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Original Article

Altering of FTO in the serum and livers of NAFLD patients: a correlation analysis

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Abstract: Nonalcoholic fatty liver disease (NAFLD), caused by a type of fatty liver without excessive alcohol intake, is characterized by steatosis, ballooning degeneration, and fatty retention of liver parenchyma cells. NAFLD's major spectrum includes nonalcoholic simple fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH);however, the relationship between NAFLD and the fat mass and obesity associated (*FTO*) gene are not fully understood. To address this, we collected clinical basic parameters of NAFLD and control patients, measured *FTO* expression levels in their serum and livers by using ELISA, qRT-PCR and Western blot, and analyzed the correlation of *FTO* expression levels to the clinical basic parameters. NAFLD more often occurs in middle-aged males, and the clinical basic parameters were significantly increased in NAFLD patients with the exception of HDL. The serum *FTO* expression level of the NAFLD patient group was significantly higher compared to the control group; however, within the NAFLD patient group, the expression level in the NASH group was significantly greater compared to the NAFL group, and was positively correlated to body mass index (BMI) in the NAFL group and total cholesterol in the NASH group. Similarly, the liver *FTO* expression level of the NAFLD patient group was significantly higher compared to the control group, and also significantly increased in the NASH group compared to the NAFL group. This study provides a significant reference for the study of *FTO* in NAFLD patients their treatment.

Keywords: Nonalcoholic fatty liver disease, nonalcoholic simple fatty liver, nonalcoholic steatohepatitis, clinical basic parameters, fat mass and obesity associated gene, correlation analysis

Introduction

The recent revelation of high-fat diets causing non-alcoholic fatty liver disease (NAFLD) has aroused the concern of people worldwide, and has therefore caused a decreased prevalence of NAFLD by 20% to 35% due to living quality improvements [1-3]. NAFLD is characterized by a series of hepatic pathological changes, such as simple steatosis, non-alcoholic steatohepatitis and cirrhosis; its major disease spectrum includes nonalcoholic simple fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH) [1, 4]. NAFL, defined as a simple steatosis [5-7], appreciably differs from NASH, which is characterized by inflammatory infiltrate, ballooning and necroapoptosis [8-11]. According to an epidemiological survey, NAFLD is closely related to several metabolic syndromes, such as obesity, diabetes mellitus, and hyperlipemia [12-14]. Of the patients with NAFL, 10%-20% will have their NAFL develop into NASH, and 1.5%-3% of patients' NAFL will develop into liver fibrosis, thereby seriously threatening their health [2, 15]. The pathogenesis of NAFLD is not yet understood; the 'two-hit' theory has been received with extensive concern [15-17]. Under metabolic stress conditions, such as obesity, type 2 diabetes mellitus, and dyslipidemia, several inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interferon (IFN), may induce insulin resistance followed by a deposition of fatty acid and triglyceride in the liver-further inducing simple steatosis [3, 18]. Liver cells and their mitochondria arethen subjected to secondary damaged by oxidative stress, cytokines, and adipocytokines.

The fat mass and obesity-associated (*FTO*) gene, discovered during a genome-wide associated study for obesity in 2007, is currently rec-

ognized as the most robust predictor of polygenic obesity [19, 20]. It encodes for a Fe(II)- and 2-oxoglutarate dependent nucleic acid demethylase present in the nucleus. Several risk alleles for *FTO*, such as the rs9939609, rs9930506, rs1421085, rs17817449, and rs1121980 polymorphisms, are known to associate with obesity and a higher body mass index (BMI) [21, 22]. *FTO* is widely expressed in the nucleus of human tissues; the brain has the highest expression level, especially in the arcuate nucleus of the hypothalamus, which is known to play a major role in controlling energy homeostasis and eating behavior [23-25]. The potential role of *FTO* in energy homeostasis, which has been well documented by several researchers in humans, mice, and rodents, is the regulation of the *FTO* mRNA expression by food intake, circulating glucose levels, weight status, and energy expenditure [26, 27]. *Fto* knockout mice exhibit normal embryonic development, but high postnatal lethality, growth retardation, lower weight and adiposity measures, and experience initial reduced weight and lean mass [28-30]. *Fto* knockout mice were protected from diet-induced obesity throughout their entire life span when consuming a high fat diet, while a high fat intake augmented weight gain in mice with *FTO* over-expression [31, 32]. A number of possible but inconclusive mechanisms for *FTO's* role in explaining these findings have been proposed. Such as, FTO may influence protein and/or fat utilization, as well as energy expenditure and adrenalin levels [33]. NAFLD as a significant disease is associated with several metabolic syndromes, such as obesity, type 2 diabetes mellitus, and dyslipidemia [34-36]; therefore, we hypothesize that *FTO* may be involved in regulating the pathogenesis of NAFLD. After measuring the *FTO* expression levels in the serum and livers of NAFLD and control patients, we found that the *FTO* expression level is significantly associated with NAFLD thus providing a significant reference for studying and treating NAFLD.

Materials and methods

Patients and basic clinical information collection

A total of 255NAFLD patients and 100 healthy patient controls were enrolled from October 2013 to October 2015 in the Six People's Hospital Affiliated to Shanghai JiaoTong University. Blood samples were collected prior to any therapeutic procedure. The NAFLD patients, after excluding those with significant alcohol consumption (female >20 g/day and male >30 g/day) and any pre-existing liver disease, were further divided into two groups according to the clinical diagnosis: 130 cases of NAFL and 125 cases of NASH. The healthy patient controls underwent a routine health examination to asses for any evidence or history of liver pathology, an unremarkable liver ultrasound, normal liver stiffness, and no elevation of serum transaminases alanine aminotransferase (AST) or aspartate aminotransferase (ALT). We also excluded patients with a history of coronary heart disease, hypertension, valvular disease, and any arrhythmia or systemic disease. In addition, the basic clinical information of the control patients, NAFL, and NASH groups were collected, including age, gender, body mass index (BMI), waistline, hipline, waist-hip ratio, fasting blood glucose (FBG), fasting insulin, total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), ALT, and AST. Subsequently, liver biopsies were also obtained and stored in -80°C refrigerator for further use. All patients provided written informed consent to participate in this study, and it was approved by the Ethics Committee of the Six People's Hospital Affiliated to Shanghai JiaoTong University.

Serum FTO levels assayed by ELISA

The collected blood samples were incubated at room temperature (RT) for 2 h followed by centrifugation at 3,000 rpm for 20 min; the supernatant was carefully collected to examine the *FTO* level in accordance with the instructions of the Human Alpha-Keto glutarate-Dependent Dioxygenase FTO (FTO) ELISA Kit (MBS918864, MyBioSource, USA). Before the assay, the Standard wells, Sample wells, and Blank wells were set; 50 µl of Standard was added to each Standard well, 50 µl of Sample was added to each Sample well, and 50 µl of Sample Diluents was added to each Blank well. Then, 100 µl of HRP-conjugate reagent was added to each well. The wells were then covered with an adhesive strip and incubated for 60 min at 37°C. This was followed by washing with the Wash Solution (1×) for four times (5 min each time). Then, 50 µl of Chromogen Solution A and 50 µl of Chromogen Solution B were added to each well, respectively. The wells were then gently mixed, protected from light, and incubated for 15 min at 37°C. Then, 50 µl of Stop Solution was added to each well. The data were recorded at 450 nm using a microplate reader for 15 min,

and analyzed by using SPSS software (version 21.0, http://spss.en.softonic.com/; Chicago, IL, USA), and histogram analysis was performed using Origin 9.5 software (http://www.originlab.com/).

Total RNA extraction of liver

A total of 100 mg of collected liver tissue from the patients was frozen in liquid nitrogen and grounded for a total RNA extraction with TRIzol, in accordance with the manufacturer's instructions. After grinding, 1 mL of TRIzol was added and mixed for 30 s; samples were incubated at room temperature for 5 min and then centrifugation at 4°C, at 12,000 rpm for 10 min. The supernatant was collected, 200 μL chloroform was added and mixed in for 15 s, incubated at room temperature for 3 min, and then centrifuged at 4°C, at 12,000 rpm for 15 min. An equal volume of isopropyl alcohol was mixed in with the absorbed aqueous phase, incubated at room temperature for 30 min, and then centrifuged at 4°C, at 12,000 rpm for 10 min; the supernatant was removed. The pellet was washed with 1 mL of 75% ethanol, centrifuged at 4°C, at 5,000 rpm for 3 min, The residual liquid was then removed, and left to air-dry at room temperature. After adding100 μL of RNAse-free ddH_2O and centrifuging at room temperature, at 12,000 rpm for 2 min, the effluent was collected, and the concentration and the purity of RNA were examined by ultraviolet spectrophotometry.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay of FTO in liver

The extracted total RNA was used for a reverse transcription reaction in accordance with the manufacturers' instruction of Rever Tra Ace-α kit (FSK-100, TOYOBO, Japan). The reverse transcription reaction mixture was prepared initially by mixing: 10 μL 2×loading buffer, 1.2 μL oligo (dT), 2 μL mRNA template, 0.2 μL MMLV reverse transcriptase, and 6.6 μL DEPC H_2O. The reverse transcription reaction was performed as following: 26°C 30 min→42°C 30 min→85°C 10 min. The reverse transcription products were used as a template to perform qRT-PCR amplification with the following primer pairs (β-actin as a internal standard): *FTO*: 5'-GGAGAATAGCTCCAGACGGG-3' (forward), 5'-TTAGCTTCGCGCTCTCGTTC-3' (reverse); β-actin: 5'-ATAACCCTGGCACCCAGCACCA-3' (forward), 5'-TGGACAGGCTGGCAAGGATGGA-3' (reverse). The qRT-PCR reaction mixture was prepared by mixing: 10 μL 2×Master Mix, 0.08 μL forward primer, 0.08 μL reverse primer, 2 μL cDNA template, 0.4 μL Taq DNA polymerase, and 7.44 μL ddH_2O. Amplification was performed as following: 95°C 3 min→(95°C 12 s→62°C 30 s), 40 cycles→72°C 30 s. The results were analyzed using the SDS 1.4 software (Applied Biosystems), and a histogram was generated using the Origin 9.5 software (http://www.originlab.com/).

Western blot assay

The total protein collected from the liver tissues was extracted and examined by using the BCA Protein Assay Kit (ZKP-C150046-1, Suzhou Zeke biotech Co., LTD, Jiangsu, China). About 35 μg of protein samples were fractionated by electrophoresis through 12.5% polyacrylamide gels, and transferred to a polyvinylidene difluoride membrane following the manufacturers' instructions. The membrane was probed with the first antibody, a mouse-derived anti-FTO antibody (ab92821, Abcam, United Kingdom), mouse-derived anti-GAPDH (ab8245, Abcam, United Kingdom) for 1.5 h at room temperature. Afterward, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 in TBST; Beijing Golden Bridge Biotechnology Co., Ltd, China) at room temperature for 1 h. The chemiluminescence luminol reagent (ZKP-C150044-1, Suzhou Zeke biotech Co., LTD, Jiangsu, China) was used to develop the immune-labeled bands on X-ray film, and the optical density of the bands was quantified using the ImageJ 1.46 software (http://rsb.info.nih.gov/ij/download.html), and a histogram was generated using the Origin 9.5 software (http://www.originlab.com/).

Statistical analysis

All data is expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 21.0, http://spss.en.softonic.com/; Chicago, IL, USA). The differences between the groups were evaluated by one-way ANOVA. Student's *t*-tests were performed in a group of two samples, and correlation analysis was performed with the bivariate Pearson's Rank correlation test; $P<0.05$ and $P<0.01$ were used to indicate sig-

Table 1. The basic parameters of NAFLD patients in the clinic

Parameters		Control (n=100)	Groups (Mean ± SD) NAFL (n=130)	NASH (n=125)
Age (year)		45.67 ± 2.22	48.71 ± 3.34**	49.70 ± 3.87**,§
Gender	Male	68	95	98
	Female	32	35	27
	Male ratio (%)	68.0	73.1	78.4
BMI (kg/m^2)		23.59 ± 1.44	26.37 ± 1.38**	30.27 ± 2.50**,§
Waistline (cm)		85.76 ± 1.85	94.03 ± 1.84**	101.12 ± 3.26**,§
Hipline (cm)		98.05 ± 2.49	100.87 ± 3.59**	101.47 ± 3.04**,§
Waist-hip ratio (%)		87.52 ± 3.00	93.33 ± 3.67**	99.74 ± 4.30**,§
FBG (mmol/L)		4.42 ± 0.18	5.46 ± 0.13**	5.71 ± 0.14**,§
Fasting insulin (μIU/mL)		7.47 ± 0.24	17.62 ± 0.203**	25.88 ± 1.59**,§
TC (mmol/L)		4.71 ± 0.48	5.02 ± 0.53**	5.23 ± 0.24**,§
TG (mmol/L)		1.05 ± 0.10	1.54 ± 0.12**	2.49 ± 1.21**,§
HDL (mmol/L)		1.41 ± 0.10	1.19 ± 0.16**	1.07 ± 0.10**,§
LDL (mmol/L)		2.67 ± 0.13	2.87 ± 0.17**	3.24 ± 0.21**,§
ALT (U/L)		24.46 ± 0.35	29.10 ± 0.92**	40.61 ± 1.01**,§
AST (U/L)		24.40 ± 1.78	26.44 ± 1.65**	31.88 ± 1.68**,§

Note: **: *P*<0.01; §: *P*<0.01; SD indicates the standard deviation; NAFL indicates thenon-alcoholic fatty liver; NASH indicates the non-alcoholic steatohepatitis; BMI indicates bodymass index; FBG indicates the fasting blood glucose; TC indicates the total cholesterol; TG indicates the triglyceride; HDL indicates the high density lipoprotein; LDL indicates the low densitylipoprotein; ALT indicates the alanine aminotransferase; AST indicates the aspartateaminotransferase.

Table 2. The content measurement of FTO in serum

Parameters	Groups (Mean ± SD) Control (n=100)	NAFL (n=130)	NASH (n=125)
FTO	18.57 ± 0.59	34.57 ± 1.734**	45.56 ± 1.58**,§

Note: **: *P*<0.01; §: *P*<0.01; FTO indicates the fat mass and obesity associated gene.

nificant differences and highly significant differences, respectively.

Results

The clinical basic parameters of NAFLD patients

NAFLD often occurred in middle-aged men; age was significantly increased in the NAFL and NASH groups when compared the control group (**: *P*<0.01), and also significantly increased in the NASH group compared to the NAFL group (§: *P*<0.01) (**Table 1**). The male ratio was significantly increased, 73.1% in the NAFL group and 78.4% in the NASH group, when compare to the 52% male ration in the control group (**: *P*<0.01); the male ration was also significantly increased in NASH group compared to the NAFL group (§: *P*<0.01). Similarly, several clinical basic parameters, including BMI, waistline, hipline, waist-hip ratio, FBG, fasting insulin, TC, TG, LDL, ALT, and AST, were significantly increased in the NAFL and NASH groups when compared to the control group (**: *P*<0.01); and also significantly increased in NASH group compared to the NAFL group (§: *P*<0.01), with the exception of HDL. The HDL level was significantly decreased, 1.19 ± 0.16 in NAFL group and 1.07 ± 0.10 in NASH group, when compared to the 1.41 ± 0.10 level of control group (**: *P*<0.01); and also significantly decreased in the NASH group compared to the NAFL group (§: *P*<0.01).

The FTO expression level was significantly increased in the serum of NAFL and NASH patients when compared control patients, and significantly correlated to BMI, waistline, and TG in the control group; to age, BMI, FBG, ALT, AST in the NAFL group; to TC, HDL in the NASH group

The *FTO* level in the serums of NAFL and NASH patients was significantly higher, 34.57 ± 1.734 and 45.56 ± 1.58, respectively, when com-

Table 3. The correlation analysis of FTO in serum to the basic index of NAFLD patients

Parameters	Correlation					
	Control		NAFL		NASH	
	p-value	r-value	*p*-value	r-value	*p*-value	r-value
Age	0.269	0.026	0.044	-0.151*	0.289	-0.050
BMI	0.013	-0.222*	0.041	0.153*	0.222	0.069
Waistline	0.003	0.272**	0.325	0.040	0.211	-0.073
Hipline	0.194	0.087	0.055	0.141	0.146	-0.095
Waist-hip ratio	0.149	0.105	0.109	-0.109	0.431	0.016
FBG	0.210	0.081	0.003	-0.240**	0.360	-0.032
Fasting insulin	0.279	-0.059	0.498	0.001	0.312	-0.044
TC	0.173	-0.095	0.379	-0.023	0.001	0.274**
TG	0.011	-0.229*	0.409	-0.020	0.150	0.093
HDL	0.157	-0.102	0.345	-0.035	0.021	-0.182*
LDL	0.280	0.059	0.092	-0.117	0.395	-0.024
ALT	0.376	0.032	0.046	-0.148*	0.268	-0.056
AST	0.244	-0.070	0.042	-0.152*	0.197	0.077

Note: *: *P*<0.05; **: *P*<0.01. NAFL indicates the non-alcoholic fatty liver; NASH indicates the non-alcoholic steatohepatitis; BMI indicates body mass index; FBG indicates the fasting blood glucose; TC indicates the total cholesterol; TG indicates the triglyceride; HDL indicates the high density lipoprotein; LDL indicates the low density lipoprotein; ALT indicates the alanine aminotransferase; AST indicates the aspartate aminotransferase.

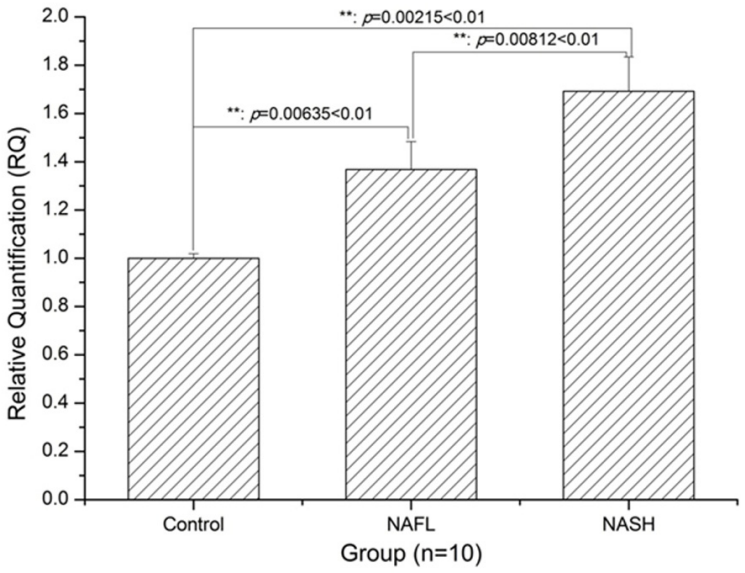

Figure 1. Histogram analysis of FTO mRNA expression level in the liver. **: *P*<0.01.

pared to the 18.57 ± 0.59 level of the control group (**: *P*<0.01); and also significantly increased in the NASH group when compared to the NAFL group (§: *P*<0.01) (**Table 2**). A correlation analysis showed that the *FTO* level in serum was positively correlated to waistline in the control group, tp BMI in the NAFL group, and to TC in the NASH group, and negatively correlated to BMI in all groups, to TG in the control group, to age, FBG, ALT, and AST in the NAFL group, and to HDL in the NASH group, and however no regression function (**Table 3**).

FTO mRNA and protein levels were significantly increased in livers of NAFL and NASH patients when compared the control patients

The FTO mRNA level was significantly increased in the livers of NAFL and NASH patients when compared to the control patients (**: *P*<0.01); and also significantly increased in the NASH group in when compared to the NAFL group (§: *P*<0.01) (**Figure 1**). Similarly, the FTO protein level was also significantly increased in livers of NAFL and NASH patients when compared the control patients (**: *P*<0.01); and also significantly increased in the NASH group when compared to the NAFL group (**: *P*<0.01) (**Figure 2**).

Discussion

The present study demonstrates that NAFLD often occurred more often middle-aged males when compared to the controls; a significantly increased number of NASH patients when compared to NAFL patients; the other clinical basic parameters, including BMI, waistline, hipline, waist-hip ratio, FBG, fasting insulin, TC, TG, LDL, ALT, and AST, exhibited the same trend with the exception of HDL. The mRNA and protein expression levels in the serum and liver were significantly increased in NASH and NAFL patients when compared to the controls, and significantly increased in NASH patients when compared to NAFL patients. The expression level of *FTO* in the serum was positively corre-

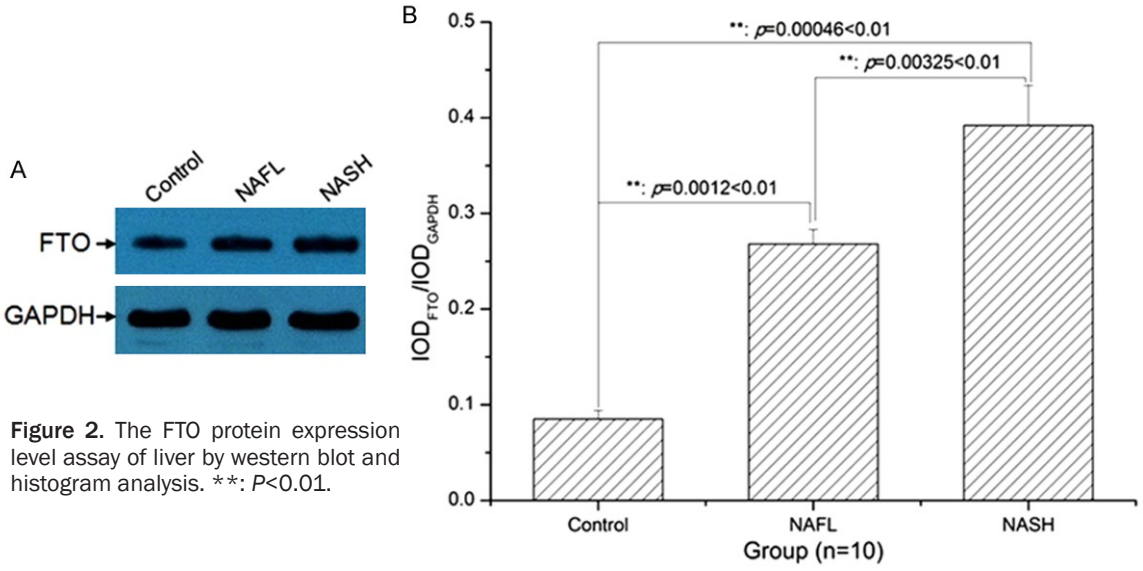

Figure 2. The FTO protein expression level assay of liver by western blot and histogram analysis. **: *P*<0.01.

lated to the waistlines of control patients, to BMI in the NAFL group, and to TC in the NASH group, and negatively correlated to BMI, TG in the control patients, to age, FBG, ALT, AST in the NAFL group, and to HDL in NASH group, and however no regression function.

NAFLD, a chronic liver disease that is often caused, worldwide, by high-fat diets, is combined with several metabolic syndromes, such as obesity, type 2 diabetes mellitus, and dyslipidemia [34-36]. As shown with the clinical basic parameters of NAFLD patients, the BMI, waistline, hipline, and waist-hip ratio were significantly increased in NASH and NAFL patients, and indicated that NAFLD is closely associated with obesity. The FBG, fasting insulin, TC, TG, and LDL were significantly increased in the NASH and NAFL patients thus indicating that NAFLD is closely associated with diabetes and hyperlipidemia. This data demonstrates that NAFLD is a metabolic syndrome with several disease associations.

FTO, a significant fat mass and obesity associated gene with a full length of 400 bp, consists of nine exons, mainly located in the 16q12-q24 of the human chromosome, and encodes for an important energy regulating protein that is closely related to lipid metabolic processes [37-40]. Therefore, we hypothesized that *FTO* may be significantly expressed in the serum and livers of NAFLD patients, and also be involved in the regulation of NAFLD. The expression levels of *FTO* in the serum were significantly increased in NAFLD patients, and positively correlated to the waistlines of control patients, to BMI in the NAFL group, and to TC in the NASH group, and negatively correlated to BMI in all groups, to TG in control patients, to age, FBG, ALT, and AST in the NAFL group, and to HDL in the NASH group. Thereby indicating that serum *FTO* may act as a significant diagnosis index for NAFLD. Significantly, the mRNA and protein expression levels of *FTO* in the liver were also increased in NAFLD patients, and especially so in NASH patients. However, the current study is preliminary, and several limitations need to be solved in the future, such as the limited sample size, detection means, and epidemiological statistics; future research should further explore the function of *FTO* in cells and animals, and help to develop a significant diagnosis index for NAFLD based on *FTO* expression levels in the serum or liver.

Conclusively, after an epidemiological survey of NAFLD patients, the associated indexes of obesity, diabetes, and hyperlipidemia were significantly changed in NASH and NAFL patients when compared to of the control patients; furthermore, *FTO* expression levels in the serum and liver were closely associated with NAFLD, and provided a significant reference for studying and treating NAFLD disease.

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Disclosure of conflict of interest

None.

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