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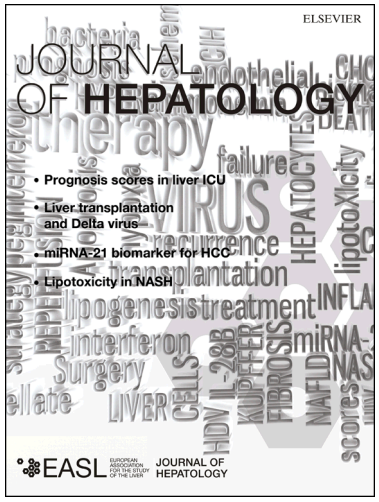

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Targeted-bisulfite sequence analysis of the methylation of CpG islands in genes encoding PNPLA3, SAMM50, and PARVB of patients with nonalcoholic fatty liver disease

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List of Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; GWAS, genome-wide association study; SNP, single-nucleotide polymorphism; *PNPLA3*, patatin-like phospholipase domain containing 3; LD, linkage disequilibrium; *SAMM50*, SAMM50 sorting and assembly machinery component; *PARVB*, parvin, β; PCR, polymerase chain reaction; NAS, NAFLD activity score; HDL, high-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; qPCR, quantitative PCR; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; bp, base position; HbA1c, hemoglobin A1c; AST, aspartate aminotransferase; ALT, alanine aminotransferase

Keywords: nonalcoholic fatty liver disease; DNA methylation; next-generation sequencing; fibrosis; *PNPLA3*; *SAMM50*; *PARVB*

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Study coordination and design: KH, AN.

Performed the experiment: TK, AK.

Statistical analysis: TK, TN, KH.

Data collection, critical review of the manuscript, approval of final version: YO, YH, KI, SS, MY.

Writing of manuscript, approval of final version: KH, TK, AK, TN, AN.

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Abstract

Background & Aims: The pathogenesis of nonalcoholic fatty liver disease (NAFLD) is affected by epigenetic factors as well as by genetic variation.

Methods: We performed targeted-bisulfite sequencing to determine the levels of DNA methylation of 4 CpG islands (CpG99, CpG71, CpG26, and CpG101) in the regulatory regions of *PNPLA3*, *SAMM50*, *PARVB* variant 1, and *PARVB* variant 2, respectively. We compared the levels of methylation of DNA in the livers of the first and second sets of patients with mild (fibrosis stages 0 and 1) or advanced (fibrosis stages 2-4) NAFLD and in those of patients with mild (F0 to F2) or advanced (F3 and F4) chronic hepatitis C infection. The hepatic mRNA levels of *PNPLA3*, *SAMM50*, and *PARVB* were measured using qPCR.

Results: CpG26, which resides in the regulatory region of *PARVB* variant 1, was markedly hypomethylated in the livers of patients with advanced NAFLD. Conversely, CpG99 in the regulatory region of *PNPLA3* was substantially hypermethylated in these patients. These differences in DNA methylation were replicated in a second set of patients with NAFLD or chronic hepatitis C. *PNPLA3* mRNA levels in the liver of the same section of a biopsy specimen used for genomic DNA preparation were lower in patients with advanced NAFLD compared with those with mild NAFLD and correlated inversely with CpG99

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methylation in liver DNA. Moreover, the levels of CpG99 methylation and *PNPLA3* mRNA were affected by the rs738409 genotype.

Conclusions: Hypomethylation of CpG26 and hypermethylation of CpG99 may contribute to the severity of fibrosis in patients with NAFLD or chronic hepatitis C infection.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) has become a common disease associated with metabolic syndrome in the population of developed countries [1, 2] and is accompanied by a gradual increase in the frequency of patients presenting with NAFLD [3, 4]. Certain people with fatty hepatocytes develop nonalcoholic steatohepatitis (NASH), in which hepatic inflammation coincides with increased hepatocyte death [3, 4]. Progressive liver fibrosis occurs in certain patients with NASH and markedly increases their risk of liver cirrhosis, primary liver cancer, and the resultant liver disease-related morbidity and mortality.

Genetic and environmental factors affect the development of NAFLD [5]. For example, genome-wide association studies (GWASs) reveal that single-nucleotide polymorphisms (SNPs) in several loci influence the course of NAFLD and the levels of liver enzymes in plasma [6-10]. Comprehensive studies on the SNP rs738409 in the patatin-like phospholipase domain containing 3 gene (*PNPLA3*) verify that this SNP plays a key role in the development of NAFLD [11-13]. We performed targeted next-generation sequence analysis and fine mapping of the linkage disequilibrium (LD) block containing *PNPLA3* rs738409 and found that polymorphisms in the genes encoding the SAMM50 sorting and assembly machinery component (*SAMM50*) and parvin, β (*PARVB*) are

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associated with the development and progression of NAFLD as well [14]. Specifically, variations in *PARVB* are associated with hepatocyte ballooning and patients' fibrosis stage [10, 14].

NAFLD is strongly associated with obesity, type 2 diabetes, and dyslipidemia, indicating that its pathogenesis is influenced by environmental factors such as excess caloric intake. Environmental factors affect DNA methylation, which is an epigenetic form of gene regulation typically associated with transcriptional repression [15]. In patients with NAFLD, insulin resistance [16] and treatment of obesity [17] affect the DNA methylation levels of several genes, and the severity of NAFLD is associated with DNA methylation levels [18]. The LD block containing *PNPLA3*, *SAMM50*, and *PARVB* is a crucial genetic locus in NAFLD, but epigenetic analysis of human liver DNA in this region has not been performed. Because recent improvements to next-generation sequencing enhances its ability to evaluate precisely of DNA methylation levels [19], we performed targeted-bisulfite sequencing coupled with polymerase chain reaction (PCR) amplification to investigate the methylation levels of 4 CpG islands in the genomic region containing the regulatory regions of *PNPLA3*, *SAMM50*, and *PARVB* in the livers of patients with NAFLD. We analyzed the association of the histological severity of NAFLD with the methylation levels of CpG99, CpG71, CpG26, and CpG101 and with the mRNA levels of *PNPLA3*,

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SAMM50, and *PARVB*.

Patients and methods

Patients and clinical variables

This study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from each study participant, and the ethics committees of Kyoto University and Yokohama City University approved the protocol. We enrolled randomly 32 (first set) and 33 (second set) Japanese patients with NAFLD who had undergone liver biopsy. The exclusion criteria of NAFLD used in this study were those reported previously [10, 14], and NAFLD was diagnosed according to standard criteria [20-22]. The degree of steatosis was graded according to the percentage of hepatocytes containing macrovesicular fat droplets [23]. The activity of NAFLD was determined according to the composite NAFLD activity score (NAS) [24]. Fibrosis severity (0–4) was scored according to the method of Brunt [23]. Patients with NAFLD were divided according to the fibrosis stage into groups with mild NAFLD (fibrosis stage 0 or 1) or advanced NAFLD (fibrosis stages 2 to 4). Patients with chronic hepatitis C (n = 30) were included in this study. They underwent liver biopsy, and liver fibrosis was staged according to the METAVIR scoring system [25]. Patients with chronic hepatitis C were divided into

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groups with mild (F0 to F2) and advanced (F3 or F4) disease. Metabolic syndrome traits were diagnosed as reported previously as follows: dyslipidemia, triglyceride level ≥150 mg/dL and/or high-density lipoprotein (HDL) cholesterol level <40 mg/dL, or under treatment for dyslipidemia; hypertension, systolic blood pressure (SBP) ≥130 mmHg and/or diastolic blood pressure (DBP) ≥85 mmHg, or under treatment for hypertension; and impaired fasting glucose, fasting glucose levels ≥110 mg/dL, or under treatment for diabetes [26]. Obesity was diagnosed according to the Japanese obesity criterion of body mass index (BMI) ≥25 kg/m^2 [27]. Clinical characteristics of patients are presented in Table 1.

Targeted-bisulfite sequencing

We isolated human genomic DNA and total RNA from the same section of frozen liver biopsy specimens and genomic DNA was isolated from whole blood as well. The bisulfite reaction was applied to 300 ng of genomic DNA by using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The genomic region covering 4 CpG islands CpG99, CpG71, CpG26, and CpG101 (Fig. 1), which reside in the regulatory regions of *PNPLA3*, *SAMM50*, and *PARVB*, were amplified from each bisulfite-treated DNA sample using region-specific primers (Supplementary Table 1). Primers were designed to avoid the CpG sequence and are unique in the entire human

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genomic sequence after bisulfite conversion. For the second set of patients with NAFLD or chronic hepatitis C, genomic regions including CpG26 and the latter part of CpG99 were amplified from each bisulfite-treated DNA sample using unrelated region-specific primers (Fig. 1 and Supplementary Table 1). Sequences were amplified using 30 ng of bisulfite-converted DNA, 1 × EpiTaq PCR Buffer (Takara Bio Inc., Ohtsu, Japan), 2.5 mM $MgCl_2$, 0.3 mM of each dNTP, 0.3 μM of each primer, and 0.375 U of TaKaRa EpiTaq HS (Takara) in a 15-μL reaction volume. The PCR was performed for 40 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 2 min. The expected size of PCR amplicons were confirmed using agarose gel electrophoresis (Supplementary Fig. 1), and the molar concentrations of amplicons were measured using a High Sensitivity NGS Fragment Analysis Kit and Fragment Analyzer (Advanced Analytical Technologies, Ames, IA). The amplicons were then pooled at an equal concentration for all patients. Pooled PCR products were sheared using a Covaris S220 (COVARIS, INC, Woburn, MA) as follows: 10% duty factor, 175 W peak incident power, 200 cycles per burst, 180-s duration, and frequency sweeping mode. Each sample was dual-indexed, and DNA libraries were prepared using the TruSeq DNA Sample Preparation kit v2 (Illumina, San Diego, CA). Samples were sequenced using a MiSeq system (Illumina) that performed 100-bp paired-end reads. Sequenced reads of each sample were checked using the FastQC software package

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(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality of the sequenced reads in all libraries was high, with a median Phred score of >31 up to the end of last sequencing cycle. Reads of bisulfite-treated DNAs were aligned to the reference human genome (UCSC hg19) using Bismark (v0.8.3, http://www.bioinformatics.babraham.ac.uk/projects/bismark/) [28], which uses the short-read aligner Bowtie 2 (Version 2.1.0, http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) [29]. Sequences were used for subsequent analysis only if both ends mapped uniquely in the correct orientation. For sequence data conversion, sorting, and indexing we used SAMtools (Version 0.1.17; http://www.samtools.sourceforge.net/) [30]. To exclude duplicate reads, we used Picard (Version 1.72; http://picard.sourceforge.net). Methylated cytosines were extracted from deduplicated reads using the Bismark methylation extractor [28]. The aligned reads and levels of methylation were visualized using an Integrative Genomics Viewer (IGV Version 1.5.65, http://www.broadinstitute.org/igv/) [31].

RNA isolation and quantitative PCR (qPCR)

Total RNA was extracted from liver tissue samples using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The quality of RNA was evaluated using an Agilent 2100 Bioanalyzer system and an Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa

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Clara, CA). The RNA Integrity Number of each total RNA sample was >8 [32]. Liver RNA samples were reverse-transcribed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). The primer sets used for qPCR are shown in Supplementary Table 2, and qPCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA) with the THUNDERBIRD SYBR qPCR Mix (TOYOBO). Gene expression levels were calculated using the Ct value. We used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control and quantified the relative mRNA levels using the $2^{-\Delta\Delta Ct}$ method. All experiments were conducted in triplicate.

SNP genotyping

Invader probes (Third Wave Technologies, Madison, WI) were constructed for rs738409 and genotyped using the genomic DNA prepared from blood.

Statistical analysis.

The clinical data and methylation levels of patients groups with mild or advanced NAFLD were compared using *t* tests, and the methylation levels in liver and blood DNAs were compared using paired *t* tests. The male:female ratio, type 2 diabetes ratios, and the presence of metabolic syndrome traits were analyzed using Fisher's exact test. Statistical analyses were performed using R software (http://www.r-project.org/). Meta-analysis to

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compare 2 sets of data was performed using fixed-effects model analysis of the standardized mean differences using the R package metafor (http://www.metafor-project.org http://www.wvbauer.com) [33]. We used the *Q*-value to address the problem of multiple testing, and the *Q*-value was calculated using Q-VALUE (http://faculty.washington.edu/~jstorey/qvalue/) [34]. A *Q*-value <0.05 was considered statistically significant.

Results

Bisulfite sequencing of CpG99, CpG71, CpG26, and CpG101

We reported that variations in the LD block containing *PNPLA3*, *SAMM50*, and *PARVB* critically affect the pathogenesis of NAFLD [10, 14]. Therefore, we determined the methylation of 4 CpG islands (CpG99, CpG71, CpG26, and CpG101) in this region (Fig. 1). First, we amplified and sequenced the genomic region containing these CpG islands from 32 liver and 29 whole-blood bisulfite-treated DNA samples (Supplementary Fig. 1). The mean coverage of the 4 CpG islands was 1567−3078× in liver and 607−1429× in blood samples (Supplementary Fig. 2). We excluded 20 CpG sites in CpG101, because the coverage was <50 in ≥2 samples. Methylation of non-CpG sites (CHH and CHG) was extremely low (<3%) in liver and blood samples (Supplementary Fig. 3), which was as low

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as optimal for methylation in a non-CpG context (Supplementary Fig. 3 and Supplementary Table 3) [35]. We used 117 (CpG99), 66 (CpG71), 42 (CpG26), and 124 (CpG101) CpG sites for the analysis (Supplementary Table 3) described below.

Methylation of the DNAs of livers of patients with mild or advanced NAFLD

We compared the methylation levels between patients with mild or advanced NAFLD in the first set (Table 1). Fig. 2 presents the results of liver DNA methylation at each CpG base position (bp) in CpG99, CpG71, CpG26, and CpG101. The DNA sequences of the 4 CpG sites in CpG99 (at bps 44,320,188; 44,320,198; 44,320,329; and 44,320,351) were hypermethylated in patients with advanced NAFLD ($P < 0.05$) (Table 2). Differential methylation was most notable in CpG26 and numerous CpG sites were hypomethylated in patients with advanced NAFLD ($P < 0.05$) (Table 3). However, the levels of DNA methylation of CpG71 and CpG101 between the 2 groups were not significantly different.

To confirm the associations between fibrosis stages and liver DNA methylation levels, we investigated methylation levels using the second set of patients with NAFLD (n = 33) and performed meta-analysis using the first and second sets. Four CpG sites in CpG99 (at bps 44,320,188; 44,320,198; 44,320,329; and 44,320,351) were significantly hypermethylated in patients with advanced NAFLD (Q-values < 0.05, Table 2). Meta-analysis indicated that 18 CpG sites in CpG26 were significantly hypomethylated in

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patients with advanced NAFLD (Q-values < 0.05, Table 3). When the first and second sets were merged to analyze the DNA methylation levels, the results were very similar to those acquired from the meta-analysis.

Because SNP rs738409 (I148M) strongly affects the pathogenesis of NAFLD [11-13], we examined the methylation levels of the rs738409 (I148M) genotype among patients with NAFLD. The first and second sets of patients with NAFLD were divided into 2 groups according to genotypes (MM vs. IM + II), and the DNA methylation levels were compared between patients with mild or advanced NAFLD in each. Methylation levels were higher at the 4 CpG sites in CpG99 (at bps 44,320,188; 44,320,198; 44,320,329; and 44,320,351) in patients with advanced NAFLD compared with those with mild disease in the MM genotype group (Supplementary Table 4). No differences in DNA methylation levels were observed between patients with mild or advanced NAFLD in the IM + II genotype groups (P > 0.05). Methylation levels were lower at the CpG sites in CpG26 in patients with advanced NAFLD compared with those with mild disease in the MM and IM+II genotype groups (P < 0.05; Supplementary Table 5).

Methylation of CpG99 and CpG26 in patients with chronic hepatitis C infection

Liver fibrosis is caused by various hepatic diseases. To determine whether the differential methylation of CpG99 and CpG26 was specific to patients with NAFLD, we measured the

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methylation levels of liver DNA of patients with chronic hepatitis C. Nine CpG sites in CpG99 including the 4 sites (at bps 44,320,188; 44,320,198; 44,320,329; and 44,320,351), which were hypermethylated in patients with advanced NAFLD described above, were significantly hypermethylated in patients with chronic hepatitis C with fibrosis stage F3 or F4 (Q-values < 0.05, Table 2). In CpG26, 26 CpG sites were significantly hypomethylated in patients with advanced chronic hepatitis C (Q-values < 0.05, Table 3). These data suggest that the differential methylation levels of CpG99 and CpG26 are related to fibrosis levels in a liver disease other than NAFLD.

Methylation of liver and blood DNA and its association with histological phenotypes of patients with NAFLD

We examined the association between histological traits other than fibrosis and the levels of liver DNA methylation in patients with mild and advanced NAFLD. None of the CpG sites showed significantly different methylation levels in patients with hepatocyte ballooning, lobular inflammation, or NAS.

To determine whether differential methylation occurred in tissues other than the livers of patients with mild or advanced NAFLD, we examined the methylation of DNA isolated from blood and found that there were no significant differences in methylation levels of CpG99, CpG71, CpG26, and CpG101 of patients with mild compared with those

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with advanced NAFLD (Fig. 3). The methylation levels of liver CpG99 were significantly lower compared with those of blood CpG99 of patients in mild or advanced NAFLD (Supplementary Fig. 4, *Q*-values <0.05). The methylation levels of CpG sites in CpG26 in liver DNA were significantly higher compared with those of DNA isolated from the blood of patients with mild or advanced NAFLD (*Q*-values <0.05).

Levels of PNPLA3, SAMM50, and PARVB mRNAs in livers of patients with NAFLD

Using qPCR, we next measured the mRNA levels of *PNPLA3*, *SAMM50*, and *PARVB* variant 1, and *PARVB* variant 2, respectively (Fig. 1). Total RNAs were prepared from the same section of a biopsy that was used to prepare genomic DNA. However, it was not possible to prepare sufficient total RNA from the one sample of the second set. The mRNA levels of *PNPLA3* were significantly lower in patients with advanced NAFLD compared with those with mild NAFLD of the first set ($P = 0.045$; Fig. 4). *PNPLA3* mRNA levels were lower in patients with advanced NAFLD of the second set ($P = 0.051$). Meta-analysis using the first and second sets of patients with NAFLD showed that the levels of *PNPLA3* mRNA were significantly lower in patients with advanced NAFLD ($P = 0.0076$). When patients with NAFLD were divided into MM and IM+II genotype groups, the levels of *PNPLA3* mRNA were significantly lower in patients with advanced NAFLD compared with those with mild disease in the MM genotype group ($P = 0.0081$, Supplementary Fig.

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5). *PNPLA3* mRNA levels were lower in patients with advanced NAFLD compared with those with mild disease in the group of IM + II genotype group, but the difference was not statistically significant. The levels of *SAMM50* mRNA did not differ between patients with mild or advanced NAFLD. Because CpG26 and CpG101 are located in the upstream regions of *PARVB* variants 1 and 2, respectively, we measured the levels of *PARVB* variant 1 and 2 mRNAs separately. We found that *PARVB* transcript variant 1 was expressed in the liver but was undetectable using qPCR because of its low level. The levels of *PARVB* variant 2 mRNA were not significantly different between patients with mild or advanced NAFLD.

We next investigated the relationship between DNA methylation and mRNA levels in patients with NAFLD. *PNPLA3* mRNA levels correlated most strongly with the methylation levels of CpG99 at bp 44,320,188 ($r = -0.442$, $P = 2.5 \times 10^{-4}$) (Fig. 4). There was a significant correlation between *PNPLA3* mRNA and DNA methylation levels in patients with NAFLD with the rs738409 MM genotype ($r = -0.606$, $P = 0.0010$) (Supplementary Fig. 5). There was no significant correlation in patients with NAFLD with the II + IM ($r = -0.245$, $P = 0.14$) genotypes. These data indicate that the rs738409 genotype affects the levels of methylation of *PNPLA3* and *PNPLA3* mRNA levels.

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Discussion

We determined that in patients with advanced NAFLD, CpG26, which lies upstream of the coding sequences of the *PARVB* transcript variant 1, was markedly hypomethylated, whereas CpG99 in the regulatory region of *PNPLA3* was hypermethylated. The database published by Murphy et al. [18] indicates that CpG26 is hypomethylated in patients with advanced NAFLD ($P = 2.8 \times 10^{-4}$, bp 44,392,384) and that the methylation levels are 67% and 73% in patients with advanced and mild NAFLD, respectively. These findings are consistent with our data reported here, and the database [18] indicates further that methylation of CpG99 does not differ between patients with mild or advanced NAFLD [18]. Because we used the bisulfite sequencing method, we were able to examine almost all CpG sites and identify those that were differentially methylated. These differentially methylated CpG sites were not included in the Illumina HumanMethylation450 Beadchip reported by Murphy et al. [18]. Thus, our approaches may be useful for precisely analyzing the DNA methylation levels of CpG islands. Our data and previous data [18] suggest that differential methylation of CpG99 and CpG26 is likely to affect the progression of NAFLD.

NAFLD often accompanies metabolic disorders such as type 2 diabetes, dyslipidemia, and hypertension is therefore considered a major manifestation of metabolic

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syndrome [3]. Metabolic syndrome is mainly caused by an excess intake of calories with a contribution from genetic factors. Diet affects DNA methylation and causes diseases such as obesity and type 2 diabetes [36], which is consistent with findings that bariatric surgery alters DNA methylation levels in the liver [17]. In the present study, a higher percentage of patients in the advanced NAFLD group exhibited impaired fasting glucose as well as dyslipidemia compared with patients with mild NAFLD. These results suggest that dyslipidemia and high blood glucose levels caused by excess caloric intake affect DNA methylation levels in the liver.

Differential methylation of CpG99 and CpG26 in patients with NAFLD was detected in patients studied here with chronic hepatitis C infection. Fibrosis progresses during chronic hepatocyte inflammation. Because differential methylation related to fibrosis stages was not specific for NAFLD, the DNA methylation levels may be caused by hepatic lipid accumulation or chronic hepatitis in contrast to serving as a cause of fibrosis. The question of whether differential methylation is a cause or an effect was not answered here, and further investigations are required.

CpG99 was hypermethylated, and *PNPLA3* mRNA levels were lower in patients with advanced NAFLD compared with those with mild NAFLD. Therefore, low *PNPLA3* expression levels correlated with disease severity observed in the liver of patients with

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NAFLD. Moreover, we show here that the rs738409 MM genotype affected the levels of DNA methylation and transcription. The methylation of CpG99 may be induced by environmental factors, causing decreased expression of *PNPLA3* mRNA and the progression of fibrosis in patients with the MM genotype. The findings that the MM genotype affecting levels of DNA methylation and transcription are novel and could contribute to establishing the association of the *PNPLA3* with fibrosis, although further analysis and larger studies are required.

The methylation levels of CpG99 and CpG26 in liver differed between patients with mild or advanced NAFLD, but not in blood, suggesting that an alternation in liver-specific DNA methylation levels leads to changes in transcriptional levels and increases the severity of the liver disease. The difference in methylation levels was highest in CpG26, which is located in the upstream region of *PARVB* transcript variant 1; however, this transcript was undetectable. The transcriptional levels of *PNPLA3* inversely correlated with the methylation levels of CpG99 near the gene. Therefore, hypomethylation of CpG26 may affect the level of the *PARVB* transcript variant 1 mRNA.

NF-κB is a key transcription factor involved in regulating inflammation [37] and ChIP-sequence data reveal that the binding of NF-κB to CpG26 [38]. The binding of NF-κB to DNA is suppressed by DNA methylation [39]. Thus, hypomethylation of CpG26

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may increase the binding affinity of NF-κB for DNA and facilitate the transcription of *PARVB* transcript variant 1 mRNA, a possibility that warrants further investigation.

In summary, our results indicate that hypermethylation of CpG99 and hypomethylation of CpG26 are related to the ultimate outcomes of patients with liver fibrosis. Genetics and epigenetic analyses suggest that *PNPLA3* and *PARVB* are involved in the progression of NAFLD.

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Figure legends

Fig. 1. PCR analysis of bisulfite-treated DNA. Amplicons (first set) were derived from bisulfite-treated DNA of the first set of patients with NAFLD, and amplicons (second set) were those from the second set of patients with NAFLD or chronic hepatitis C.

Fig. 2. DNA methylation status of CpG99, CpG77, CpG26, and CpG101 in the livers of patients with mild or advanced NAFLD of the first set. The numbers in the figure indicate the positions of the CpG sites.

Fig. 3. DNA methylation status of CpG99, CpG77, CpG26, and CpG101 in the blood of patients with mild or advanced NAFLD of the first set.

Fig. 4. Liver mRNA levels in the first set (A) and second set (B) of patients with NAFLD and the correlation between DNA methylation status and mRNA levels (C). Bars represent the mean ± SD. White and black bars represent mild and advanced NAFLD.

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Fig. 1

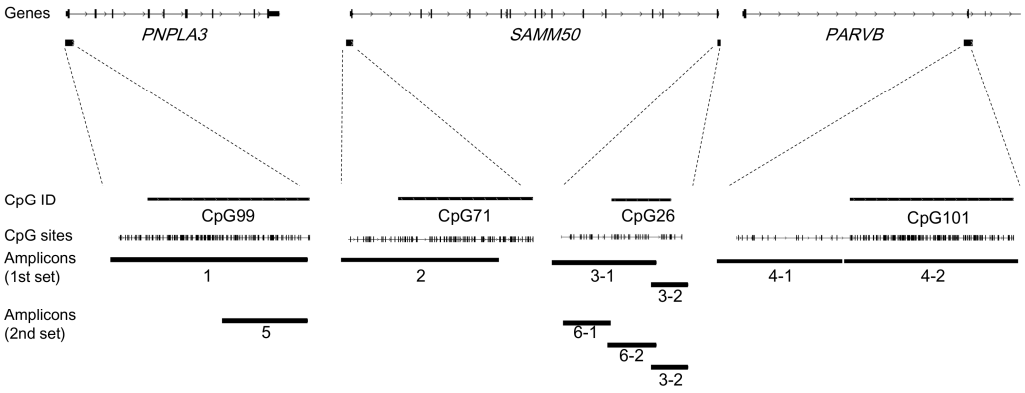

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Fig. 2

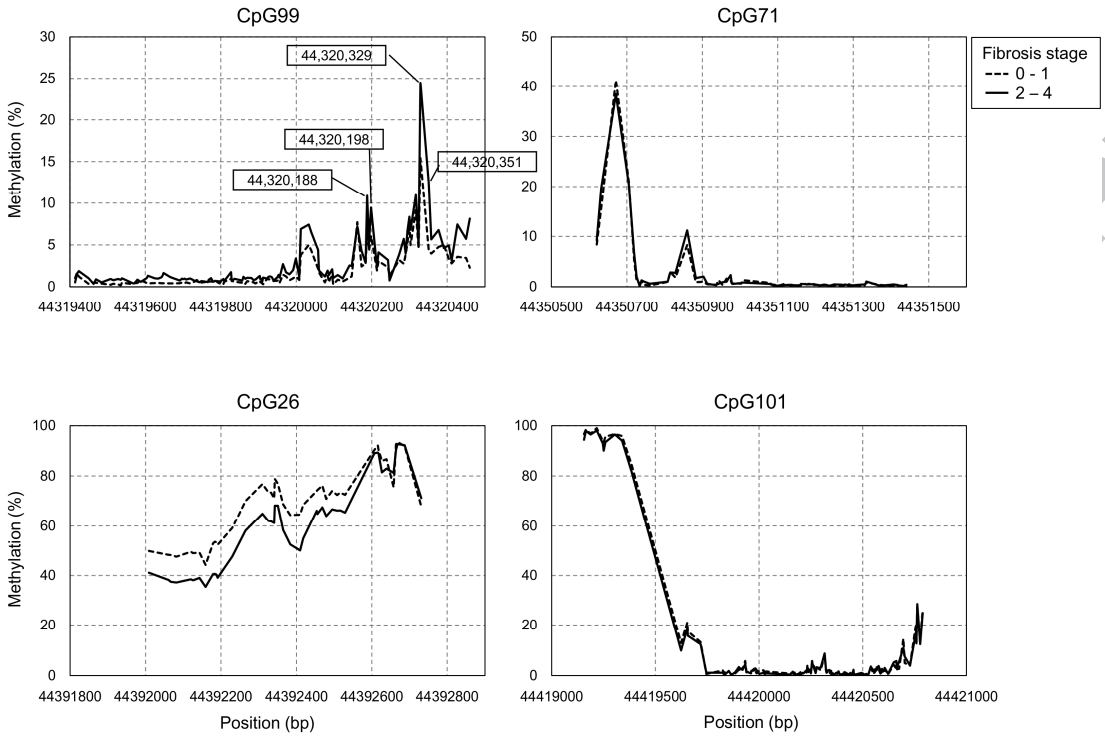

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Fig. 3

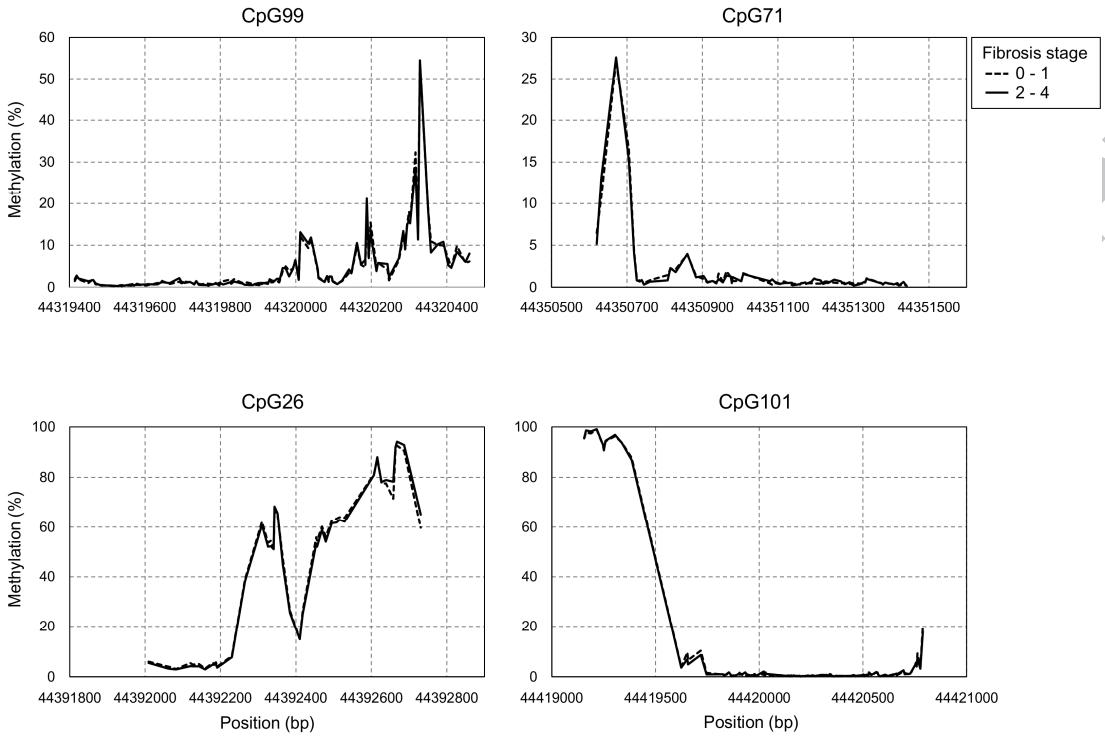

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Fig. 4

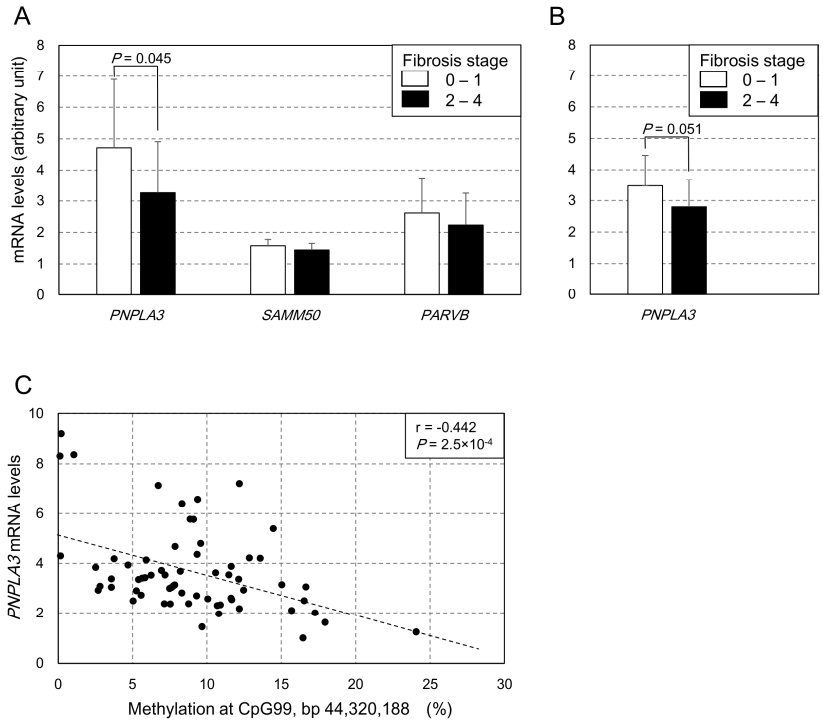

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Table 1. Clinical characteristics of study participants.

	NAFLD						Chronic hepatitis C		
	1st set			2nd set					
	Mild	Advanced	*P*-value	Mild	Advanced	*P*-value	Mild	Advanced	*P*-value
n	21	11	-	15	18	-	19	11	-
Male/Female	15 / 6	4 / 7	0.072*	9/6	12/6	0.73*	11/8	8/3	0.47*
Age (years)	47.3 ± 18.1	59.5 ± 13.5	0.042	49.8 ± 15.1	53.3 ± 14.5	0.51	54.8 ± 12.8	64.3 ± 9.7	0.031
BMI (kg/m^2)	27.7 ± 3.9	26.9 ± 4.3	0.61	29.5 ± 3.6	28.8 ± 4.6	0.65	23.1 ± 3.1	25.1 ± 6.3	0.39
Plasma glucose (mg/dL)	99.9 ± 12.6	116.4 ± 25.2	0.12	104.7 ± 18.3	109.8 ± 19.2	0.44	107.3 ± 29.4	111.8 ± 24.6	0.67
Hb.A1c (%)	5.8 ± 1.2	6.1 ± 1.2	0.57	5.8 ± 0.7	6 ± 0.8	0.38	5.7 ± 1.2	5.6 ± 0.8	0.78
Fasting insulin (μU/mL)	17.4 ± 28.3	13.9 ± 7.5	0.59	13.3 ± 6	20.6 ± 9.8	0.016	10.3 ± 5.7	11.2 ± 7.1	0.73
Total cholesterol (mg/dL)	203.2 ± 28.4	186.3 ± 37	0.21	198.6 ± 37.6	199.6 ± 30.2	0.93	195.5 ± 29.4	177.1 ± 42.1	0.24
Triglycerides (mg/dL)	148.6 ± 69.8	124.9 ± 33.9	0.21	154.5 ± 51.1	155.4 ± 84.7	0.97	170.4 ± 125.8	116.6 ± 53.8	0.12
HDL-cholesterol (mg/dL)	53.6 ± 15.2	54.7 ± 14.3	0.84	51.4 ± 9.8	49.3 ± 20.3	0.70	52.6 ± 16.1	52 ± 14.5	0.91
SBP (mmHg)	127.9 ± 17.1	145.0 ± 8.9	0.07	129.9 ± 14.6	122.9 ± 12.6	0.16	125.3 ± 16.6	137.2 ± 18.8	0.10
DBP (mmHg)	76.6 ± 12.9	84.7 ± 13.8	0.44	78.4 ± 12.2	74.4 ± 11.5	0.35	75.2 ± 10.2	82.1 ± 12.7	0.14
AST (IU/L)	38.2 ± 19.6	67.6 ± 31.2	0.013	44.6 ± 19.7	46.7 ± 17.3	0.75	42.2 ± 19.6	73.3 ± 48.8	0.066
ALT (IU/L)	65.0 ± 48.5	86.1 ± 51.4	0.28	69.9 ± 44.5	57.4 ± 29.9	0.36	50.3 ± 30	68.8 ± 52.2	0.30
Ferritin (ng/ml)	240.8 ± 178.5	271.1 ± 211.9	0.69	183.2 ± 90.7	200 ± 90.5	0.60	161.5 ± 122.1	336.4 ± 375.4	0.16
Hyaluronic acid (ng/dL)	36.6 ± 38.2	101.3 ± 99.4	0.061	32.5 ± 24	82.6 ± 91	0.037	72 ± 51.2	235.6 ± 240.6	0.049
Type IV collagen 7s (ng/dL)	4.2 ± 0.8	6.4 ± 2.3	0.010	4.4 ± 0.8	6.3 ± 2.2	0.0015	5 ± 1.5	7.9 ± 2.5	0.0037
Steatosis grade (0–3)	1.5 ± 0.7	1.5 ± 0.7	0.93	1.9 ± 0.7	1.5 ± 0.7	0.16	-	-	-
Lobular inflammation (0–3)	0.9 ± 0.6	2.0 ± 0.4	4.6×10^{-6}	1.1 ± 0.6	1.3 ± 0.5	0.27	-	-	-
Hepatocyte ballooning (0–2)	0.7 ± 0.6	1.5 ± 0.5	0.0012	0.3 ± 0.5	0.5 ± 0.5	0.18	-	-	-

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NAS (0–8)	3.1 ± 1.5	5.0 ± 1.0	2.9×10^{-4}	3.2 ± 1.4	3.3 ± 1.1	0.86	-	-	-
Fibrosis stage (0–4)	0.7 ± 0.5	2.5 ± 0.5	1.3×10^{-8}	0.8 ± 0.4	2.6 ± 0.6	3.4×10^{-11}	1.2 ± 0.7	3.3 ± 0.5	3.2×10^{-10}
Obesity (%)	17 (81.0)	9 (81.8)	1.00	12 (80.0)	13 (72.2)	0.70	5 (26.3)	5 (45.5)	0.43
Type 2 diabetes (%)	4 (19.0)	4 (36.4)	0.40	3 (20.0)	6 (33.3)	0.46	3 (15.8)	5 (45.5)	0.10
Impaired fasting glucose (%)	5 (23.8)	5 (45.5)	0.25	5 (33.3)	8 (44.4)	0.72	5 (26.3)	5 (45.5)	0.43
Dyslipidemia (%)	12 (57.1)	8 (72.7)	0.46	7 (46.7)	10 (55.6)	0.73	2 (10.5)	0 (0.0)	0.52
Hypertension (%)	7 (33.3)	5 (45.5)	0.70	11 (73.3)	9 (50.0)	0.28	9 (47.4)	8 (72.7)	0.26
Genotype rs738409 (II/IM/MM)	2/9/10	2/4/5	0.77	4/7/4	2/8/8	0.48	7/5/7	2/3/6	0.56

Values are shown as means ± SD.

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Table 2. Comparison of liver genomic methylation levels in the CpG99 region in patients with NFALD and those with chronic hepatitis C according to the stage of fibrosis.

Position (bp)	1st set of NAFLD			2nd set of NAFLD			NAFLD Meta-analysis		Chronic hepatitis C			
	Stages 0-1 (n = 21)	Stages 2-4 (n = 11)	*P*-value	Stages 0-1 (n = 15)	Stages 2-4 (n = 18)	*P*-value	*P*-value	*Q*-value	Stages 0-2 (n = 19)	Stages 3-4 (n = 11)	*P*-value	*Q*-value
44,320,033	5 ± 4	7 ± 4	0.14	4 ± 2	4 ± 2	0.96	0.33	0.41	5 ± 3	8 ± 3	0.05	0.067
44,320,040	4 ± 4	6 ± 6	0.31	4 ± 2	5 ± 3	0.26	0.12	0.20	4 ± 3	6 ± 5	0.18	0.21
44,320,162	8 ± 5	7 ± 4	0.77	8 ± 2	8 ± 2	0.58	0.83	0.84	10 ± 3	10 ± 2	0.74	0.76
44,320,188	7 ± 5	11 ± 5	0.034	8 ± 2	11 ± 5	0.079	0.0075	0.035	11 ± 5	16 ± 5	0.010	0.031
44,320,198	6 ± 5	9 ± 3	0.036	6 ± 2	9 ± 5	0.028	0.0059	0.029	8 ± 4	14 ± 4	8.4×10^{-4}	0.016
44,320,295	5 ± 6	7 ± 4	0.43	4 ± 2	8 ± 4	0.0077	0.022	0.062	7 ± 4	11 ± 4	0.0038	0.021
44,320,300	7 ± 6	8 ± 6	0.68	5 ± 2	9 ± 6	0.022	0.069	0.14	8 ± 4	13 ± 5	0.011	0.031
44,320,303	5 ± 5	7 ± 5	0.35	5 ± 2	7 ± 4	0.075	0.061	0.12	8 ± 4	11 ± 4	0.028	0.046
44,320,317	9 ± 6	11 ± 8	0.51	10 ± 4	12 ± 3	0.11	0.10	0.18	12 ± 5	19 ± 5	0.0012	0.016
44,320,329	15 ± 9	24 ± 10	0.017	18 ± 4	21 ± 7	0.22	0.012	0.043	21 ± 8	33 ± 9	0.0013	0.016
44,320,351	4 ± 5	12 ± 7	0.0036	7 ± 2	9 ± 5	0.079	4.4×10^{-4}	0.010	8 ± 4	15 ± 5	0.0021	0.016
44,320,377	5 ± 4	7 ± 5	0.26	5 ± 2	6 ± 2	0.028	0.021	0.061	6 ± 4	9 ± 2	0.011	0.031

Data represent the mean ± SD. Mean methylation levels >5% are indicated.

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Table 3. Comparison of liver genomic methylation levels in the CpG26 region in patients with NFALD and those with chronic hepatitis C according to the stage of fibrosis.

Position (bp)	1st set of NAFLD			2nd set of NAFLD			NAFLD Meta-analysis		Chronic hepatitis C			
	Stages 0-1 (n = 21)	Stages 2-4 (n = 11)	*P*-value	Stages 0-1 (n = 15)	Stages 2-4 (n = 18)	*P*-value	*P*-value	*Q*-value	Stages 0-2 (n = 19)	Stages 3-4 (n = 11)	*P*-value	*Q*-value
44,392,061	48 ± 10	38 ± 8	0.0048	33 ± 5	30 ± 7	0.14	0.0046	0.024	27 ± 6	23 ± 4	0.065	0.081
44,392,067	48 ± 10	37 ± 9	0.0045	34 ± 5	30 ± 7	0.13	0.0032	0.020	28 ± 6	24 ± 4	0.029	0.046
44,392,082	48 ± 9	37 ± 9	0.0060	36 ± 5	32 ± 7	0.11	0.0025	0.020	28 ± 6	24 ± 3	0.0094	0.030
44,392,120	50 ± 11	39 ± 8	0.0039	45 ± 7	43 ± 9	0.41	0.018	0.060	37 ± 6	30 ± 5	0.0021	0.016
44,392,126	49 ± 9	38 ± 8	0.0014	45 ± 7	43 ± 8	0.47	0.0093	0.041	37 ± 5	30 ± 6	0.0037	0.021
44,392,143	49 ± 10	39 ± 7	0.0027	47 ± 6	44 ± 8	0.36	0.012	0.043	39 ± 5	31 ± 6	0.0014	0.016
44,392,159	44 ± 9	35 ± 8	0.0085	43 ± 6	41 ± 7	0.47	0.027	0.074	36 ± 5	27 ± 6	7.3×10^{-4}	0.016
44,392,179	53 ± 14	41 ± 7	0.0025	51 ± 8	50 ± 9	0.63	0.040	0.098	44 ± 6	34 ± 7	0.0017	0.016
44,392,184	54 ± 14	41 ± 7	0.0014	51 ± 7	50 ± 9	0.69	0.038	0.096	45 ± 6	34 ± 7	0.0010	0.016
44,392,188	53 ± 13	40 ± 8	0.0014	51 ± 8	50 ± 9	0.85	0.051	0.11	44 ± 7	34 ± 8	0.0018	0.016
44,392,191	52 ± 13	39 ± 8	0.0010	51 ± 8	49 ± 8	0.55	0.019	0.060	43 ± 6	33 ± 7	6.3×10^{-4}	0.016
44,392,230	59 ± 11	48 ± 8	0.0018	56 ± 7	53 ± 8	0.42	0.014	0.049	48 ± 6	40 ± 10	0.018	0.035
44,392,265	70 ± 11	58 ± 8	0.0023	70 ± 9	65 ± 8	0.16	0.0035	0.020	63 ± 8	50 ± 11	0.0047	0.023
44,392,310	77 ± 10	65 ± 10	0.0058	51 ± 16	41 ± 9	0.035	3.4×10^{-4}	0.010	38 ± 8	31 ± 9	0.044	0.062
44,392,326	74 ± 9	62 ± 8	0.0016	46 ± 8	40 ± 8	0.055	4.8×10^{-4}	0.010	36 ± 7	30 ± 8	0.028	0.046
44,392,333	73 ± 9	62 ± 10	0.0042	49 ± 8	43 ± 9	0.038	3.9×10^{-4}	0.010	38 ± 7	32 ± 8	0.038	0.055
44,392,341	71 ± 9	61 ± 7	0.0030	50 ± 9	44 ± 9	0.070	0.0013	0.015	39 ± 7	34 ± 7	0.063	0.080
44,392,343	79 ± 10	68 ± 7	0.0020	57 ± 14	50 ± 9	0.079	0.0012	0.015	45 ± 7	39 ± 8	0.034	0.051
44,392,351	77 ± 9	68 ± 8	0.0088	58 ± 8	52 ± 9	0.077	0.0031	0.020	47 ± 7	41 ± 8	0.070	0.087
44,392,365	69 ± 9	58 ± 7	0.0017	54 ± 8	50 ± 10	0.25	0.0042	0.023	45 ± 8	38 ± 8	0.016	0.035

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44,392,384	64 ± 11	52 ± 8	0.0011	58 ± 8	54 ± 10	0.15	0.0027	0.020	47 ± 7	39 ± 9	0.018	0.035
44,392,410	65 ± 10	50 ± 9	2.6×10^{-4}	58 ± 10	54 ± 11	0.29	0.0021	0.019	48 ± 8	38 ± 10	0.012	0.032
44,392,418	68 ± 10	55 ± 9	6.7×10^{-4}	63 ± 10	58 ± 11	0.17	0.0014	0.015	53 ± 10	41 ± 11	0.0089	0.030
44,392,454	74 ± 12	66 ± 10	0.061	67 ± 9	65 ± 10	0.43	0.079	0.15	58 ± 10	47 ± 11	0.018	0.035
44,392,456	74 ± 11	65 ± 10	0.022	68 ± 11	63 ± 11	0.15	0.011	0.043	57 ± 11	47 ± 11	0.020	0.038
44,392,469	76 ± 12	68 ± 9	0.028	71 ± 11	65 ± 10	0.14	0.015	0.051	59 ± 10	49 ± 12	0.021	0.039
44,392,479	71 ± 13	64 ± 13	0.16	65 ± 13	59 ± 12	0.20	0.060	0.12	53 ± 11	43 ± 9	0.0069	0.026
44,392,495	74 ± 14	67 ± 12	0.13	65 ± 10	59 ± 13	0.17	0.048	0.11	54 ± 9	47 ± 8	0.035	0.052
44,392,508	72 ± 15	66 ± 15	0.26	62 ± 13	59 ± 10	0.54	0.23	0.30	51 ± 9	44 ± 7	0.015	0.035
44,392,516	73 ± 15	66 ± 14	0.20	61 ± 13	58 ± 9	0.50	0.17	0.25	51 ± 8	43 ± 7	0.012	0.032
44,392,529	73 ± 15	65 ± 16	0.24	58 ± 9	56 ± 9	0.52	0.20	0.27	49 ± 9	42 ± 7	0.018	0.035
44,392,607	90 ± 6	89 ± 3	0.38	82 ± 8	81 ± 11	0.63	0.40	0.48	76 ± 10	69 ± 10	0.085	0.10
44,392,616	92 ± 4	89 ± 5	0.14	86 ± 7	85 ± 11	0.74	0.18	0.26	80 ± 10	73 ± 9	0.053	0.071
44,392,618	90 ± 5	89 ± 4	0.36	84 ± 7	83 ± 12	0.87	0.49	0.55	78 ± 10	70 ± 10	0.053	0.071
44,392,627	86 ± 7	81 ± 6	0.060	80 ± 9	77 ± 12	0.49	0.10	0.18	72 ± 11	61 ± 12	0.017	0.035
44,392,639	87 ± 6	83 ± 7	0.14	80 ± 7	79 ± 13	0.73	0.21	0.28	74 ± 11	63 ± 13	0.027	0.046
44,392,658	76 ± 26	81 ± 18	0.48	78 ± 19	81 ± 14	0.62	0.43	0.50	73 ± 20	68 ± 18	0.46	0.48
44,392,664	93 ± 4	90 ± 4	0.16	88 ± 5	87 ± 9	0.58	0.18	0.26	84 ± 8	77 ± 10	0.047	0.066
44,392,668	94 ± 5	93 ± 4	0.59	89 ± 5	91 ± 7	0.38	0.78	0.81	88 ± 8	81 ± 9	0.054	0.071
44,392,687	92 ± 5	92 ± 3	0.83	90 ± 6	88 ± 7	0.40	0.64	0.71	86 ± 7	79 ± 9	0.032	0.050
44,392,731	68 ± 24	71 ± 14	0.62	73 ± 14	75 ± 8	0.55	0.46	0.53	69 ± 17	66 ± 15	0.61	0.63

Data represent the mean ± SD.

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