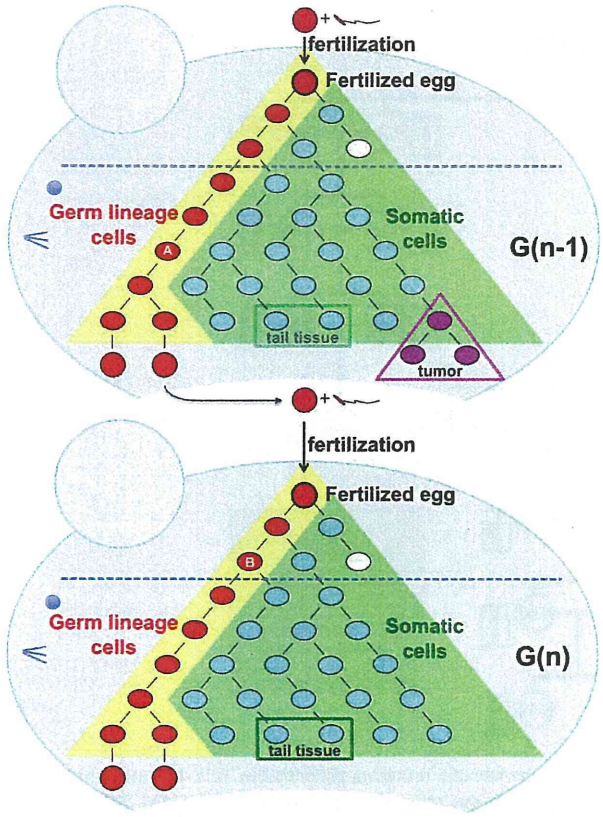

Figure 7 | Germ line mutations occur at different stages of the germ cell lineage. Mutations detected in the tail DNA of the first mutant mouse had occurred either in the germ lineage cells of the previous generation or during the very early developmental stage of the mutant mouse. Mutations start to accumulate from the first replication of fertilized egg DNA; however, each mutation is diluted out in the tissue DNA. Therefore, we used the tail DNA sequence as a reference sequence of fertilized egg DNA. In contrast to tail tissue, differentiated gametes can transmit their sequence information monoclonally to offspring. If the original mutated allele was mapped in multiple mice of the same generation, such as mutation #54 (in Fig. 4, Supplementary Data S1 online), the mutation probably occurred in the germ lineage cells of the parents (indicated as A). For mutations in the X chromosome (such as mutation #261), which began in the male with a heterozygous status (see Supplementary Fig. S4 online), the mutation probably occurred in a cell during the early stage of embryonic development (shown as B), resulting in mosaicism of tail tissue. These results indicate that germline mutations occur at different developmental stages of the germ cell lineage.

and inflammation, and is widely present in the DNA of various organisms. It is likely that the oxidative environment expands the genetic diversity of species by increasing the mutation rate of the germ lineage cells to accelerate the evolutionary process. MTH1, OGG1 and MUTYH, which are well conserved among species, may have contributed coordinately to control the germline mutation rate to an appropriate level for each species during evolution by controlling the amount of 8-oxoG in the genome (Supplementary Fig. S1 online).

Methods

Animals. *Mth1*$^{+/-}$, *Ogg1*$^{+/-}$, and *Mutyh*$^{+/-}$ mice were established[13,14,16] and backcrossed to C57BL/6J:Jcl (Clear Japan, Tokyo, Japan) for more than 16 generations. By crossing the C57BL/6J-background *Ogg1*$^{+/-}$, *Mth1*$^{+/-}$, and *Mutyh*$^{+/-}$ mice, we obtained *Mth1*$^{+/-}$/*Ogg1*$^{+/-}$ mice and *Ogg1*$^{+/-}$/*Mutyh*$^{+/-}$ mice. *Mth1*$^{+/-}$/*Ogg1*$^{+/-}$ mice were then mated with *Ogg1*$^{+/-}$/*Mutyh*$^{+/-}$ mice to obtain *Mth1*$^{+/-}$/*Ogg1*$^{-/-}$/*Mutyh*$^{+/-}$ mice. Finally, by crossing the *Mth1*$^{+/-}$/*Ogg1*$^{-/-}$/*Mutyh*$^{+/-}$ mice, we obtained a pair of *Mth1*$^{-/-}$/*Ogg1*$^{-/-}$/*Mutyh*$^{-/-}$ mice (TOY32M and TOY44F). All animals were maintained in a temperature-controlled (22 ± 2°C, 55 ± 5% humidity), specific pathogen-free room with a 12-h light-dark cycle. The care and use of all animals were performed in accordance with prescribed national guidelines, and the Animal Care and Use Committee of Kyushu University granted ethical approval for the study.

Quantification of 8-oxo-dG by LC-MS/MS. To detect the level of nuclear 8-oxodG, C57BL/6J:Jcl and TOY-KO mice (12–14 weeks old) were euthanized by cervical dislocation, and tissues were immediately removed and frozen in liquid nitrogen. DNA was extracted using a DNA Extractor TIS Kit (# 296-67701, Wako Pure Chemical Industries, Osaka, Japan), according to the manufacturer's instructions, with a slight modification: 10 mM 2, 2, 6, 6-tetramethylpiperidine-N-oxyl (Wako Pure Chemical Industries) was added to all reagents at all stages of manipulation[29]. Extracted DNA was hydrolyzed with 0.17 mg/ml nuclease P1 (Yamasa, Chiba, Japan) and 1.7 μM acid phosphatase (P-1435, Sigma-Aldrich Japan Inc., Tokyo, Japan) in 17 mM sodium acetate buffer (pH 4.5) at 37°C for 30 min, followed by filtration at 12,000 × *g* for 3 min (Ultrafree-MC probind 0.45 μm, Millipore, Billerica, MA). The digested samples (100 μl) were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using a Shimadzu VP-10 HPLC system connected to an API3000 MS/MS system (PE-SCIEX, SpectraLab Scientific Inc, Ontario, Canada).

Statistical analyses. Statistical analyses were conducted using JMP 9.02 (SAS Institute Japan, Tokyo, Japan).

Detection of germ line mutations by whole exome sequencing. Exome sequencing libraries for three TOY-KO mice (TOY365F, TOY450F and TOY609F) and five DBF1 (DBA/2J:Jcl × C57BL/6J:Jcl F1) mice as controls were prepared using a SureSelect^{XT} Mouse All Exon Kit (Agilent Technologies Japan, Tokyo, Japan), according to the manufacturer's instructions. Briefly, 3 μg of genomic tail DNA was sonicated into 150–180 bp fragments using a Covaris S2 System (Covaris, Woburn, MA, USA). The adaptors were ligated to the sonicated DNA after blunting and ~200 bp fragments were extracted using a 2% E-Gel (Life Technologies Japan, Tokyo, Japan). The extracted fragments were amplified with 2.5 mM SureSelect Pre-Capture primers and Platinum PCR Amplification Mix (Life Technologies), under the following conditions: 72°C for 20 min and 95°C for 5 min; 12 cycles of 95°C for 15 sec, 54°C for 45 sec and 70°C for 1 min; and a final extension at 70°C for 5 min. The PCR products were purified with a PureLink column (Life Technologies Japan). Purified PCR products (500 ng) were hybridized for 36 h at 65°C with SureSelect baits, according to the manufacturer's protocol. The captured libraries were amplified with the SureSelect Barcoding primer (BC1-8) for SOLiD with Herculase II Fusion DNA Polymerase (Agilent Technologies Japan), under the following conditions: 95°C for 5 min; 8 cycles of 95°C for 15 sec, 54°C for 45 sec and 70°C for 1 min; final extension at 70°C for 5 min. The captured barcoding libraries were quantified with an Agilent QPCR NHS Library Quantification Kit (Agilent Technologies Japan) and pooled. The four pooled libraries (1 pM) were amplified and purified with an EZ bead system (Life Technologies Japan). Purified P2-enriched beads were sequenced on one full slide of a SOLiD4 system (Life Technologies Japan). About 130 million paired-end sequencing reads (50 bp and 35 bp) were obtained from each library. Bioscope1.3.1 (Life Technologies Japan) was used to map the SOLiD paired-end reads to the mm9 reference mouse genome sequence (MGSCv37) using default parameters for Targeted resequencing methods. BEDtools v2.16.2 were used to calculate the coverage depth statistics and target enrichment efficiency. Avadis-NGS v1.3 (Strand Scientific Intelligence Inc., Karnataka, India) was used to carry out single nucleotide variant (SNV) calling with eight BAM format files (three TOY-KO lines and five control samples). The cutoff parameters of the SNV call were as follows: filtered sequencing quality ≤20, filtered PCR duplications, consensus base quality ≤50, total coverage <10, variants read depth <3, and the Decibel Score by Avadis-NGS v1.3 <50. The Decibel Score, read depth of the SNV allele and SNV allele frequency were used to sort these candidates. The iterative genomic viewer was used to check the candidates sequentially to eliminate apparent false positives. Finally, MassARRAY was used to select 286 mutation candidates for validation experiments (Supplementary Table S1 online).

Confirmation of mutations by sequencing. A MassARRAY3 Analyzer (Sequenom Inc, San Diego, CA) with iPLEX Gold Genotyping Reagent (Sequenom Inc) was used to validate the 286 candidates, according to the manufacturer's instructions. Briefly, MassARRAY Typer4 Assay Designer (Sequenom Inc) designed the 286 PCR primer pairs and 286 iPLEX primers as single-base extension primers for each candidate. We used 37 genomic DNA samples, including 35 samples from the TOY-KO pedigree and two control samples, as well as C57BL/6J and the original ES cell DNA to determine the origin of the *de novo* mutations in the TOY-KO pedigree. Ten nanograms of genomic DNA were used in each multiplex PCR for the MassARRAY. After dephosphorylation, single-base extension with the iPLEX primer and desalting were performed. The reaction products were spotted onto a 384-format SpectroCHIP with a MassARRAY Nanodispenser (Sequenom Inc) and then subjected to a MassARRAY 3 analyzer (Sequenom Inc). MassARRAY Typer 4.0 software (Sequenom Inc) was used to analyze the mass spectrum data.

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Association of polymorphisms in *GCKR* and *TRIB1* with nonalcoholic fatty liver disease and metabolic syndrome traits

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Abstract. In several genome-wide association studies, nonalcoholic fatty liver disease and alanine aminotransferase susceptibility variants have been identified in several genes, including *LYPLAL1*, *ZP4*, *GCKR*, *HSD17B13*, *PALLD*, *PPP1R3B*, *FDFT1*, *TRIB1*, *COL13A1*, *CPN1*, *ERLIN1*, *CWF19L1*, *EFCAB4B*, *PZP*, and *NCAN*. To investigate the relationship between these genes and nonalcoholic fatty liver disease in the Japanese population, we genotyped 540 patients and 1012 control subjects for 18 variations. We performed logistic regression analyses to characterize the association between the tested variations and nonalcoholic fatty liver disease. Metabolic syndrome and histological traits were also analyzed by linear regression. We also examined *GCKR* rs780094, *TRIB1* rs2954021, and *PNPLA3* rs738409 for epistatic effects. The A-allele of rs780094 in *GCKR* ($P = 0.0024$) and the A-allele of rs2954021 *TRIB1* ($P = 4.5\times10^{-5}$) were significantly associated with nonalcoholic fatty liver disease. *GCKR* rs780094 was also associated with decreased plasma glucose, and increased triglycerides in the patient and control groups. *GCKR* rs780094 was also associated with an increased ratio of visceral to subcutaneous fat area in the patients with nonalcoholic fatty liver disease. Variations in *GCKR*, *TRIB1*, and *PNPLA3* independently influenced nonalcoholic fatty liver disease and had no epistatic effects. Our data suggest variations in *GCKR* and *TRIB1* are involved in the development of nonalcoholic fatty liver disease.

Key words: *GCKR*, *TRIB1*, Nonalcoholic fatty liver disease, Metabolic syndrome, Japanese

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) has been recognized as an important health concern [1, 2]. NAFLD is a spectrum of liver diseases ranging from simple steatosis, through steatohepatitis (NASH), to fibrosis and ultimately cirrhosis. The frequency of patients presenting with NAFLD has increased in Japan in proportion to the increase in the population with metabolic syndrome [3]. NAFLD is observed in 20–30% of the population in Japan and approximately 1–3% of them are considered to have NASH, similar to American and European populations [3, 4].

In addition to environmental factors, genetic factors are important in the development of NAFLD [5]. In a previous search for these genetic factors, we found that variations in peroxisome proliferator-activated receptor γ coactivator 1α (*PPARGC1A*), angiotensin II type 1 receptor (*AGTR1*), and nitric oxide synthase 2 (inducible) (*NOS2*) are associated with NAFLD in Japanese individuals [6-8]. Genome-wide association studies (GWAS) have shown that SNPs in the patatin-like phospholipase domain containing 3 (*PNPLA3*) influence NAFLD and plasma liver enzymes [9-12]. We reported that the risk allele (G) of rs738409 in *PNPLA3* is strongly associated with NAFLD, as well as with

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increases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and fibrosis stage in Japanese patients with NAFLD [13]. In a recent GWAS, we found that polymorphisms in the SAMM50 sorting and assembly machinery component (*SAMM50*), parvin, β (*PARVB*), and *PNPLA3* were associated with the development and progression of NAFLD [14].

Several other susceptibility loci for NAFLD [9-12, 14] and ALT [15-17] have been reported in GWAS, but these loci have not been confirmed in the Asian population. We investigated the association between SNPs identified by GWAS and NAFLD in the Japanese population.

Materials and Methods

Study subjects

The entire study was conducted in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from each subject, and the protocol was approved by the ethics committee of Kyoto University, Yokohama City University, Hiroshima University, and Kurume University.

We enrolled 1012 control subjects (general population) from among Japanese volunteers undergoing medical examinations for common disease screening. Control subjects were retained from our previous study (control-2) [14]. Japanese patients with NAFLD who underwent liver biopsy (488 with NASH and 52 with simple steatosis) were enrolled; 392 (NAFLD-1) and 98 (NAFLD-2) of these subjects were retained from the previous study [14]. Control and NAFLD subjects were collected at Yokohama City University Hospital, Hiroshima University Hospital, and Kurume University Hospital. Patients with the following diseases were excluded from the study: viral hepatitis (hepatitis B and C, Epstein–Barr virus infection), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, drug-induced hepatitis, and alcoholic hepatitis (present or past daily consumption of more than 20 g alcohol per day). None of the patients showed clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding, or a serum bilirubin level greater than two-fold the normal upper limit.

Liver biopsy tissues were stained with hematoxylin and eosin, reticulin, and Masson's trichrome stain. The histological criterion for NAFLD diagnosis is macrovesicular fatty change in hepatocytes with displacement of the nucleus toward the cell edge [18]. When more than 5% of hepatocytes are affected by macrovesicular steatosis, patients are diagnosed as having either steatosis or NASH; minimal criteria for the diagnosis of NASH included the presence of >5% macrovesicular steatosis, inflammation, and liver cell ballooning, typically with predominantly centrilobular (acinar zone 3) distribution [19, 20]. The degree of steatosis was graded as follows, based on the percentage of hepatocytes containing macrovesicular fat droplets: grade 0, no steatosis; grade 1, <33% hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets [21]. Hepatitis activity (necroinflammatory grade) was also determined on the basis of the composite NAS, as described by Kleiner *et al.* [22]. NAS is the unweighted sum of the scores for steatosis, lobular inflammation, and hepatocellular ballooning, and ranges from 0 to 8. Fibrosis severity was scored according to the method of Brunt [18] and was expressed on a 4-point scale as follows: 0, none; 1, perivenular and/or perisinusoidal fibrosis in zone 3; 2, combined pericellular portal fibrosis; 3, septal/bridging fibrosis; and 4, cirrhosis.

Clinical and laboratory evaluation

Patient weight and height were measured using a calibrated scale after removing shoes and heavy clothing, if present. Venous blood samples were obtained after overnight fasting (12 h) to measure plasma glucose, hemoglobin A1c (HbA1c), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, serum AST, and ALT. All blood chemistry was measured using conventional methods.

The patients underwent CT imaging (in the supine position) to measure visceral fat area (VFA) and subcutaneous fat area (SFA) at the umbilical level (L4–L5); these values were calculated using the FatScan software program (N2system, Osaka, Japan) [23]. Clinical characteristics are shown in Table 1.

DNA extraction and SNP genotyping

Genomic DNA was extracted using Genomix (Talent Srl, Trieste, Italy) for blood samples collected from each subject. Invader probes (Third Wave Technologies, Madison, WI, USA) were designed for 18 SNPs previously identified as susceptibility loci for NAFLD [10, 11] or ALT [15-17]. The SNPs were

Table 1 Clinical characteristics

	NAFLD	Control	*P*
n	540	1012	–
Men/Women	285/255	500/512	0.21*
Type 2 diabetes (%)	250 (46.3%)	66 (6.5%)	1.1×10^{-76}*
Age (year)	50.5 ± 14.3	53.1 ± 15.3	0.0013
BMI (kg/m^2)	28.0 ± 5.0	22.7 ± 3.2	1.9×10^{-107}
Plasma glucose (mg/dL)	118.5 ± 36.1	98.2 ± 19.0	1.2×10^{-61}
HbA1c (%)	6.4 ± 1.3	5.5 ± 0.7	6.2×10^{-63}
Total cholesterol (mg/dL)	212.4 ± 39.3	208.5 ± 36.2	0.21
Triglycerides (mg/dL)	167.0 ± 105.2	110.0 ± 88.5	1.4×10^{-57}
HDL-cholesterol (mg/dL)	52.9 ± 14.8	62.7 ± 15.5	3.3×10^{-37}
SBP (mmHg)	128.1 ± 14.7	124.5 ± 19.1	1.0×10^{-4}
DBP (mmHg)	78.5 ± 11.6	76.3 ± 11.6	2.2×10^{-4}
AST (IU/L)	50.1 ± 29.9	23.0 ± 10.2	1.2×10^{-119}
ALT (IU/L)	81.0 ± 56.3	20.3 ± 11.8	4.1×10^{-171}
VFA (cm^2)†	128.6 ± 58.3	–	–
SFA (cm^2)†	209.9 ± 102.6	–	–
V/S ratio†	0.71 ± 0.39	–	–
Steatosis grade (1-3)	1.6 ± 0.7	–	–
Lobular inflammation (0-3)	1.3 ± 0.7	–	–
Hepatocyte ballooning (0-2)	1.1 ± 0.7	–	–
NAS (0-8)	4.0 ± 1.6	–	–
Fibrosis stage (0-4)	1.7 ± 1.0	–	–

P-values for comparison of the clinical data between the simple steatosis and NASH groups were obtained by the Mann–Whitney *U*-test. *, ratios were analyzed by χ^2-test. †, n = 439

as follows: rs12137855 near lysophospholipase-like 1 (*LYPLAL1*); rs2499604 near zona pellucida glycoprotein 4 (*ZP4*); rs780094 in glucokinase (hexokinase 4) regulator (*GCKR*); rs6834314 near hydroxysteroid (17-β) dehydrogenase 13 (*HSD17B13*); rs2710833 near palladin, cytoskeletal associated protein (*PALLD*); rs343062 in chromosome 7, position 35,549,066; rs4240624 and rs2126259 near protein phosphatase 1, regulatory subunit 3B (*PPP1R3B*); rs2645424 in farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*); rs2954021 near tribbles homolog 1 (*TRIB1*); rs1227756 in collagen, type XIII, α 1 (*COL13A1*); rs10883437 and rs11597390 near carboxypeptidase N, polypeptide 1 (*CPN1*); rs2862954 in ER lipid raft associated 1 (*ERLIN1*); rs17668255 in CWF19-like 1, cell cycle control (*CWF19L1*); rs887304 in EF-hand calcium binding domain 4B (*EFCAB4B*); rs6487679 near pregnancy-zone protein (*PZP*); and rs2228603 in neurocan (*NCAN*). SNPs were genotyped by Invader assay as described [24], with a success rate of >98.0%.

Statistical analysis

We categorized the genotypes as 0, 1, or 2, depending on the number of risk alleles present. Odds ratios (OR) and *P*-values, adjusted for age, sex, logarithmically transformed body mass index (BMI), and the presence of type 2 diabetes mellitus (DM), were calculated by multiple logistic regression analysis. Multiple linear regression analyses were performed to test the independent effect of each allele on biochemical traits, and histological and anthropometric parameters, accounting for the effects of other variables (i.e., age, sex, and logarithmically transformed BMI). BMI, fasting plasma glucose, triglycerides, AST, ALT, VFA, SFA, and VFA to SFA (V/S) ratio values were logarithmically transformed before performing multiple linear and logistic regression analyses. Simple comparisons of the clinical data between NAFLD and control groups were carried out using the Mann–Whitney *U*-test. Male: female and the presence of DM ratios were analyzed by χ^2-test. To test SNP×SNP epistasis for case–control population-based samples, we used the logistic regression model for each SNP1 and SNP2, and fit the model in the form of $Y=\beta_0+\beta_1\times SNP1+\beta_2\times SNP2+\beta_3\times SNP1\times SNP2+\beta_4\times age+\beta_5\times sex+\beta_6\times \log_{10}(BMI)+\beta7\times DM$. Statistical analyses were performed using PLINK 1.07 (http://pngu.mgh.harvard.edu/purcell/plink) [25] and R software (http://www.r-project.

org/). *P*-values less than 2.8×10^{-3} (0.05/18) was considered significant.

Results

First, we examined the association of 18 SNPs with NAFLD and control subjects. We performed multiple logistic regression analysis using genotypes, age, sex, BMI, and the presence of DM as independent variables. Two SNPs, rs780094 in *GCKR* ($P = 0.0024$) and rs2954021 in *TRIB1* ($P = 4.5 \times 10^{-5}$), were significantly associated with NAFLD (Table 2). No other SNPs showed significant associations with NAFLD. Minor allele frequencies (MAFs) of eight SNPs were no more than 0.06. The lack of a significant association, especially in those SNPs with small MAF, is most likely due to the relatively low power of this study. All SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$), with the exception of rs12137855 ($P = 0.025$) in the NAFLD patients.

Next, we examined the association of rs780094 and rs2954021 with metabolic syndrome traits in NAFLD. As reported previously [11], rs780094 in *GCKR* is associated with lower plasma glucose ($P = 0.0047$), higher triglycerides ($P = 0.0029$), and higher diastolic blood pressure ($P = 0.018$) (Table 3). An association

Table 2 Association tests of SNPs in patients with NAFLD and control subjects

Chr	SNP ID	position (build 132)	Nearby gene	Allele1/ allele2	Risk allele	Risk allele frequency		*P*-value	OR (95%CI)
						NAFLD	Control		
1	rs12137855	219,448,378	*LYPLAL1*	T/C	C	0.94	0.95	0.72	0.93 (0.61 - 1.40)
1	rs2499604	238,103,501	*ZP4*	A/G	A	0.46	0.48	0.59	0.95 (0.77 - 1.16)
2	rs780094	27,741,237	*GCKR*	G/A	A	0.62	0.55	**0.0024**	1.37 (1.12 - 1.68)
4	rs6834314	88,213,808	*HSD17B13*	G/A	A	0.69	0.66	0.30	1.12 (0.91 - 1.38)
4	rs2710833	169,409,958	*PALLD*	T/C	T	0.13	0.12	0.53	0.91 (0.67 - 1.23)
7	rs343062	35,549,066	no gene	T/C	T	0.46	0.47	0.92	0.99 (0.81 - 1.22)
8	rs4240624	9,184,231	*PPP1R3B*	G/A	A	0.99	0.99	0.40	0.64 (0.23 - 1.79)
8	rs2126259	9,185,146	*PPP1R3B*	A/G	G	0.99	0.99	0.37	0.62 (0.22 - 1.74)
8	rs2645424	11,684,463	*FDFT1*	C/T	C	0.23	0.21	0.30	1.13 (0.90 - 1.43)
8	rs2954021	126,482,077	*TRIB1*	A/G	A	0.52	0.45	**4.5×10^{-5}**	1.53 (1.25 - 1.88)
10	rs1227756	71,588,504	*COL13A1*	A/G	G	0.72	0.72	0.82	1.03 (0.82 - 1.29)
10	rs10883437	101,795,361	*CPN1*	A/T	T	0.83	0.82	0.64	0.94 (0.73 - 1.21)
10	rs11597390	101,861,435	*CPN1*	A/G	G	0.96	0.95	0.80	0.94 (0.59 - 1.51)
10	rs2862954	101,912,064	*ERLIN1*	C/T	T	0.97	0.95	0.58	1.16 (0.69 - 1.94)
10	rs17668255	102,000,701	*CWF19L1*	T/C	C	0.97	0.95	0.54	1.17 (0.70 - 1.97)
12	rs887304	3,757,548	*EFCAB4B*	A/G	A	0.005	0.001	0.70	1.46 (0.22 - 9.64)
12	rs6487679	9,371,332	*PZP*	C/T	C	0.11	0.11	0.75	1.05 (0.77 - 1.43)
19	rs2228603	19,329,924	*NCAN*	T/C	T	0.05	0.06	0.41	0.82 (0.51 - 1.32)

The OR for each SNP was adjusted simultaneously for age, sex, logarithmically transformed BMI, and the presence of DM. Bold entries indicate *P*-value < 0.05.

Table 3 Tests of association between significant SNPs and metabolic traits in NAFLD

SNP ID	rs780094				rs2954021			
	NAFLD		Control		NAFLD		Control	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
Plasma glucose	-0.018	**0.0047**	-0.007	**0.014**	-0.003	0.67	-0.003	0.29
Total cholesterol	0.182	0.94	1.464	0.34	2.663	0.28	0.187	0.90
Triglycerides	0.037	**0.0029**	0.039	**2.5×10^{-5}**	0.013	0.30	0.005	0.58
HDL-cholesterol	-0.588	0.49	-0.946	0.12	0.750	0.39	0.014	0.98
SBP	0.000	1.00	-0.280	0.69	-0.180	0.88	-1.102	0.13
DBP	2.222	**0.018**	-0.082	0.86	-1.115	0.24	-0.311	0.50
AST	0.005	0.72	0.009	0.21	-0.009	0.51	0.007	0.33
ALT	-0.008	0.58	0.019	**0.036**	-0.017	0.27	0.012	0.18

Data were derived from linear regression analysis. Values of FPG, triglycerides, AST, and ALT were logarithmically transformed. Each metabolic phenotype was adjusted simultaneously for age, sex, and logarithmically transformed BMI. Bold entries indicate *P*-value < 0.05.

Table 4 Association between significant SNPs and histological traits in NAFLD subjects

SNP ID	Steatosis grade		Lobular inflammation		Hepatocyte ballooning		NAS		Fibrosis stage	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
rs780094	0.013	0.76	0.006	0.89	0.004	0.92	0.002	0.99	0.049	0.42
rs2954021	0.005	0.92	-0.140	**0.0016**	-0.072	0.09	-0.214	**0.029**	-0.152	**0.014**

Data were derived from linear regression analysis. Each phenotype was adjusted for age, sex, logarithmically transformed BMI, and the presence of DM. Bold entries indicate *P*-value < 0.05.

Table 5 Association between significant SNPs and anthropometric parameters in NAFLD subjects

SNP ID	BMI		VFA*		SFA*		V/S ratio*	
	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*-value
rs780094	-0.004	0.37	0.023	0.051	-0.011	0.26	0.036	**0.0067**
rs2954021	-0.007	0.11	-0.024	**0.048**	-0.023	**0.021**	0.002	0.87

Data were derived from linear regression analysis. Values of BMI, VFA, SFA, and V/S ratio were logarithmically transformed. BMI and V/S ratio were adjusted for age and sex. VFA and SFA were adjusted for age, sex, and logarithmically transformed BMI. Bold entries indicate *P*-value < 0.05. *, n = 439.

Table 6 Logistic analysis of 3 SNPs on the association between NAFLD and control subjects

Explanatory valuables	*P*-value	OR (95% CI)
rs738409	4.1×10^{-13}	2.20 (1.78 - 2.72)
rs2954021	9.7×10^{-5}	1.52 (1.23 - 1.88)
rs780094	0.0011	1.42 (1.15 - 1.76)

The *P*-value and OR were derived from the following logistic regression model:
$Y=\beta_0+\beta_1\times$rs738409$+\beta_2\times$rs2954021$+\beta_3\times$rs780094$+\beta_4\times$age$+\beta_5\times$sex$+\beta_6\times\log_{10}$(BMI)$+\beta7\times$DM.

between rs780094 with lower plasma glucose (P = 0.014) and higher triglycerides ($P = 2.5\times10^{-5}$) was also observed in the control subjects. Originally, rs2954021 in *TRIB1* was reported to be associated with ALT [16]; however, we observed no association with ALT or AST in the NAFLD and control subjects. The A-allele of rs2954021 in *TRIB1*, which is a risk allele of NAFLD, was associated with lower lobular inflammation, NAS, and fibrosis grade, and rs780094 in *GCKR* was not associated with histological phenotype (Table 4). We also examined anthropometric parameters in the NAFLD patients and found that rs780094 in *GCKR* was associated with increased V/S ratio (P = 0.0067) (Table 5).

Next, we tested SNP×SNP epistasis, including rs738409 in *PNPLA3*, which was an SNP for NAFLD susceptibility. No SNP pairs showed significant epistatic effects on NAFLD (data not shown). We performed multiple logistic regression analysis of three genotypes (rs780094, rs2954021, and rs738409), age, sex, logarithmically transformed BMI, and the presence of DM as independent variables and found that the effects of these SNPs were independent (Table 6).

Discussion

After the first report of an association of *PNPLA3* rs738409 with NAFLD [9], many replication studies and meta-analysis were performed in various populations, verifying the importance of rs738409 in the development of NAFLD [11-14, 26, 27]. GWAS for NAFLD and ALT yielded SNPs in genes other than *PNPLA3* [15-17]; however, a few replication studies produced conflicting results. Among the 18 SNPs in this study, only six SNPs (rs2499604, rs780094, rs2645424, rs1227756, rs2862954, and rs6487679) were included in our previous GWAS [14]. The *P*-values for six SNPs exceeded the cut-off levels (5.0×10^{-5}) and proceeded to the second stage of analysis. The JSNP database (http://snp.ims.u-tokyo.ac.jp/) was used as a control; we made no adjustment for age, sex, BMI, or the presence of DM because clinical information was not available. The NAFLD sample size in our GWAS was relatively small (n = 392). Therefore, we investigated 18 SNPs associated with NAFLD and ALT susceptibility, including the six SNPs described above, in a larger

set of NAFLD patients and control subjects for whom clinical information was available.

In this study, we confirmed the association of rs780094 in *GCKR* and rs2954021 in *TRIB1*. The A-allele of rs780094 in *GCKR* was associated with NAFLD in subjects of European descent [11, 28, 29]. No association between the A-allele of rs780094 and NAFLD was observed in African American and Hispanic Americans [28, 29]. A study in Asian populations (Indian, Malay, and Chinese) was not conclusive, due to small sample size [30]. GWAS in the Japanese population reported by Kawaguchi *et al.* showed a weak association ($P = 0.011$) [12]. Therefore, rs780094 in *GCKR* is associated with NAFLD in the Japanese population. Other NAFLD susceptibility SNPs in *LYPLAL1*, *PPP1R3B,* and *NCAN* were not associated with NAFLD in our study. *LYPLAL1* rs12137855 and *NCAN* rs2228603 were not associated with NAFLD in another Japanese study [12]. This may be due to the relatively lower power of this study, since the MAFs were no more than 0.05. These results may be also due to ethnic differences in linkage disequilibrium (LD) patterns, ethnic-specific association, and gene/environment interactions.

The A-allele of rs780094 in *GCKR* was associated with decreased fasting plasma glucose and increased triglycerides, as reported by Speliotes *et al.* [11]. An association between increased triglycerides and rs780094 has been reported in previous GWAS [31]. The A-allele of rs780094 in *GCKR* was associated with an increased V/S ratio in our study. These data suggest the A-allele of rs780094 in *GCKR* is related to the development of NAFLD through the increased serum triglycerides caused by visceral fat accumulation.

TRIB1 rs2954021 is associated with increased ALT [16]. *TRIB1* rs17321515, which is in LD ($r^2 = 1.00$), is associated with increased triglycerides [32]. Although rs2954021 was associated with NAFLD, this SNP was not associated with ALT or triglycerides in our study. Although further study is necessary, these and previous results suggest *TRIB1* rs2954021 is related to the development of NAFLD through increased triglycerides.

We previously showed that *PNPLA3* rs738409 is associated with NAFLD severity [14]. *GCKR* rs780094 and *TRIB1* rs2954021 were not associated with histological traits and ALT levels, suggesting these SNPs are not related to NAFLD severity. *PNPLA3* rs738409 was not associated with metabolic syndrome traits [14]. These results were confirmed in this study (490 subjects overlapped) and *PNPLA3* rs738409 was not associated with VFA ($P = 0.32$) or V/S ratio ($P = 0.14$). The effects of *GCKR*, *TRIB1*, and *PNPLA3* on NAFLD were different. Indeed, *GCKR* rs780094, *TRIB1* rs2954021, and *PNPLA3* rs738409 were independently associated with NAFLD.

In conclusion, *GCKR* rs780094 may be involved in the development of NAFLD but does not affect disease severity. Our study suggests *GCKR* rs780094, *TRIB1* rs2954021, and *PNPLA3* rs738409 affect NAFLD through different mechanisms.

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Disclosure Statement

The authors have nothing to disclose.

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Original Article

Early Effect of Single-dose Sitagliptin Administration on Gastric Emptying: Crossover Study Using the ^{13}C Breath Test

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Background/Aims
The gastrointestinal motility effects of endogenous incretin hormones enhanced by dipeptidyl peptidase-IV (DPP-IV) inhibitors have not yet been sufficiently investigated. The aim of this study was to determine whether single pre-prandial sitagliptin, the DPP-IV inhibitor, administration might have an effect on the rate of liquid gastric emptying using the ^{13}C-acetic acid breath test.

Methods
Ten healthy male volunteers participated in this randomized, two-way crossover study. The subjects fasted for overnight and were randomly assigned to receive 50 mg sitagliptin 2 hours before ingestion of the liquid test meal (200 kcal per 200 mL, containing 100 mg ^{13}C-acetate) or the test meal alone. Under both conditions, breath samples were collected for 150 minutes following the meal. Liquid gastric emptying was estimated by the values of the following parameters: the time required for 50% emptying of the labeled meal ($T_{1/2}$), the analog to the scintigraphy lag time for 10% emptying of the labeled meal (T_{lag}), the gastric emptying coefficient and the regression-estimated constants (β and κ), calculated by using the $^{13}CO_2$ breath excretion curve using the conventional formulae. The parameters between the 2 test conditions were compared statistically.

Results
No significant differences in the calculated parameters, including $T_{1/2}$, T_{lag}, gastric emptying coefficient or β and κ, were observed between the 2 test conditions.

Conclusions
The present study revealed that single-dose sitagliptin intake had no significant influence on the rate of liquid gastric emptying in asymptomatic volunteers.
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Key Words
Breath tests; Gastric emptying; Sitagliptin

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Introduction

The incretin hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are peptides secreted from the intestine into the circulation in response to food ingestion, and they help manage glycemic control by regulating insulin and glucagon release, slowing gastric emptying, and reducing caloric intake.[1-4] Physiologically, the clinical utility of native GLP-1 and GIP is limited because they are rapidly degraded and inactivated by the enzyme dipeptidyl peptidase-IV (DPP-IV).[5,6]

Inhibition of this enzyme leads to an increase in circulating endogenous GLP-1 and GIP levels. Therefore, DPP-IV inhibitors are a novel therapeutic strategy for type 2 diabetes. Since the release of sitagliptin in 2006, numerous studies have documented the advantages of DPP-IV inhibitors in the management of type 2 diabetes mellitus.[7-10] However, the effect of DPP-IV inhibitor-induced enhancement of endogenous incretin hormones on gastrointestinal motility has not yet been sufficiently investigated.[11,12] In the present study, the pharmacological effects of pre-prandial single-dose sitagliptin administration on the rate of liquid gastric emptying were examined in healthy volunteers using a ^{13}C-acetic acid breath test.

Materials and Methods

Subjects

The subjects were 10 asymptomatic male volunteers (median age 34 years, range 27-50 years). The height and weight of the subjects were as follows: median height, 169 cm; height range, 162-181 cm; median weight, 64.5 kg; and weight range, 60-92 kg. None of the subjects were habitual drinkers. All were non-smokers and none had a history of gastrointestinal disease or abdominal surgery. None of the subjects was on any routine medication at the time of the study.

The study (Clinical trial registry number: UMIN 000006213) was conducted in accordance with the Declaration of Helsinki. Prior to study initiation, written informed consent was obtained from all participants. The study protocol using the ^{13}C-acetic acid breath test was approved by the Ethics Committee of Yokohama City University School of Medicine.

^{13}C-acetic Acid Breath Test

Ten subjects participated in this randomized, two-way crossover study (Fig. 1). After overnight fasting (at least 8 hours), the subjects received 50 mg sitagliptin orally 2 hours before ingestion of the test meal (sitagliptin condition) or the test meal alone (control condition) in a random sequence. The 2 test conditions were separated by a washout period of at least 7 days.

The test meal was a 200 kcal per 200 mL liquid meal (Racol with milk flavor, Otsuka Pharmaceutical, Co., Ltd., Tokyo, Japan) containing 100 mg of ^{13}C-acetic acid (Cambridge Isotope Laboratories, Inc., USA), and the subjects were requested to consume the meal within 5 minutes.

Gastric emptying was measured using the ^{13}C-acetic acid breath test while the subjects were seated. Breath samples were collected in air bags at baseline (before test meal) and at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 135 and 150 minutes after completion of the test meal ingestion. The $^{13}CO_2/^{12}CO_2$ ratio in collected breath samples was determined as the difference above baseline using non-dispersive infrared spectrophotometry (POCone, Otsuka Electronics Co., Ltd., Osaka, Japan).

Data Analysis

In accordance with the method reported by Ghoos et al,[13] the percentage of $^{13}CO_2$ recovery in expired breaths per hour (percent dose per hour) against time was fitted to the formula $y(t) = at^{b}e^{-ct}$ by non-linear regression analysis, where y is the percentage of ^{13}C excretion in breath per hour, t is time in hours, and a, b, and c are constants. The time-course of cumulative $^{13}CO_2$ recovery in expired breaths can be fitted to another formula, $z(t) =$

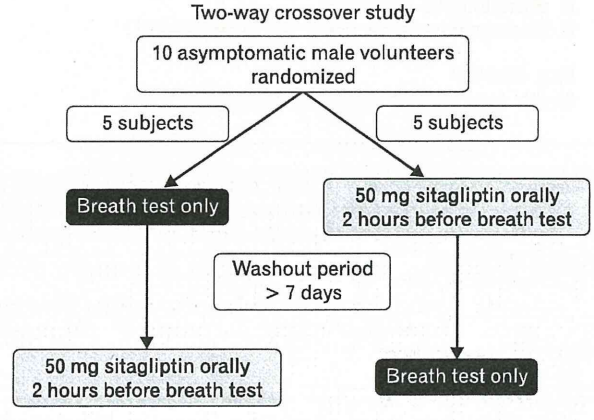

Figure 1. The flow of volunteers throughout the trial: two-way crossover study.