

World Journal of *Gastroenterology*

World J Gastroenterol 2017 June 14; 23(22): 3945-4134





EDITORIAL

- 3945 Bleeding with the artificial heart: Gastrointestinal hemorrhage in CF-LVAD patients
Gurvits GE, Fradkov E

REVIEW

- 3954 Role of non-steroidal anti-inflammatory drugs on intestinal permeability and nonalcoholic fatty liver disease
Utzeri E, Usai P
- 3964 Molecular mimicry in *Helicobacter pylori* infections
Chmiela M, Gonciarz W
- 3978 Gallbladder cancer epidemiology, pathogenesis and molecular genetics: Recent update
Sharma A, Sharma KL, Gupta A, Yadav A, Kumar A

ORIGINAL ARTICLE

Basic Study

- 3999 Serelaxin increases the antifibrotic action of rosiglitazone in a model of hepatic fibrosis
Bennett RG, Simpson RL, Hamel FG
- 4007 Bcl-2 degradation is an additional pro-apoptotic effect of polo-like kinase inhibition in cholangiocarcinoma cells
Sydor S, Jafoui S, Wingerter L, Swoboda S, Mertens JC, Gerken G, Canbay A, Paul A, Fingas CD
- 4016 Effect of *CXCR3/HO-1* genes modified bone marrow mesenchymal stem cells on small bowel transplant rejection
Yin ML, Song HL, Yang Y, Zheng WP, Liu T, Shen ZY

Case Control Study

- 4039 Systemic interleukin-9 in inflammatory bowel disease: Association with mucosal healing in ulcerative colitis
Matusiewicz M, Neubauer K, Bednarz-Misa I, Gorska S, Krzystek-Korpacka M
- 4047 Association of keratin 8/18 variants with non-alcoholic fatty liver disease and insulin resistance in Chinese patients: A case-control study
Li R, Liao XH, Ye JZ, Li MR, Wu YQ, Hu X, Zhong BH

Retrospective Study

- 4054** Barcelona Clinic Liver Cancer outperforms Hong Kong Liver Cancer staging of hepatocellular carcinoma in multiethnic Asians: Real-world perspective

Li JW, Goh BBG, Chang PE, Tan CK

Observational Study

- 4064** Single-operator cholangioscopy for biliary complications in liver transplant recipients

Hüsing-Kabar A, Heinzow HS, Schmidt HHJ, Stenger C, Gerth HU, Pohlen M, Thölking G, Wilms C, Kabar I

- 4072** Efficacy and safety of combined directly acting antivirals for treatment of Chinese chronic hepatitis C patients in a real-world setting

Chen JH, Zeng Z, Zhang XX, Zhang Y, Zhang RW, Wang S, Wu CH, Yu M, Liu D, Xi HL, Zhou YX, An YY, Xu XY

- 4080** Observation of the effect of targeted therapy of 64-slice spiral CT combined with cryoablation for liver cancer

Yan QH, Xu DG, Shen YF, Yuan DL, Bao JH, Li HB, Lv YG

Prospective Study

- 4090** Inflammatory bowel disease incidence in Czech children: A regional prospective study, 2000-2015

Schwarz J, Šykora J, Cvalínová D, Pomahačová R, Klečková J, Kryl M, Včelák P

- 4102** Drug-induced liver injury in inflammatory bowel disease: 1-year prospective observational study

Koller T, Galambosova M, Filakovska S, Kubincova M, Hlavaty T, Toth J, Krajcovicova A, Payer J

SYSTEMATIC REVIEWS

- 4112** Can fecal microbiota transplantation cure irritable bowel syndrome?

Halkjær SI, Boolsen AW, Günther S, Christensen AH, Petersen AM

CASE REPORT

- 4121** Application of novel magnified single balloon enteroscopy for a patient with Cronkhite-Canada syndrome

Murata M, Bamba S, Takahashi K, Imaeda H, Nishida A, Inatomi O, Tsujikawa T, Kushima R, Sugimoto M, Andoh A

- 4127** Synchronous triple occurrence of MALT lymphoma, schwannoma, and adenocarcinoma of the stomach

Choi KW, Joo M, Kim HS, Lee WY

LETTERS TO THE EDITOR

- 4132** Is tremor related to celiac disease?

Ameghino L, Rossi MD, Cerquetti D, Merello M

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World Journal of Gastroenterology (*WJG*) is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. The 2015 edition of Journal Citation Reports® released by Thomson Reuters (ISI) cites the 2015 impact factor for *WJG* as 2.787 (5-year impact factor: 2.848), ranking *WJG* as 38 among 78 journals in gastroenterology and hepatology (quartile in category Q2).

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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PUBLICATION DATE
June 14, 2017

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Case Control Study

Association of keratin 8/18 variants with non-alcoholic fatty liver disease and insulin resistance in Chinese patients: A case-control study

Rui Li, Xian-Hua Liao, Jun-Zhao Ye, Min-Rui Li, Yan-Qin Wu, Xuan Hu, Bi-Hui Zhong

Rui Li, Xian-Hua Liao, Jun-Zhao Ye, Min-Rui Li, Yan-Qin Wu, Xuan Hu, Bi-Hui Zhong, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

Author contributions: Li R and Liao XH wrote the manuscript and performed the data analysis; Ye JZ designed the research; Li MR, Wu YQ and Hu X performed the research; Zhong BH revised the paper.

Supported by National Natural Science Foundation of China, No. 81670518 and No. 81170392; The Science and Technology Project of Guangdong Province, China, No. 2013B021800290 and No. 2014A020212118; and Guangzhou Science and Technology Innovation Commission, China, No. 201604020155.

Institutional review board statement: The study was reviewed and approved by The First Affiliated Hospital of Sun Yat-Sen University Institutional Review Board.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: No additional data are available.

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Manuscript source: Unsolicited manuscript

Correspondence to: Bi-Hui Zhong, MD, PhD, Department of Gastroenterology, The First Affiliated Hospital of Sun Yat-Sen University, No. 58 Zhongshan Road II, Guangzhou 510080, Guangdong Province, China. sophiazhong@medmail.com.cn
Telephone: +86-20-87755766
Fax: +86-20-87332916

Received: January 31, 2017
Peer-review started: February 8, 2017
First decision: February 23, 2017
Revised: March 10, 2017
Accepted: May 4, 2017
Article in press: May 4, 2017
Published online: June 14, 2017

Abstract**AIM**

To test the hypothesis that K8/K18 variants predispose humans to non-alcoholic fatty liver disease (NAFLD) progression and its metabolic phenotypes.

METHODS

We selected a total of 373 unrelated adult subjects from our Physical Examination Department, including 200 unrelated NAFLD patients and 173 controls of both genders and different ages. Diagnoses of NAFLD were established according to ultrasonic signs of fatty liver. All subjects were tested for population characteristics, lipid profile, liver tests, as well as glucose tests. Genomic DNA was obtained from peripheral blood with a DNeasy Tissue Kit. K8/K18 coding regions were analyzed, including 15 exons and exon-intron boundaries.

RESULTS

Among 200 NAFLD patients, 10 (5%) heterozygous

carriers of keratin variants were identified. There were 5 amino-acid-altering heterozygous variants and 6 non-coding heterozygous variants. One novel amino-acid-altering heterozygous variant (K18 N193S) and three novel non-coding variants were observed (K8 IVS5-9A→G, K8 IVS6+19G→A, K18 T195T). A total of 9 patients had a single variant and 1 patient had compound variants (K18 N193S+K8 IVS3-15C→G). Only one R341H variant was found in the control group (1 of 173, 0.58%). The frequency of keratin variants in NAFLD patients was significantly higher than that in the control group (5% *vs* 0.58%, $P = 0.015$). Notably, the keratin variants were significantly associated with insulin resistance (IR) in NAFLD patients (8.86% in NAFLD patients with IR *vs* 2.5% in NAFLD patients without IR, $P = 0.043$).

CONCLUSION

K8/K18 variants are overrepresented in Chinese NAFLD patients and might accelerate liver fat storage through IR.

Key words: Keratin; Variant; Non-alcoholic fatty liver disease; Insulin resistance; Chinese population

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Core tip: This study presents the first investigation of the association between keratin 8 and 18 (K8/K18) variants and non-alcoholic fatty liver disease (NAFLD) in a Chinese population. We found an increased frequency of variants in NAFLD patients *vs* controls. We also identified a new amino acid-altering variant of K18. The results demonstrate that keratin variants are overrepresented in Chinese NAFLD patients and might accelerate liver fat storage through insulin resistance.

Li R, Liao XH, Ye JZ, Li MR, Wu YQ, Hu X, Zhong BH. Association of keratin 8/18 variants with non-alcoholic fatty liver disease and insulin resistance in Chinese patients: A case-control study. *World J Gastroenterol* 2017; 23(22): 4047-4053 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i22/4047.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i22.4047>

INTRODUCTION

Keratin (K) proteins form intermediate filaments (IFs) in epithelial cells, which constitute the cytoskeleton along with actin filaments and microtubules^[1,2]. Keratin proteins are primarily expressed in epithelial tissues, hair and skin appendages, and they are classified into relatively acidic type I (K9-K28, K31-K40) and relatively basic type II keratins (K1-K8, K71-K86)^[3,4]. Type I and type II keratins exist as paired polymeric filaments that display a tripartite structure containing a conserved α -helical central rod domain flanked by less conserved N-terminal head and C-terminal tail

domains^[3,5]. For example, K5/K14 and K1/K10 are found in basal and suprabasal keratinocytes, whereas K8/K18 are found in adult hepatocytes and other simple epithelial cells^[6]. Moreover, human association studies have identified mutations in keratins that can cause or predispose carriers to a broad range of human diseases, including multiple liver diseases^[1,7]. Finally, the importance of K8/K18 in protecting hepatocytes from apoptosis was clearly demonstrated in keratin-related genetically engineered animal models^[6,8].

Non-alcoholic fatty liver disease (NAFLD), which is regarded as a hepatic manifestation of metabolic syndrome, has become the most common chronic liver disease worldwide, as it affects 20%-50% of the general population in affluent countries^[9,10], and 15% of individuals in China^[11]. NAFLD is strongly concomitant with obesity, hypertriglyceridemia, type 2 diabetes mellitus, and insulin resistance (IR)^[12]. It is generally accepted that the initiating events in NAFLD depend on the development of obesity and IR in adipose tissue and the liver.

There are accumulating examples of K8/K18 involvement in the glucose-insulin cross-talk, which support the impact of K8/K18 IFs on insulin-dependent glucose metabolism regulation in the liver and its implication in glucose- or insulin-associated diseases^[12,13]. Lower fasting glucose levels, increased glucose tolerance and insulin sensitivity, reduced glucose-stimulated insulin secretion and decreased pancreatic insulin content have been shown to occur in K8 knockout mice^[14]. The mislocalization of glucose transporter (GLUT) and hexokinase (HK) status may contribute to the modulation of IFs in hepatocytes and hepatoma cells that lack K8/K18^[14,15].

Subsequent studies have established that keratin mutation plays an important role in liver diseases and glucose metabolism. Therefore, we hypothesized that K8/K18 variants may contribute to susceptibility to NAFLD. No studies have been reported on the association between K8/K18 mutations and NAFLD. Therefore, we tested our hypothesis by sequencing the K8/K18 coding regions in genomic DNA from 200 NAFLD subjects. Herein, we report our positive findings.

MATERIALS AND METHODS

Subjects

A total of 373 unrelated adult subjects were selected from our Physical Examination Department from January 2011 to December 2012, including 200 unrelated Chinese patients of both genders and different ages (164 males, 36 females; mean age, 40.85 \pm 9.9 years) and 173 healthy controls who were matched for sex and age (130 males, 43 females; mean age, 39.55 \pm 9.9 years). Diagnoses of NAFLD were performed under standard clinical evaluation conditions by ultrasonography according to the AASLD

Table 1 Demographics and clinical information of non-alcoholic fatty liver disease patients and controls

	Controls (<i>n</i> = 173)	NAFLD patients (<i>n</i> = 200)	<i>P</i> value
Sex (male, %)	130 (75.14)	164 (82.00)	0.107
Age (yr)	39.55 ± 9.92	40.85 ± 9.89	0.101
ALT (U/L)	21.65 ± 12.69	43.63 ± 29.16	< 0.0001
AST (U/L)	24.74 ± 6.52	33.85 ± 12.93	< 0.0001
FBG (mmol/L)	4.89 ± 0.62	5.40 ± 1.45	< 0.0001
TC (mmol/L)	4.71 ± 0.77	5.68 ± 1.09	< 0.0001
TG (mmol/L)	1.29 ± 0.99	2.86 ± 2.14	< 0.0001
HDL (mmol/L)	1.22 ± 0.29	1.07 ± 0.23	< 0.0001
LDL (mmol/L)	2.80 ± 0.61	3.32 ± 0.95	< 0.0001
UA (μmol/L)	331.33 ± 88.61	412.42 ± 96.60	< 0.0001

Data are presented as mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FBG: Fasting blood glucose; TC: Total cholesterol; TG: Triglyceride; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; NAFLD: Non-alcoholic fatty liver disease.

criteria^[16]. Other causes of liver disease were excluded, including increased alcohol intake (> 210/140 g weekly for males/females), viral and autoimmune hepatitis, hereditary hemochromatosis, Wilson's disease and alpha1-antitrypsin deficiency. Controls were confirmed as healthy based on a medical history, general examinations and laboratory examinations at the same hospital. Each participant underwent an anthropometric assessment, including measurements of weight and stature. Body mass index (BMI) was calculated as weight (kg)/stature (m²). All subjects received blood tests after an overnight 12 h fast, including serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), serum uric acid (UA), fasting blood glucose (FBG), fasting insulin (FIN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). IR was defined by the homeostasis model assessment-IR (HOMA-IR) index. As reported in an epidemiology survey carried out in China, IR was defined as HOMA-IR > 2.69 (*i.e.*, exceeding the 75% percentile of HOMA-IR in normal glucose tolerance subjects)^[17,18].

Genomic DNA extraction and genotyping

Genomic DNA was obtained from peripheral blood (EDTA anticoagulation) using a DNeasy Tissue Kit (Qiagen, Biotech, Beijing, China). The entire K8/K18 coding regions (15 exons and their adjacent exon-intron boundaries) of the DNA fragments were analyzed, which had been amplified with a Touchdown polymerase chain reaction protocol using Premix PrimeSTAR HS (TaKaRa, Biotechnology, Dalian, China) and previously described primers^[19] to obtain a high amplification specificity. Messenger RNA sequences of K8 (NM002273) and K18 (NM000224) were used to localize coding variants, while genomic sequences (hKRT8 [M34482] and hKRT18 [AF179904]) were employed for noncoding variants.

Statistical analysis

Statistical analyses were performed using SPSS statistical software, version 20.0 for Windows (SPSS Inc., Chicago, IL, United States). Continuous variables are expressed as mean ± SD. For continuous variables, a two-tailed *t*-test was used for two-group comparisons, whereas Kruskal-Wallis nonparametric one-way analysis of variance was used to compare qualitative variables. K8/K18 variant frequencies in the NAFLD patient and control groups were compared by the two-tailed Fisher exact probability test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Demographics and characteristics of NAFLD patients

Demographics and clinical information for the NAFLD patients and control groups are summarized in Table 1. A total of 200 patients with NAFLD were included. As expected, most (82%) patients were male. Compared with the control group, NAFLD patients showed higher ALT, AST, FBG, TC, TG, LDL and UA levels and lower levels of HDL.

Keratin variants in NAFLD patients and controls

We identified keratin heterozygous variants in 10 (5%) of 200 NAFLD patients, including 5 carriers of amino-acid-altering heterozygous variants and 6 carriers of non-coding heterozygous variants (Table 2). Among 200 NAFLD patients, 2 (1%) carried R341H heterozygous exonic variants (Figure 1A), which is lower than the previously reported frequency. Additionally, one novel amino acid-altering heterozygous variant (K18 N193S) and three novel non-coding variants (K8 IVS5-9A→G, K8 IVS6+19G→A, K18 T195T) were observed (Figure 1B). A total of 9 patients had a single variant and 1 patient had compound variants (K18 N193S+K8 IVS3-15C→G). A previously described silent and common heterozygous K8 L227L variant, which we detected in 43% of patients (not shown), is not included in Table 2 or any subsequent analysis.

Keratin variant is associated with biochemical parameters in patients with NAFLD

We found an increased frequency of variants in NAFLD patients vs controls (5% vs 0.58%, *P* = 0.015). To explore whether K8/K18 variants could affect the biochemical parameters, we compared non-carriers with variant carriers in all NAFLD patients. We found that there was no significant difference in clinical features, including BMI, liver biochemistry, glucose, lipids or HOMA-IR (Table 3). However, after dividing patients into those with or without IR, keratin variants showed a significant association with IR in NAFLD patients (Table 2; 8.86% in NAFLD patients with IR vs 2.5% in NAFLD patients without IR, *P* = 0.043).

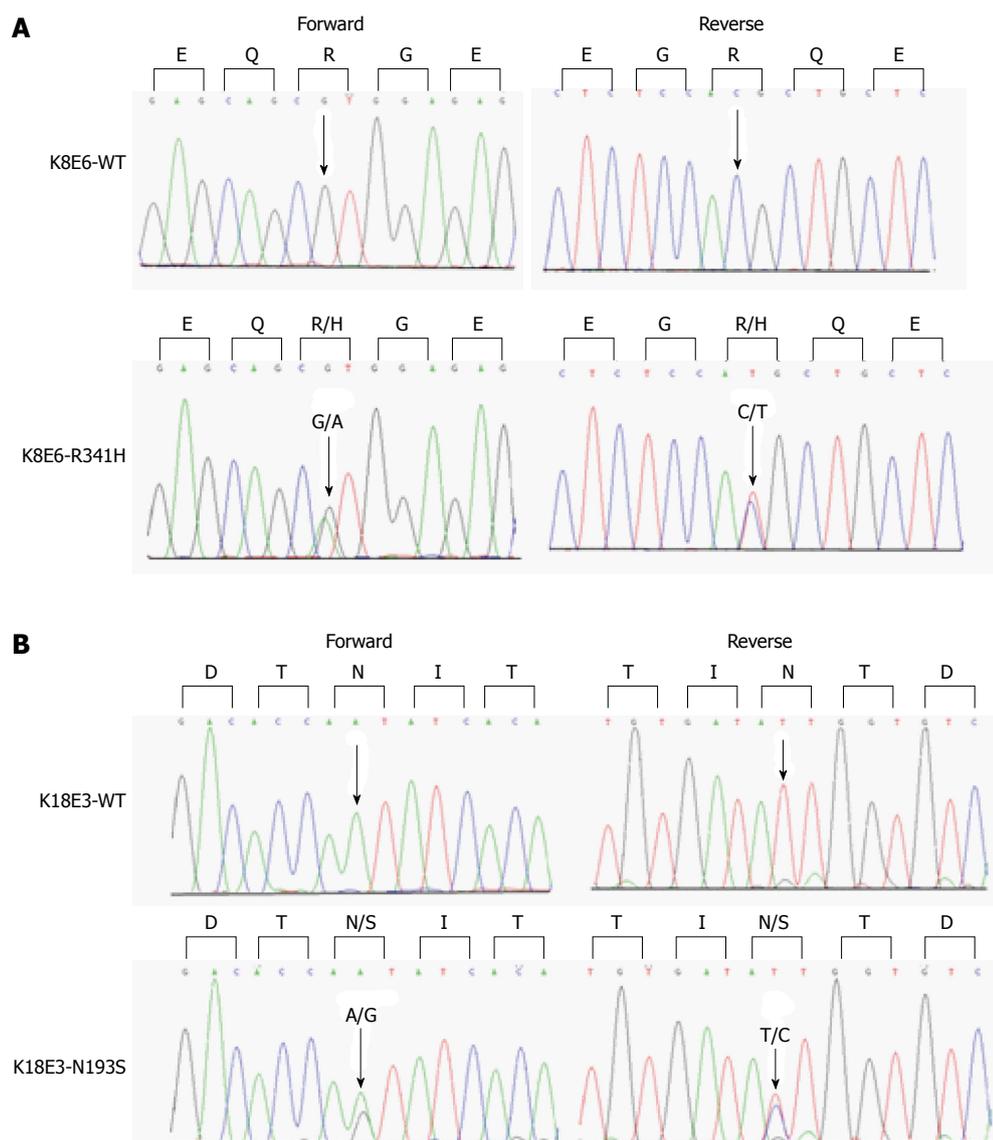


Figure 1 Detection of keratin variants in non-alcoholic fatty liver disease patients and controls. Genomic DNA was sequenced in the forward and reverse directions. The nucleotide locations of the variants were determined as described in “Patients and Methods”. The heterozygous K8 exon 6-R341H (A) and novel heterozygous variant K18 exon 3-N193S (B) are presented in comparison with their corresponding wild type (WT) samples.

DISCUSSION

Previous reports suggested that K8/K18 variants predispose carriers to various types of liver injury, but it is not known whether K8 or K18 variants show an association with NAFLD. For the first time, our present study investigated the association between K8/K18 variants and NAFLD in a Chinese population. We analyzed K8/K18 variants in NAFLD patients and observed the potential association of keratin variants with NAFLD.

Our results demonstrate that keratin variants are overrepresented in NAFLD patients. Moreover, K8 has the most keratin variants, which is consistent with data from patients with chronic hepatitis C, primary biliary cirrhosis and acute liver failure. This finding highlights the importance of *K8/K18* gene variants in NAFLD. The frequency of keratin variants presented here is

somewhat lower compared with those in patients who suffer from other types of liver disease (e.g., 13.1% and 12.4%^[20,21]), as previously reported, but it is close to that observed in an Asian cohort in a United States study^[22]. Multiple K8/K18 variants have been verified and R341H represents the most common amino acid-altering K8/K18 variant in Chinese populations. As confirmed herein, the K8 variant R341H also exclusively associates with the intronic K8 IVS7+10delC deletion^[19]. We also found a new amino acid-altering variant N193S of K18 in a patient who simultaneously carried an intronic variant IVS3-15C→G. It remains to be determined whether this nucleotide error at one site acts in conjunction with the other.

Hepatic steatosis occurs when insulin signaling is impaired, with the development of IR driven by adipose tissue and the liver, along with the sustained excess delivery of fatty acids to the liver. Here, we

Table 2 Distribution of keratin variants in non-alcoholic fatty liver disease patients and controls

Keratin gene	Variant		NAFLD			
	Nucleotide	Amino acid	Control (<i>n</i> = 173)	Total (<i>n</i> = 200)	NAFLD with IR (<i>n</i> = 79)	NAFLD without IR (<i>n</i> = 121)
K8	1022G→A	R341H	1	2	2	0
	904C→T	R302C	0	1	1	0
	1381G→A	V461M	0	1	0	1
	IVS5-9A→G (new)		0	1	1	0
	IVS3-26 C→T		0	1	1	0
K18	IVS6+19G→A (new)		0	1	1	0
	IVS6+17C→T		0	1	0	1
	IVS3-15C→G		0	1 ¹	1 ¹	0
	1590C→G	N193S (new)	0	1 ¹	1 ¹	0
	1448A→G	T195T (new)	0	1	0	1
Total (%)			1 (0.58)	10 (5.00)	7 (8.86)	3 (2.48)

¹One patient carries two K8/K18 variants (K18 N193S+K8 IVS3-15C→G). The table displays the number of patients with NAFLD and controls harboring the listed keratin variants. IR: Insulin resistance; NAFLD: Non-alcoholic fatty liver disease.

Table 3 Clinical features of non-alcoholic fatty liver disease patients harboring significant keratin variants

	Keratin variant carriers		<i>P</i> value
	No (<i>n</i> = 190)	Yes (<i>n</i> = 10)	
Sex (male, %)	155 (81.58)	9 (90.00)	0.436
Age (yr)	40.75 ± 9.87	42.60 ± 10.63	0.603
Diastolic pressure (mmHg)	82.67 ± 10.47	85.80 ± 9.38	0.330
Systolic pressure (mmHg)	130.79 ± 14.82	134.20 ± 17.07	0.550
BMI (kg/m ²)	26.26 ± 2.91	26.10 ± 3.09	0.877
ALT (U/L)	35.93 ± 18.35	38.40 ± 25.30	0.767
AST (U/L)	34.03 ± 13.08	30.40 ± 9.31	0.265
TC (mmol/L)	5.67 ± 1.02	6.10 ± 1.69	0.453
TG (mmol/L)	2.85 ± 2.14	3.00 ± 2.35	0.850
HDL (mmol/L)	1.09 ± 0.24	1.10 ± 0.18	0.828
LDL (mmol/L)	3.31 ± 0.94	3.65 ± 1.12	0.374
FBG (mmol/L)	5.38 ± 1.43	5.91 ± 1.44	0.281
FINS (mmol/L)	11.17 ± 4.52	12.73 ± 6.00	0.439
HOMA-IR	2.64 ± 1.14	3.25 ± 1.50	0.237
UA (μmol/L)	414.06 ± 96.89	381.50 ± 89.88	0.292

Data are presented as mean ± SD. BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TC: Total cholesterol; TG: Triglyceride HDL: High-density lipoprotein; LDL: Low-density lipoprotein; FBG: Fasting blood glucose; FINS: Fasting insulin; UA: Uric acid.

observed a correlation of IR with keratin variants. This association suggests that K8/K18 mutations result in increased liver fat, probably through the hepatic IR, in accordance with other studies. For example, early work using K8-null mice found reduced fasting blood glucose levels, increased glucose tolerance and insulin sensitivity, altered insulin vesicle morphology, and reduced pancreatic insulin levels^[14]. Similarly, a study conducted in Finland shows that K8/K18 loss in hepatoma cells leads to distinct alterations in HK status, which are associated with a differential modulation of insulin signaling-dependent regulation of glucose-mediated glycogen formation and proliferative capacity^[23]. However, differences in K8/K18 IF loss cause the mislocalization of the GLUT 1-4 transport proteins. Additionally, it is important to consider the cellular context, namely, cultured hepatic cells^[23] *vs in*

vivo embryonic^[24] conditions. This reveals distinctive increases in glucose uptake, glucose-6-phosphate formation, lactate release, and glycogen formation in K8/K18 IF- deficient hepatocytes *vs* respective IF-containing counterparts. Moreover, compelling evidence suggests that the shape and distribution of mitochondria are modulated by cytoskeletal proteins, including keratins^[25]. Together, these findings suggest that hepatocyte keratins have a central role in the systemic regulation of glucose. Although the molecular mechanisms that underlie these metabolic perturbations are unclear, K8/K18 IFs likely modify liver fat content via the insulin signaling pathway^[26,27].

This study was limited by the absence of liver biopsies to evaluate the severity of hepatic steatosis and fibrosis stage. The absence of histological evidence may weaken the clinical relevance of these genetic effects, although abdominal ultrasound is generally applied in larger surveys as a noninvasive and convenient tool to diagnose NAFLD^[12]. Moreover, cell-based and transgenic mouse studies with genetic K8/K18 mutants or knockouts are needed to verify that keratin variants can cause or predispose carriers to the development of pathologies.

As studies of Asian populations have been limited, we are the first to discuss an association between keratin variants and NAFLD. Our findings provide novel evidence for keratin proteins as modulators in the development of NAFLD and in the pathophysiology of IR. Further studies of K8/K18 mutations as drivers of IR and liver fat accumulation are needed. These observations raise the question of how naturally occurring keratin mutations affect liver fatty in humans, and whether such mutations may similarly affect *in vitro* models or animals.

COMMENTS

Background

Keratins 8 and 18 (K8/K18) protect hepatocytes from various types of injury, and previous reports have suggested that K8/K18 variants predispose carriers

to various forms of liver injury. However, it is not known whether K8 or K18 variants have an association with non-alcoholic fatty liver disease (NAFLD). Thus, we tested the hypothesis that K8/K18 variants predispose humans to NAFLD progression and its metabolic phenotypes.

Research frontiers

The relationship between keratin variants and liver disease varies in different populations. Therefore, studies enrolling histological evidence and more ethnic groups are required. Further studies are also needed to determine the associations and functional consequences of these variants in NAFLD.

Innovations and breakthroughs

This is the first published study to investigate the relationship between keratin variants and NAFLD. These findings also provide novel evidence for keratin proteins as modulators in the development of NAFLD and in the pathophysiology of insulin resistance.

Applications

The association of keratin variants with NAFLD suggests a prominent gene influence on human NAFLD. They should pay more attention to individuals who carries keratin variant, and provide early intervention before disease progression.

Terminology

Keratins constitute the largest subgroup of intermediate filaments and are expressed primarily in epithelial tissues, hair and skin appendages. NAFLD is the most common cause of liver dysfunction in the Western world. It is a spectrum of liver disease that includes simple steatosis, fatty infiltration plus inflammation, and hepatocellular ballooning degeneration, progressing to fibrosis and ultimately cirrhosis.

Peer-review

This is a very interesting human study that suggests an association between keratin 8/18 variants and NAFLD. Also the authors successfully showed that IR is a potential mediator of this association. These are completely novel viewpoints in NAFLD research. This study is a good start for future research in this field.

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P- Reviewer: Higuera-de la Tijera MF, Ikura Y **S- Editor:** Gong ZM
L- Editor: Wang TQ **E- Editor:** Zhang FF





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ISSN 1007-9327

