relationships of clinical and anthropometric parameters with histological changes; steatosis seems to be determined by insulin resistance, whereas the progression to cirrhosis includes several factors such as mytocondrial disfunction, oxidant stress, cytokines, iron overload, bacterial overgrowth, excess intracellular fatty acids and genetic background[32]. This genetic background may be a little piece in this puzzle.

In conclusion, the present study demonstrates that the polymorphism Ala54Thr of FABP predict neither liver histological changes nor insulin resistance and adipokines in patients with NAFLD.

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