Genome-wide analysis of DNA methylation in human peripheral leukocytes identifies potential biomarkers of nonalcoholic fatty liver disease

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Abstract. The aim of the present study was to uncover the role of leukocytic DNA methylation in the evaluation of nonalcoholic fatty liver disease (NAFLD). Patients with biopsy-proven NAFLD (n=35) and normal controls (n=30) were recruited from Chinese Han population. Their DNA methylation in peripheral leukocytes was subjected to genome-wide profiling. The association between differential methylation of CpG sites and NAFLD was further investigated on the basis of histopathological classification, bioinformatics, and pyrosequencing. A panel of 863 differentially methylated CpG sites dominated by global hypomethylation, characterized the NAFLD patients. Hypomethylated CpG sites of Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552) and carnitine palmitoyltransferase 1C (CPT1C) (cg21604803) associated with the increased risk of NAFLD [cg15536552, odds ratio (OR): 11.44, 95% confidence interval (CI): 1.04-125.37, P=0.046; cg21604803, OR: 6.57, 95% CI: 1.02-42.15, P=0.047] at cut-off β-values of <3.36 (ACSL4 cg15536552) and <3.54 (CPT1C cg21604803), respectively, after the adjustment of age, sex, body mass index (BMI) and homeostasis model assessment of insulin resistant (HOMA-IR). Their methylation levels also served as biomarkers of NAFLD (ACSL4 cg15536552, AUC: 0.80, 95% CI: 0.62-0.98, P=0.009; CPT1C cg21604803, AUC: 0.78, 95% CI: 0.65-0.91, P=0.001). Pathologically, lowered methylation level (β-values <3.26) of ACSL4 (cg15536552) conferred susceptibility to nonalcoholic steatohepatitis (NASH). Taken together, genome-wide hypomethylation of peripheral leukocytes may differentiate NAFLD patients from normal controls. The leukocytic hypomethylated ACSL4 (cg15536552) was suggested to be a biomarker for the pathological characteristics of NAFLD.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is a metabolic-related liver disease associated with obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM), and other components of the metabolic syndrome (MetS). Proportionally, it is now the most common chronic liver disease worldwide (1). The spectrum of NAFLD ranges from nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma (HCC). Multiple mechanisms are considered to underlie NAFLD, such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and bacterial endotoxins (1).

It is well known that genetic factors play an important role in NAFLD. Recently, epigenetic factors (DNA methylation, histone modifications and noncoding RNA) have also been uncovered to serve as the molecular basis of NAFLD (2,3). Of these epigenetic factors, DNA methylation reflects a level of epigenetic regulation that is closely linked to transcription factor (TF) binding and chromatin accessibility. Upregulated DNA methylation at the gene promoter usually leads to transcriptional repression (4,5). Accumulating proofs have revealed that DNA methylation takes a critical role in the regulation of IR, obesity and T2DM, which are major risk factors for NAFLD. Moreover, methyl-depleted diets are reported to promote NASH in animal models, whereas methyl-rich diets prevent NASH (6,7). Locus-specific and global hypomethylation are also associated with NAFLD (8-10). Thus, DNA methylation may be of great importance in the initiation and progression of NAFLD.

Despite its accuracy in NAFLD diagnosis, liver biopsy is limited in clinical application because of its invasiveness. Ultrasound, another widely used method for NAFLD diagnosis, displays poor sensitivity in patients with mild steatosis (<33%) (11,12). Thus, biomarkers for noninvasive, early diagnosis of NAFLD remained to be explored until

now. Fortunately, DNA methylation of peripheral leukocytes is currently employed, serving as alternative for that of organic tissue, in the diagnosis of various diseases (13-16). For example, lowered level of DNA methylation in peripheral blood reflects early T2DM (13). A nested case-control study showed that global hypomethylation in leukocytes may be useful biomarker of HCC susceptibility (16). Genomic DNA methylation in peripheral leukocytes is associated with gastric cancer in a population-based, case-control study (15).

We, therefore, enrolled NAFLD patients and normal controls from Chinese population so as to profile leukocytic DNA methylation. The association between DNA methylation and hepatic pathology was then subjected to evaluation. Characteristic sites of NAFLD-related methylation were filtered, and further assessed for noninvasive diagnosis.

Patients and methods

Study population. A total of 65 unrelated adults (aged 18-70 years) were recruited from March, 2012 to May, 2013. Of these, NAFLD patients were enrolled from Xinhua Hospital, Shanghai, China (n=14); Tianjin Hospital of Infectious Diseases, Tianjin, China (n=4) and Zhengxing Hospital, Zhangzhou, Fujian, China (n=17), respectively. Thirty healthy controls were recruited accordingly. All subjects were Han Chinese in origin. Each patient underwent both ultrasound-guided percutaneous liver biopsy and FibroScan® 502 (Echosens, Paris, France) examination, and met the diagnostic criteria for NAFLD (1). The exclusion criteria were as follows: i) excessive alcohol consumption (>30 g/day for males and >20 g/day for females); ii) other diseases that led to fatty liver, such as chronic hepatitis C, drug-induced liver injury, Wilson's disease, total parenteral nutrition, and autoimmune hepatitis; iii) previous liver transplantation; and iv) other end-stage disease or malignancy. All control subjects were confirmed to be free of liver diseases by B-mode ultrasound and FibroScan examination [controlled attenuation parameter (CAP) <240 dB/m and liver stiffness measurement (LSM) values <7.0 kPa] (17,18). These subjects demonstrated normal liver function without evidence of liver injury. The study protocol was approved by the Ethics Committee of Xinhua Hospital in accordance with the Declaration of Helsinki. All participants were enrolled under informed consent.

Clinical and laboratory evaluation. Demographic and anthropometric measurements were carried out in NAFLD patients and normal controls, including sex, age, body mass index [BMI, weight (kg)/height $(m)^2$], and waist-to-hip ratio [WHR, waist circumference (cm)/hip circumference (cm)]. Biochemical tests were conducted, including fasting blood glucose (FBG), fasting serum insulin, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl-transpeptidase (GGT), total bilirubin (TBIL), direct bilirubin (DBIL), and uric acid (UA). All biochemical parameters were measured using a conventional automated analyzer (7600; Hitachi, Tokyo, Japan). Homeostasis model assessment of insulin resistant (HOMA-IR) was used to evaluate IR: HOMA-IR = fasting serum insulin (μIU/ml) x fasting plasma glucose (mmol/l)/22.5.

Liver histology. Percutaneous liver biopsy was performed in all NAFLD patients under real-time ultrasound guidance. Biopsy specimens were then formalin-fixed, paraffin-embedded, sectioned, and treated with hematoxylin and eosin (H&E), Masson's and reticulin staining. Histological changes were assessed according to Kleiner's classification, with the NALFD activity score (NAS) based on steatosis, lobular inflammation and hepatocyte ballooning (19). Patients with NAS <3 were excluded of NASH. While those with NAS of 3-4 (at least 1 for ballooning degeneration), and ≥5 were diagnosed to be borderline NASH, and NASH, respectively. Liver fibrosis was staged as follows: F0, none; F1, perisinusoidal or portal fibrosis; F2, perisinusoidal and periportal fibrosis without bridging; F3, bridging fibrosis; and F4, cirrhosis (20).

DNA extraction and bisulfite conversion. Genomic DNA was extracted from peripheral blood samples obtained from each subject using the nucleic acid extraction kit (Qiagen, Hilden, Germany). The DNA quality was determined by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Bisulfite conversion of DNA (500 ng/sample) was then performed by the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol, with a modified thermo-cycling procedure as suggested by Illumina (San Diego, CA, USA). The protocol included 16 cycles of denaturing at 95˚C for 30 sec, incubation at 50˚C for 60 min, and a final holding step at 4˚C.

Methylation analysis. The Human Methylation 450K BeadChip (Illumina) was used to analyze the genome-wide DNA methylation profile across 485 577 loci distributed in promoters, gene bodies, 3'-untranslated regions (3'-UTR), and intergenic regions, respectively. In detail, bisulfite-converted DNA (4 μl) was hybridized with the Methylation 450K BeadChip following the Illumina Infinium HD Methylation protocol. Illumina's Genome Studio® Methylation module version 1.0 (Illumina) was employed to calculate the methylation level at each CpG site by β-value [β=intensity of the methylated allele (M)/(intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100)]. The obtained β-values, ranging from 0 (fully unmethylated) to 1 (fully methylated), was further normalized by R package (Partek Inc., St. Louis, MO, USA) (21). Resultantly, average Δ-β-value was calculated to indicate the differential methylation between NAFLD patients and controls. Quality control inclusion valuation depended on hybridization detection P-values of <0.05. DNA loci with differential methylation were filtered by Δ-β-value and DiffScore as follows (21,22): $\text{DiffScore}=10\text{Sign}(\beta_{\text{NAFLD}}-\beta_{\text{controls}})\log_{10}P$.

Bioinformatics analysis. The effect of differentially methylated sites on biological functions was analyzed by gene ontology (GO). Moreover, KEGG algorithm was used to identify signaling pathways that significantly related to the differentially methylated sites. The false discovery rate (FDR) was employed to exclude false positive results (23,24).

Pyrosequencing. Acyl-CoA synthetase long-chain family member 4 (ACSL4) was filtered for validation using pyrosequencing. In detail, forward, GTGATGGATTTTGGGGTTTT,

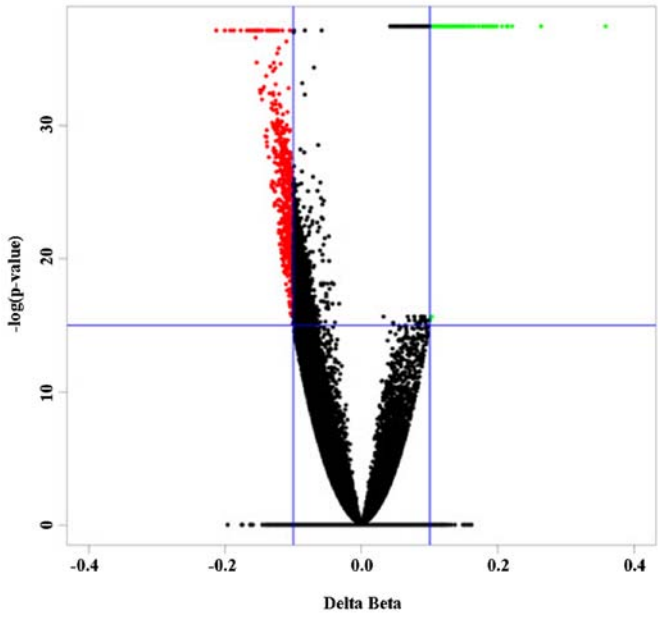

Figure 1. Volcano plot of DNA methylation profile in the peripheral leukocytes of both nonalcoholic fatty liver disease (NAFLD) patients and normal controls. Δ-β-values (NAFLD vs. controls) are shown on the x-axis. Lg-converted P-values are shown on the y-axis. The red and green dots are hypomethylated and hypermethylated CpG sites, respectively.

reverse, AAAACTCCCTAACCCTCAATTAC and sequencing primers, GTATTTAGAGGGTTAGAAGTTAT were designed with PyroMark Assay Design software (version 2.0; Qiagen). Bisulfite treated DNA was amplified by PCR using the PyroMark PCR kit (Qiagen). The pyrosequencing assay was then performed on a PyroMark Q96 instrument using the PyroMark Gold Q96 kit (both from Qiagen). The sequencing results were analyzed using the PyroMark CpG software (Qiagen).

Statistical analysis. Continuous variables were determined by the unpaired Student's t-test (data with normal distribution) or Mann-Whitney U test (data with skewed distribution). Comparative analyses of categorical variables were carried out using the Chi-square test. Fisher's exact test and Chi-square test were employed to classify the GO category and significant signaling pathways, respectively. Two-way ANOVA was used to compare groups with two independent variables (age and sex). Because of its non-normal distribution, methylation level of CpG sites was analyzed after $\log_2$ transformation. The optimal cut-off value for each CpG site was determined by maximizing the Youden index. CpG sites with methylated levels ≥ and < optimal cut-off were defined as high, and low methylation, respectively. Multivariate regression analysis was then performed to identify independent risk CpG sites for NAFLD or NASH. In addition, the diagnostic efficiency for risk CpG sites was analyzed by area under the receiver operating characteristic curves (AUC) and 95% confidence interval (CI). All statistical analyses were performed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). A two-tailed P<0.05 was considered statistically significant. The statistical methods used in this study were reviewed by Dr Guang-Yu Chen from Clinical Epidemiology Center, Shanghai Jiao Tong University.

Table I. Clinical characteristics of NAFLD patients and normal controls.

Characteristics	NAFLD (n=35)	Controls (n=30)	P-value
Gender (M/F)	27/8	18/12	0.118
Age (years)	37.72±12.25	46.63±7.24	0.002
WHR	0.95±0.04	0.86±0.06	<0.0001
BMI (kg/m^2)	27.58±3.54	23.02±2.63	<0.0001
ALT (U/l)	71.99±53.46	15.60±4.42	<0.0001
ALP (U/l)	95.30±41.02	90.20±23.46	0.885
GGT (U/l)	81.46±65.23	14.67±5.49	<0.0001
TG (mmol/l)	2.25±1.55	0.82±0.34	<0.0001
TC (mmol/l)	4.94±0.91	4.34±0.61	0.004
HDL-C (mmol/l)	1.21±0.30	1.43±0.25	0.003
LDL-C (mmol/)	2.97±0.96	2.29±0.49	<0.0001
UA (μmol/l)	378.62±113.51	257.83±62.14	<0.0001
FBG (mmol/l)	5.90±2.80	5.29±0.39	0.595
Insulin (μU/ml)	7.29±5.24	3.86±2.48	<0.0001
HOMA-IR	1.68±1.03	0.90±0.56	<0.0001
CAP (dB/m)	310 (277-359)	195 (174-228)	<0.0001
LSM (kPa)	7.60 (5.40-12)	4.15 (3.70-4.70)	<0.0001

NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; WHR, Waist-to-hip ratio; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; UA, uric acid; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment of IR; CAP, controlled attenuation parameter; LSM, liver stiffness measurement.

Results

Clinical characteristics of the study population. The clinical characteristics of the study population are outlined in Table I. When compared to those of control group, levels of BMI, WHR, ALT, GGT, TG, TC, HDL-C, LDL-C, UA, CAP and LSM were significantly higher in the NAFLD group (P<0.01-0.05).

DNA methylation profile of peripheral leukocytes differentiated NAFLD patients from normal controls. When compared to those of normal controls, a panel of 863 differentially methylated (DM) CpG sites in peripheral blood cells characterized the NAFLD patients (Fig. 1). Among these DM sites, 183 (21.2%) were hypermethylated in NAFLD patients, whereas 680 sites (78.8%) were hypomethylated.

According to genomic location, CpG sites distribute in CpG islands (CGIs), shores [0-2 kilobase (kb) from CpG islands], shelves (2-4 kb from CpG islands) and other/open sea, respectively. On the other hand, the location of CpG sites is classified as promoter, gene body, 3'-UTR and intergenic, respectively. The promoter is further divided into surrounding transcription sites (TSS -200 to -1500 bp, TSS200 and TSS1500), 5'-UTR and 1st exon, respectively (Fig. 2).

In the present study, most DM CpG sites (57.36%) localized in CGIs, followed by 20.97 and 15.99% in the other/open sea and shore regions, respectively. On the contrary, these CpG sites

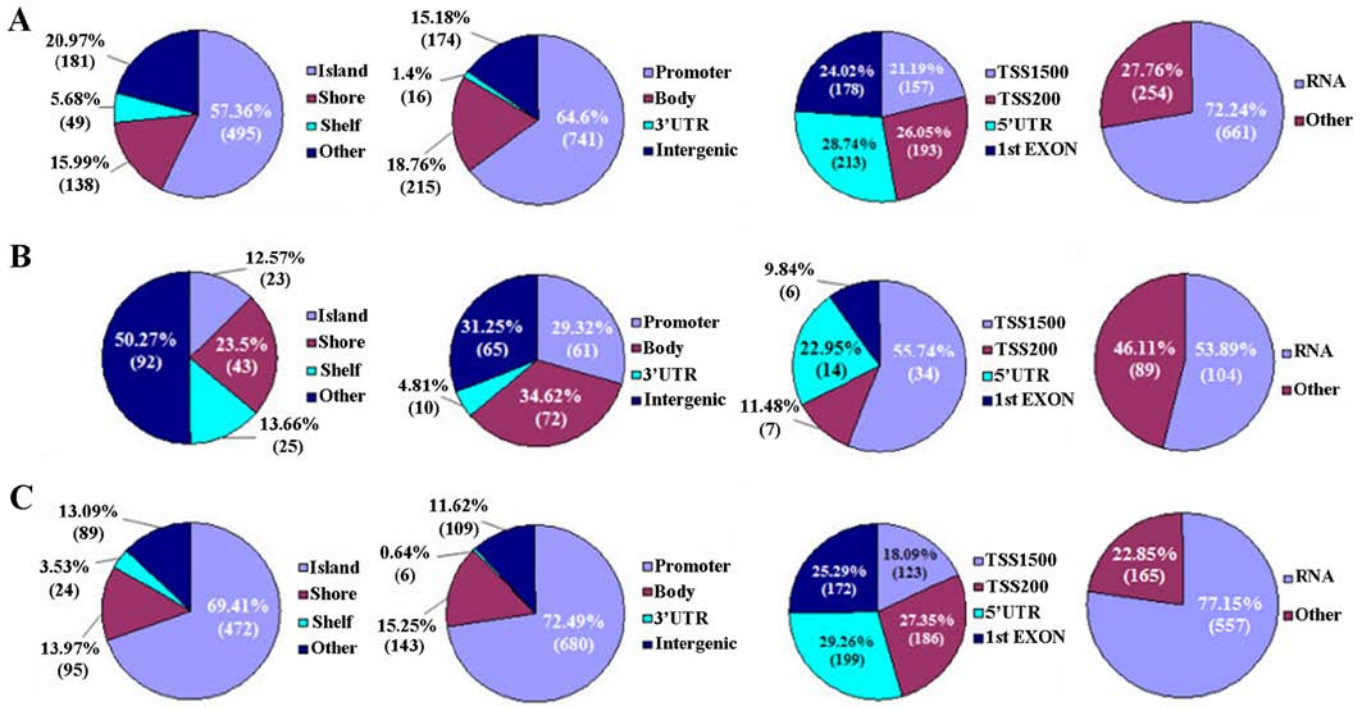

Figure 2. Genomic location of differentially methylated loci in the peripheral leukocytes of nonalcoholic fatty liver disease (NAFLD) patients. (A-C) Percentage of CpG methylation location (island, shore, shelf and other); functional location (promoter, body, 3'-untranslated region (3'-UTR), and intergenic regions); sub-location of promoter regions (TSS1500, TSS200, 5'-UTR and 1st exon) and methylation differences by RNA coding and noncoding regions in total [(A) hypermethylated and hypomethylated loci], (B) hypermethylated and (C) hypomethylated loci groups, respectively.

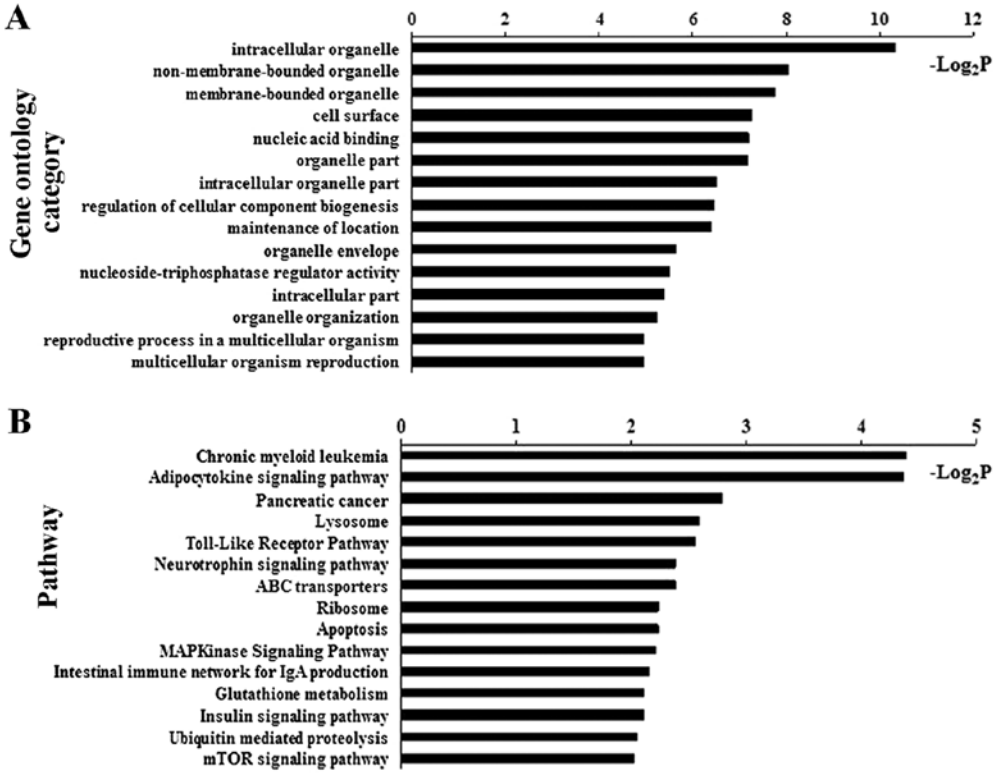

Figure 3. Functional analysis of differentially methylated loci in nonalcoholic fatty liver disease (NAFLD) patients. (A) GO analysis of genes with differentially methylated loci in NAFLD patients. (B) Pathway analysis of genes with differentially methylated loci. x-axis, negative logarithm of the P-value ($-\log_2 P$). The larger the value, the smaller the P-value. y-axis, the name of the gene ontology category, significant GO terms, and pathways of differentially methylated genes.

were enriched in promoter (64.60%), and less likely to be in the gene body (18.76%), intergenic regions (15.18%), and 3'-UTR (1.4%). In the promoter, the DM CpG sites usually distributed in the TSS1500, TSS200, 5'-UTR and 1st exon regions (Fig. 2A).

Table II. Signaling pathways related to differentially methylated genes.

Pathways	P-value	Genes
Chronic myeloid leukemia	0.047	GAB2, ARAF, IKBKG, TGFB3, SMAD3
Adipocytokine signaling pathway	0.048	ACSL4, CPT1C, IRS4, IKBKG
Pancreatic cancer	0.144	ARAF, IKBKG, TGFB3, SMAD3
Lysosome	0.165	LAMP2, AP4E1, IDS, ATP6AP1, GAA
Toll-like receptor pathway	0.170	IRAK1, IKBKG, ELK1

GAB2, GRB2-associated binding protein 2; ARAF, A-Raf proto-oncogene, serine/threonine kinase; IKBKG, inhibitor of κ light polypeptide gene enhancer in B-cells, kinase γ; TGFB3, transforming growth factor β3; SMAD3, SMAD family member 3; ACSL4, acyl-CoA synthetase long-chain family member 4; CPT1C, carnitine palmitoyltransferase 1C; IRS4, insulin receptor substrate 4; LAMP2, lysosomal-associated membrane protein 2; AP4E1, adaptor-related protein complex 4, epsilon 1 subunit; IDS, iduronate 2-sulfatase; ATP6AP1, ATPase, H^+ transporting, lysosomal accessory protein 1; GAA, glucosidase α; acid; IRAK1, interleukin-1 receptor-associated kinase 1; ELK1, member of ETS oncogene family.

Table III. Difference in DNA methylation between NAFLD patients and normal controls.

	NAFLD (n=35)	Control (n=30)	P-value
ACSL4			
cg15536552	3.29 (3.09-3.60)	3.86 (3.37-6.12)	0.009
cg06822229	2.57 (2.23-3.45)	3.11 (2.75-6.14)	0.023
cg08855111	3.31 (3.12-3.75)	3.49 (3.18-6.15)	0.354
cg19635884	0.87 (0.08-1.71)	1.00 (0.46-5.86)	0.119
cg26119746	2.89 (2.70-3.15)	3.06 (2.57-6.09)	0.767
cg09091181	0.82 (0.34-1.36)	1.01 (0.32-5.86)	0.506
cg10721440	0.62 (0.13-1.44)	0.80 (0.17-5.88)	0.354
CPT1C			
cg21604803	3.22 (2.87-3.53)	3.64 (3.24-4.11)	0.004
IRS4			
cg06779802	3.28 (2.79-4.52)	3.93 (3.09-6.10)	0.061
IKBKG			
cg08417382	2.42 (2.06-3.17)	2.71 (2.15-6.35)	0.248
cg02869694	2.13 (1.79-2.61)	2.16 (1.75-6.19)	0.418
cg00813156	2.27 (2.08-2.64)	2.35 (2.10-6.18)	0.549
cg08560117	2.70 (2.55-3.02)	2.92 (2.60-6.01)	0.266
cg08873063	3.22 (3.09-3.64)	3.51 (3.17-6.06)	0.068

The methylated level (%) was used after $\log_2$ transformation and expressed as medians (IQR). NAFLD, nonalcoholic fatty liver disease; ACSL4, acyl-CoA synthetase long-chain family member 4; CPT1C, carnitine palmitoyltransferase 1C; IRS4, insulin receptor substrate 4; IKBKG, inhibitor of κ light polypeptide gene enhancer in B-cells, kinase γ.

Methylatively, hypermethylated CpG sites dominated the other/open sea (50.27%) and gene body (34.62%), respectively. In the promoter, most hypermethylated CpG sites (55.74%) localized in TSS500 (Fig. 2B). In contrast, the hypomethylated CpG sites predominantly located in CGIs (69.41%) and promoter (72.49%), respectively. In the promoter, these hypomethylated CpG sites were likely to localize in 5'-UTR (29.26%) (Fig. 2C). Taken together, the DM CpG sites (hyper- or hypomethylated sites) were predominantly distributed in the RNA coding regions of the genome (Fig. 2).

Differential methylation of the adipocytokine signaling pathway characterized NAFLD patients. Bioinformatic analysis identified GOs and signaling pathways significantly associated with the DM CpG sites in NAFLD patients (Fig. 3 and Table II). Of these, adipocytokine signaling pathway demonstrated one of the top-ranking pathways. Four critical genes, including ACSL4, carnitine palmitoyltransferase 1C (CPT1C), insulin receptor substrate 4 (IRS4) and inhibitor of κ light polypeptide gene enhancer in B-cells, kinase γ (IKBKG), of the adipocytokine signaling pathway were verified to be hypomethylated in their promoter (Table II).

Hypomethylated ACSL4 (cg15536552) and CPT1C (cg21604803) in adipocytokine signaling pathway conferred susceptibility to NAFLD. When compared to those in the control group, β-values of 2 loci in the ACSL4 gene (cg15536552 and cg06822229) and 1 locus the CPT1C gene (cg21604803) were markedly lower in the NAFLD group (ACSL4 cg15536552, P=0.009; ACSL4 cg06822229, P=0.023; CPT1C cg21604803, P=0.004) (Table III). No significant differences could be found in other loci between the NAFLD patients and controls.

After grouping by sex and age, the ACSL4 (cg15536552 and cg06822229) methylation level was proved to be significantly higher in females than in males, regardless whether NAFLD patients (ACSL4 cg15536552, P<0.0001; ACSL4 cg06822229, P<0.0001) or normal controls (ACSL4 cg15536552, P<0.0001; ACSL4 cg06822229, P<0.0001) (Fig. 4A and B). Nevertheless, male NAFLD patients demonstrated methylation levels of ACSL4 (cg15536552 and cg06822229) much lower than those in the normal controls correspondingly (ACSL4 cg15536552, P=0.007; ACSL4 cg06822229, P=0.035) (Fig. 4C and D). As compared to that of normal controls, the methylation values of ACSL4 (cg15536552) and CPT1C (cg21604803) were also significantly lower in NAFLD patients ≤50 years (ACSL4 cg15536552, P=0.009; CPT1C cg21604803, P=0.001) (Fig. 5).

Moreover, hypomethylation of ACSL4 (cg15536552) and CPT1C (cg21604803) was proved to increase the risk of NAFLD (ACSL4 cg15536552, OR: 10.50, 95% CI: 1.70-64.99, P=0.014; CPT1C cg21604803, OR: 7.67, 95% CI: 2.14-27.49, P=0.001). After adjusting for BMI and HOMA-IR, the hypomethylated ACSL4 (cg15536552) and CPT1C (cg21604803) still conferred susceptibility to NAFLD (ACSL4 cg15536552, OR: 11.44, 95% CI: 1.04-125.37, P=0.046; CPT1C cg21604803, OR: 6.57, 95% CI: 1.02-42.15, P=0.047) (Table IV). Their methylation levels, with cut-off β-values of 3.36 (ACSL4 cg15536552) and

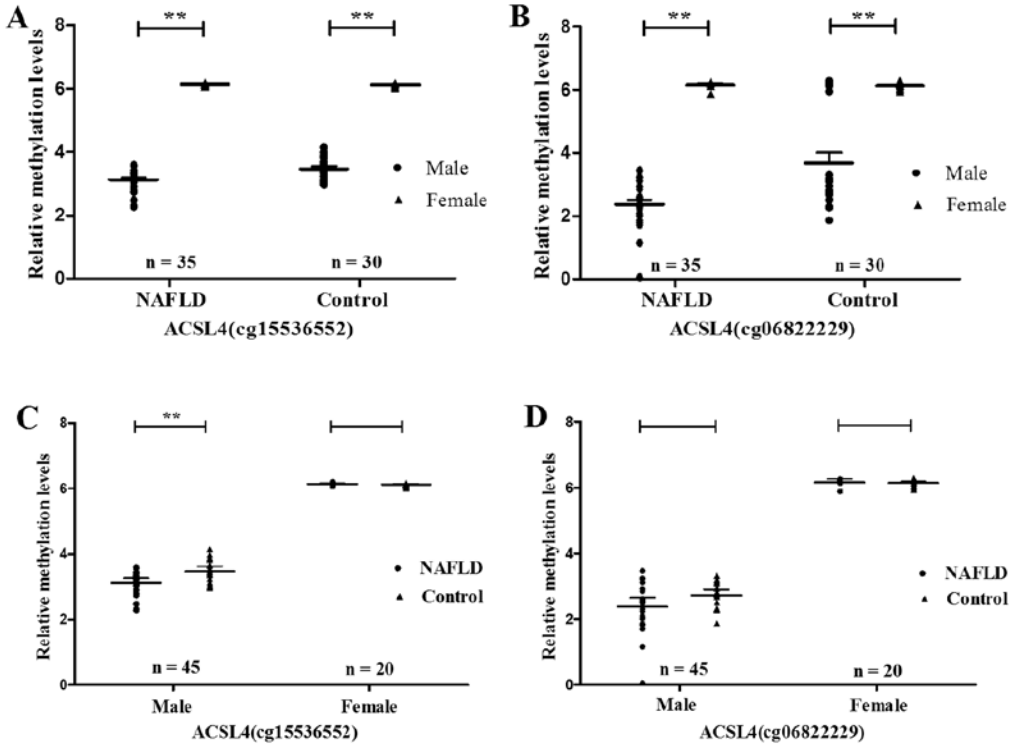

Figure 4. Comparison of methylated levels of Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552 and cg06822229) between sex of subjects. (A and B) Methylation levels of ACSL4 (cg15536552 and cg06822229) between sexs in both nonalcoholic fatty liver disease (NAFLD) and control groups. (C and D) Methylated levels of ACSL4 (cg15536552 and cg06822229) between NAFLD and control groups in both sexes. **P<0.01, statistically significant difference.

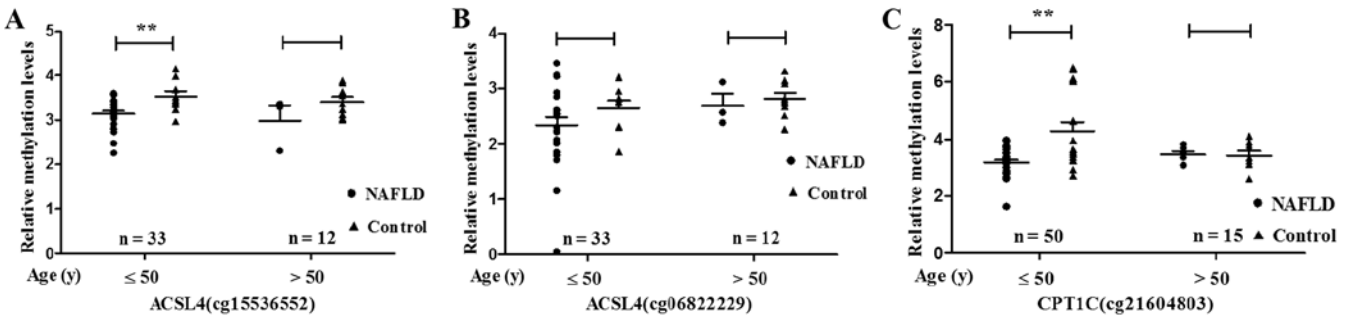

Figure 5. Methylation levels of (A and B) Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552 and cg06822229) and (C) CPT1C (cg21604803) in nonalcoholic fatty liver disease (NAFLD) patients and normal controls with different age (≤50 and >50 years). **P<0.01, statistically significant difference.

3.54 (CPT1C cg21604803), well differentiated NAFLD patients from normal controls (ACSL4 cg15536552, AUC: 0.80, 95% CI: 0.62-0.98, P=0.009, n=33; CPT1C cg21604803, AUC: 0.78, 95% CI: 0.65-0.91, P=0.001, n=50) (Fig. 6A and B).

Hypomethylation of ACSL4 (cg15536552) and CPT1C (cg21604803) associate with histopathological classification. Histopathologically, hypomethylation of ACSL4 (cg15536552) significantly associated with hepatic steatosis (S2-3 vs. S0, P=0.004) and NAS (≥3 vs. <3, P=0.021) (Fig. 7A and B), respectively, in male subjects. However, no significant correlation was found between ACSL4 (cg15536552) methylation and liver fibrosis (F2-4 vs. F0-1, P=0.501) (Fig. 7C). Despite its noncorrelation with NAS and fibrosis, hypomethylation of CPT1C (cg21604803) also conferred high risk to hepatic steatosis (S2-3 vs. S0, P=0.007) (Fig. 7D).

Moreover, patients with ACSL4 (cg15536552) hypomethylation showed increased risk for borderline/definitive NASH (OR: 8.56, 95% CI: 1.33-54.95, P=0.024). After adjusting for BMI and HOMA-IR, ACSL4 (cg15536552) hypomethylation qualify itself for the risk factor of borderline/definitive NASH (OR: 8.72, 95% CI: 1.29-58.78, P=0.026) (Table IV). By receiver operating characteristic (ROC) curve, decreased level of ACSL4 (cg15536552) methylation serve as an index, with the optimum cut-off β-values of 3.26, for borderline/definitive NASH in male NAFLD patients (AUC: 0.79, 95% CI: 0.60-0.97, P=0.019, n=24) (Fig. 6C).

Validation of ACSL4 (cg15536552) methylation by pyrosequencing. For the sake of its close association with borderline/definitive NASH, ACSL4 (cg15536552) was subjected to validation in male NAFLD patients aged ≤50 (n=24).

Table IV. ACSL4 (cg15536552) and CPT1C (cg21604803) methylation associated with NAFLD and borderline/definitive NASH.

Methylation level	NAFLD, n (%)	Control, n (%)	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
ACSL4 (cg15536552)						
High (≥3.36)	6 (25)	7 (77.78)	-			
Low (<3.36)	18 (75)	2 (22.22)	10.50 (1.70-64.99)	0.014	11.44 (1.04-125.37)[a]	0.046
CPT1C (cg21604803)						
High (≥3.54)	6 (20.69)	14 (66.67)	-		-	
Low (<3.54)	23 (79.31)	7 (33.33)	7.67 (2.14-27.49)	0.001	6.57 (1.02-42.15)1	0.047
Methylation level	NAS ≥3, n (%)	NAS <3, n (%)	OR (95% CI)	-	Adjusted OR (95% CI)	-
ACSL4 (cg15536552)						
High (≥3.26)	3 (21.43)	7 (70)	-		-	
Low (<3.26)	11 (78.57)	3 (30)	8.56 (1.33-54.95)	0.024	8.72 (1.29-58.78)[a]	0.026

[a]Adjusted OR, odds ratios adjusted for BMI, HOMA-IR; 95% CI, 95% confidence interval; NAFLD, nonalcoholic fatty liver disease; ACSL4, acyl-CoA synthetase long-chain family member 4; CPT1C, carnitine palmitoyltransferase 1C.

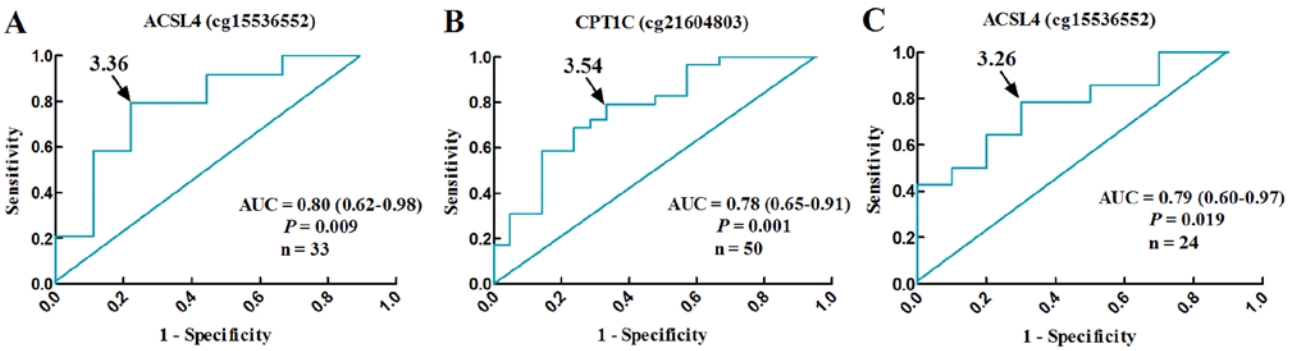

Figure 6. Receiver operating characteristic (ROC) curves for Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552) and CPT1C (cg21604803). (A) ROC curve for ACSL4 (cg15536552) methylation in the diagnosis of nonalcoholic fatty liver disease (NAFLD) in male subjects aged ≤50 (n=33). (B) ROC curve for CPT1C (cg21604803) methylation in the diagnosis of NAFLD in subjects aged ≤50 (n=50). (C) ROC curve for ACSL4 (cg15536552) methylation in the diagnosis of borderline/definite nonalcoholic steatohepatitis (NASH) in male NAFLD patients aged ≤50 (n=24). The arrow shows the optimum cut-off β-value.

Indeed, pyrosequencing displayed methylation level of ACSL4 (cg15536552) in parallel to that of methylation 450K BeadChip (r=0.756, P<0.0001) (Fig. 8A). The methylation level of ACSL4 (cg15536552) was also statistically decreased in patients with borderline/definite NASH (NAS ≥3) (P=0.004) (Fig. 8B).

Discussion

NAFLD is generally considered to be the result of environmental, genetic, and epigenetic disorders. DNA methylation reflects one of the most important patterns of epigenetic modification, and mediates cross-talk between environmental and epigenetic factors. To shed light on its role in NAFLD, DNA methylation was recently subjected to profiling in liver tissue. First, hepatic DNA methylation was analyzed by genome-wide profiling in patients with mild to advanced NAFLD (10). Second, global analysis of DNA methylation was carried out, before and after bariatric surgery, in morbidly obese patients with NAFLD (25). Strikingly, altered methylation in CpG sites of genes (i.e., *PC*, *ACLY*, *PLCG1*, *IGF1*, *IGFBP2*, *PRKCE*, *ZNF274*, *FGFR2*, *MAT1A* and *CASP1*), which regulated glycolipid metabolism, steatohepatitis, fibrosis and carcinogenesis, demonstrated close association with NAFLD in both studies (10,26). Hypermethylated promoter of peroxisome proliferator-activated receptor γ co-activator 1a (PPARGC1A) significantly correlated to plasma fasting insulin and HOMA-IR (8). Hypermethylation in the promoter of glucokinase and L-type pyruvate kinase, which lead to down-regulation of their transcription, is related to both impaired insulin sensitivity and NAFLD in high-fat diet induced animal models (9,27). Differences in DNA methylation even distinguish patients with advanced versus mild NAFLD (10,26). DNA methylation, therefore, is suggested to be an epigenetic factor deeply involved in NAFLD.

In spite of its critical role in NAFLD, DNA methylation of liver tissue is prevented from experimental and clinical application due to the difficulty in sample collection. Fortunately, peripheral leukocytes instead of organic tissue, has now been employed in the profiling of DNA methylation related to various diseases, such as cancers and myelodysplastic syndrome (13,14,16,25,28). As compared to that of normal controls, a CpG site in the first intron of *FTO* shows significant hypomethylation in the peripheral leukocytes of patients with type 2 diabetes (13). Increased risk for different types of cancer

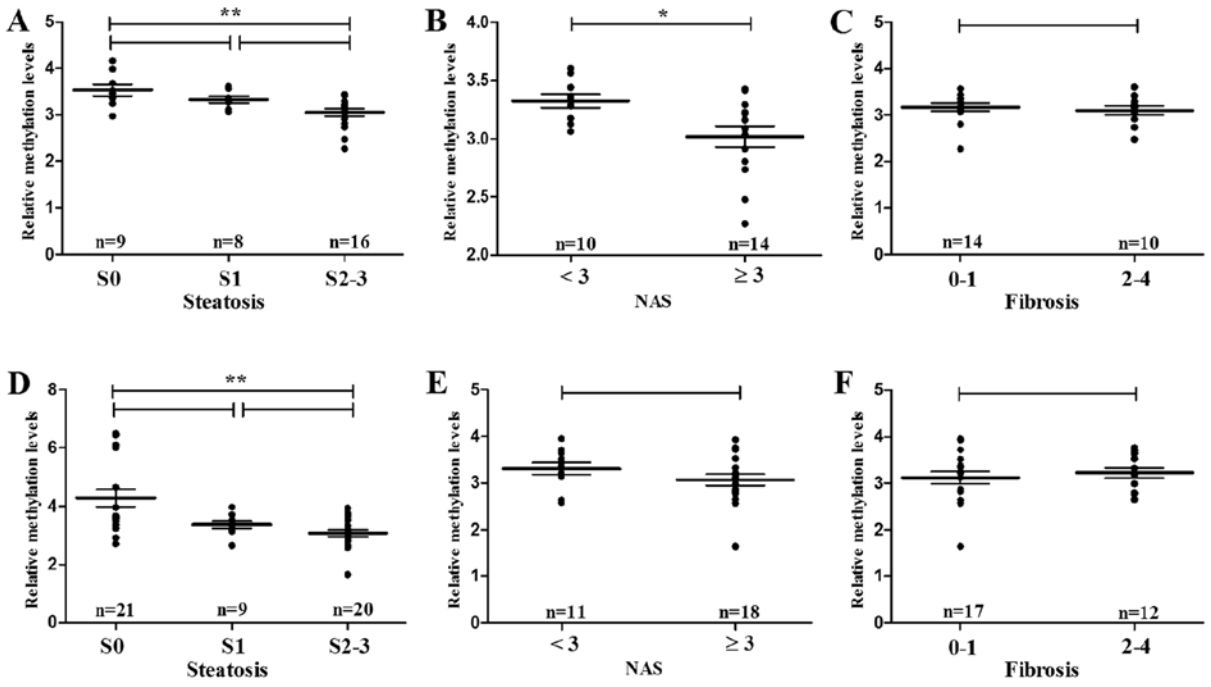

Figure 7. Methylation levels of Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552) and CPT1C (cg21604803) were associated with the histopathologic characteristics of nonalcoholic fatty liver disease (NAFLD). (A-C) Methylation level of ACSL4 (cg15536552) between different grades of (A) steatosis, (B) NAS and (C) fibrosis in males subjects aged ≤50 (n=33). (D-F) Methylation level of CPT1C (cg21604803) between different grades of (D) steatosis, (E) NAS and (F) fibrosis in subjects aged ≤50 (n=50). **P<0.01 and *P<0.05, statistically significant difference.

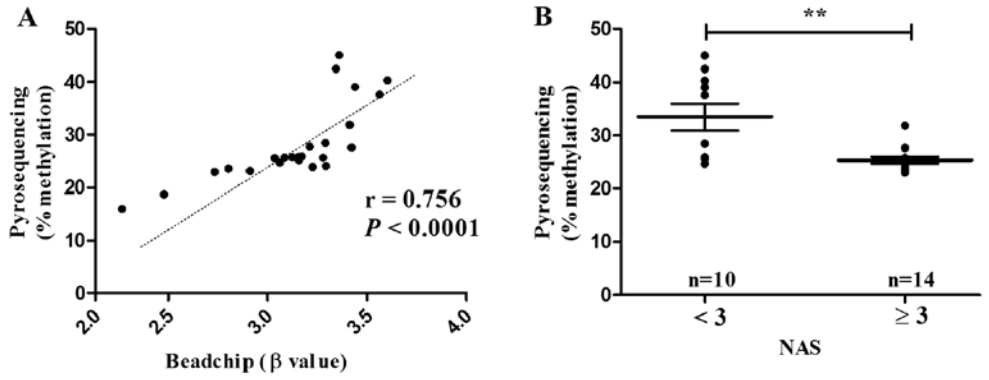

Figure 8. Validation of Acyl-CoA synthetase long-chain family member 4 (ACSL4) (cg15536552) methylation. (A) The correlation between results of methylation 450K BeadChip (β-value) and bisulfite pyrosequencing. (B) Comparison of ACSL4 (cg15536552) methylation between nonalcoholic fatty liver disease (NAFLD) patients with NAS ≥3 and <3. The β-value (% methylation) was employed after $\log_2$ transformation. **P<0.01, statistically significant difference.

(colon, bladder, stomach, breast, head and neck cancer) has been found in cohorts with the lowest quartile of leukocytic DNA methylation (15,29-35). Thus DNA methylation of peripheral leukocytes, serving as alternative for organic tissue to some extent, may be potential in the identification of NAFLD.

In the present study, genome-wide profiling of DNA methylation was performed in the peripheral leukocytes obtained from both NAFLD patients and normal controls. In total, 863 DM CpG sites characterized the groups of NAFLD patients. In detail, the percentage of hyper- and hypomethylated loci in the coding region was much higher than that in other regions of the genome. According to the non-coding region of genome, hypo- and hypermethylated loci were abundant in the CGIs of promoter and intergenic region, respectively. These findings indicate that the DM loci, although numerically limited, are highly enriched in the biological regions of the genome.

Dramatically, genomic hypomethylation, representing 78.8% of DM loci, reflected the methylatic characteristics of peripheral leukocytes in NAFLD patients. Hypo- rather than hypermethylation, with 76% of DM loci, already reported to dominate the genome of liver tissues in patients with advanced NAFLD (10). Hypomethylation also serves as the epigenetic characteristics of alcohol-related fatty liver disease (10). It is further uncovered to underlie the animal model of NASH induced by methyl-deficient diet (36). Taken together, hepatocytes and leukocytes of NAFLD patients share the same pattern of DNA methylation, that of global hypomethylation (10).

NAFLD-related DM loci of peripheral leukocytes were then subjected to bioinformatic analysis on the basis of GO and KEGG algorithm. Interestingly, multiple hypomethylated CpG sites were focused in critical genes, including ACSL4 (cg15536552, cg06822229, cg08855111, cg19635884, cg26119746, cg09091181

and cg10721440), CPT1C (cg21604803), IRS4 (cg06779802) and IKBKG (cg08417382, cg02869694, cg00813156, cg08560117 and cg08873063), of adipocytokine signaling pathway. When compared to those in the control group, NAFLD patients suffered from significantly lowered β-values in 2 loci of the ACSL4 gene (cg15536552 and cg06822229) and 1 locus of the CPT1C gene (cg21604803). ACSL4, an important enzyme regulating the intracellular level of unesterified arachidonic acid (AA), has been reported overexpressed in African-American patients with NAFLD or NASH (37). Hepatic fat content and steroid synthesis are also significantly associated with ACSL4 even after adjustment for BMI (38). Functional studies have shown that upregulated ACSL4 accelerates lipogenesis, whereas downregulated ACSL4 prevents the accumulation of cellular cholesterol (39). On the other hand, CPT1C (isoform of CPT1) plays a vital role in the mitochondrial β-oxidation, energy balance, and hepatic glucose homeostasis. These results are supposed to reflect the differentially methylated effect of both peripheral leukocytes and liver tissue in response to lipogenetic stimuli, and potentiate leukocytic DNA methylation to be noninvasive biomarker for NAFLD.

Indeed, statistically hypomethylated ACSL4 (cg15536552) and CPT1C (cg21604803) in peripheral leukocytes associated with the grade of liver steatosis in this study. Both loci were verified to be independent variables and risk factors of NAFLD, even after the adjustment for age, sex and BMI and HOMA-IR. Their accuracy for the diagnosis of NAFLD, as evaluated by ROC curves, was 0.80 of ACSL4 cg15536552, and 0.78 of CPT1C cg21604803, respectively. Moreover, the hypomethylation of ACSL4 (cg15536552) served as an independent risk factor for borderline/definitive NASH (NAS ≥3) after the adjustment for BMI and HOMA-IR. Its AUC for the detection of NAS (≥3) was proved to be 0.79. DNA methylation of peripheral leukocytes is qualified for the serum biomarker of NAFLD/NASH with moderate diagnostic efficiency. Noninvasive biomarker for hepato-pathological identification, especially NASH, has long been the focus of research and clinical interference. Although multiple indexes [i.e., cytokertin 18 (CK18), procollagen III, adiponectin, ferritin, tumor necrosis factor-α (TNF-α)] have been explored (40-42), limited diagnostic efficiency has made it a difficult task until now. The serum concentration of CK18, an index for hepatocyte apoptosis, exhibits the most promising AUC (0.82) for NASH diagnosis (40-42). Dramatically, hypomethylation of ACSL4 (cg15536552) showed comparable efficacy. Then combination of these 2 biomarkers may provide us with a better solution for the noninvasive diagnosis of NAFLD/NASH.

Some limitations of the study should be considered. First, the DNA methylation was only measured in peripheral leukocytes. Methylation pattern of whole blood has been reported to serve as a good proxy for methylation levels from a specific site of action (43,44). Consistently, both liver tissue and peripheral leukocytes exhibited global hypomethylation. Despite the similarity in methylation pattern, there were still different loci between liver tissue and peripheral leukocytes on the basis of tissue-specific methylation. Second, sample size of the present study was relative limited. Clinical trial with large cohorts would be preferable for the validation of our findings.

In conclusion, NAFLD patients demonstrate global DNA hypomethylation in peripheral leukocytes when compared to that of normal controls. Hypomethylated CpG sites of ACSL4 (cg15536552) and CPT1C (cg21604803), critical genes of adipocytokine signaling pathway, are associated with the increased risk for NAFLD. Lowered methylation level of ACSL4 (cg15536552) serves as an index for borderline/definitive NASH. DNA methylation profiling of peripheral leukocytes, therefore, may identify noninvasive biomarkers with potential for the pathological evaluation of NAFLD.

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Availability of data and material

Material described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

RNZ and QP contributed equally to this work; RNZ, QP, RDZ, YQM, FS, DZ, GYC and CYZ enrolled the cohorts and collected blood samples. RNZ and QP performed methylation analysis. RNZ interpreted the data and was involved in the manuscript preparation. JGF designed and supervised the study and wrote the manuscript.

Ethics approval and consent to participate

The study was reviewed and approved by the Ethics Committee of Xinhua Hospital, Shanghai Jiaotong University, School of Medicine. Informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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