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Two novel susceptibility loci for type 2 diabetes mellitus identified by longitudinal exome-wide association studies in a Japanese population

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ABSTRACT

Recent genome-wide association studies identified genetic variants that confer susceptibility to type 2 diabetes mellitus (T2DM). However, few longitudinal genome-wide association studies of this metabolic disorder have been reported to date. Therefore, we performed a longitudinal exome-wide association study of T2DM, using 24,579 single nucleotide polymorphisms (SNPs) and repeated measurements from 6022 Japanese individuals. The generalized estimating equation model was applied to test relations of SNPs to three T2DM-related parameters: prevalence of T2DM, fasting plasma glucose level, and blood glycosylated hemoglobin content. Three SNPs that passed quality control were significantly ($P < 2.26 \times 10^{-7}$) associated with two of the three T2DM-related parameters in additive and recessive models. Of the three SNPs, rs6414624 in *EVC* and rs78338345 in *GGA3* were novel susceptibility loci for T2DM. In the present study, the SNP of *GGA3* was predicted to be a genetic variant whose minor allele frequency has recently increased in East Asia.

1. Introduction

According to a global report on diabetes from the World Health Organization (WHO), the estimated number of patients with diabetes was 422 million in 2014 [1]. This number is approximately four-fold greater than that in 1980. The WHO estimated that in 2030, diabetes would be the 7th leading cause of death worldwide [2]. In Japan, the Ministry of Health, Labour, and Welfare reported that the number of deaths (per 100,000 population) from diabetes was 13,327 in 2015 (http://www.e-stat.go.jp/SG1/estat/, [3]).

Genome-wide association studies (GWASs) for genetic variants linked to type 2 diabetes mellitus (T2DM) revealed > 80 susceptibility loci for T2DM [4]. Particularly, a large number of GWASs has examined T2DM in populations with European ancestry. However, previous studies reported inter-ethnic differences in the genetic contribution to T2DM [4–11]. A large-scale GWAS meta-analysis identified seven novel loci that commonly conferred susceptibility to T2DM across a variety of ethnic groups (European, East Asian, South Asian, and Mexican/Mexican American). However, of the seven loci, the effects of six loci on T2DM were not remarkable in a Japanese population, and a single nucleotide polymorphism (SNP) near the remaining locus, *LPP*, was monomorphic in the population examined [8]. The discrepancy in disease-susceptible variants among populations could be due to differences in genetic and environmental factors among the ethnic groups, sample sizes, or statistical methods used.

Although previous GWASs identified numerous T2DM susceptibility loci, most studies were conducted in a cross-sectional manner, which commonly measures traits at a single point in time. The longitudinal study can evaluate temporal changes in fasting plasma glucose (FPG) level and blood glycosylated hemoglobin (HbA_{1c}) content as well as the prevalence of T2DM related to age. Therefore, we performed longitudinal exome-wide association studies (EWASs) to explore novel

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Table 1
Characteristics of 6022 study subjects in the Inabe cohort.

Characteristic	Controls[a]	Subjects with T2DM[a]
No. of subjects	5267	755
Sex (male/female, %)	53.4/46.6	71.3/28.7
Age (years)	51.5 ± 0.08 (25,000)	59.7 ± 0.17 (3520)
Height (cm)	162.5 ± 0.06 (24,744)	163.2 ± 0.17 (3168)
Weight (kg)	60.5 ± 0.07 (24,744)	65.4 ± 0.23 (3166)
Body mass index (kg/m^2)	22.8 ± 0.02 (24,744)	24.4 ± 0.07 (3166)
Waist circumference (cm)	80.2 ± 0.07 (18,924)	85.5 ± 0.20 (2423)
Smoking (%)	37.8 (25,000)	43.0 (3520)
Hypertension (%)	30.1 (25,000)	60.1 (3520)
Systolic blood pressure (mm Hg)	119.8 ± 0.10 (24,737)	127.7 ± 0.30 (3163)
Diastolic blood pressure (mm Hg)	74.3 ± 0.08 (24,737)	78.0 ± 0.22 (3163)
Fasting plasma glucose (mmol/L)	5.27 ± 0.005 (25,000)	7.41 ± 0.039 (3520)
Blood hemoglobin A_{1c} (%)	5.57 ± 0.003 (18,290)	6.64 ± 0.023 (2717)
Dyslipidemia (%)	55.7 (25,000)	76.8 (3520)
Serum triglycerides (mmol/L)	1.26 ± 0.006 (24,618)	1.26 ± 0.016 (3411)
Serum HDL-cholesterol (mmol/L)	1.61 ± 0.003 (24,592)	1.60 ± 0.008 (3402)
Serum LDL-cholesterol (mmol/L)	3.19 ± 0.005 (23,521)	3.11 ± 0.015 (3301)
Chronic kidney disease (%)	9.1 (25,000)	24.7 (3520)
Serum creatinine (μmol/L)	62.0 ± 0.41 (25,000)	125.3 ± 3.78 (3520)
eGFR (mL min^{-1} 1.73^{-1} m^{-2})	79.6 ± 0.11 (22,466)	71.1 ± 0.46 (3293)
Hyperuricemia (%)	16.5 (25,000)	26.8 (3520)

[a] Values in parentheses indicate the numbers of measurements taken. Quantitative data are means and standard errors. Categorical data are percentages. T2DM, type 2 diabetes mellitus; HDL, high density lipoprotein; LDL, low density lipoprotein; eGFR, estimated glomerular filtration rate.

susceptibility genes for T2DM. Environmental factors, including lifestyle and diet, affect the prevalence of T2DM, and insulin secretion and sensitivity differ among ethnic groups [12]. Therefore, we also focused on genetic variants showing recent expansion across East Asian populations, because these variants may have been acquired to adapt to specific environments.

2. Results

2.1. Characteristics of subjects

Our research group has traced physiological data for 6026 community-dwelling individuals who visited the health care center of Inabe General Hospital for an annual health checkup in Inabe City, Japan from April 2003 to March 2014 (mean followed-up period, 5 years). We refer to this cohort as the "Inabe cohort." The characteristics of 6022 subjects with respect to longitudinal data, omitting four outliers identified by principal component analysis, are shown in Table 1. The Inabe cohort consists of 755 subjects with T2DM and 5267 controls. The proportion of T2DM was higher in males (8.9%, 538 subjects) than in females (3.6%, 217 subjects), while the mean age was nearly equal between the sexes (approximately 52.5 years old). This result is consistent with a report from the Ministry of Health, Labour, and Welfare in Japan [13]. Values for most medical examinations [age, height, weight, body mass index (BMI), waist circumference, systolic and diastolic blood pressure, FPG, HbA_{1c}, serum concentration of creatinine, and the prevalence of hypertension, dyslipidemia, chronic kidney disease, and hyperuricemia] were higher in subjects with T2DM than in controls, except that for estimated glomerular filtration rate.

2.2. Association of SNPs with longitudinal data on T2DM-related parameters

After removing monomorphic sites of ~244,000 genetic variants among 6022 individuals, genotype data were converted into suitable formats for three genetic models. After quality controls (see Sections 5.3 and 5.4 in Materials and methods), we examined the relations of 24,579 SNPs to repeated outcomes of the following three T2DM-related parameters: prevalence of T2DM, FPG level, and HbA_{1c} content. The relations were assessed using the generalized estimating equation (GEE) model [14,15] after adjusting for age, gender, BMI, and smoking status. In our longitudinal EWASs, the recessive model yielded 76 and three SNPs that were significantly ($P < 2.26 \times 10^{-7}$) associated with the prevalence of T2DM and FPG levels, respectively (Fig. 1, Table 2, and Supplementary Table S1). Of these SNPs, two showed relations to both the prevalence of T2DM and FPG levels. In the additive model, one SNP showed significant ($P < 2.26 \times 10^{-7}$) relations to both FPG and HbA_{1c} (Fig. 1, Table 2, and Supplementary Table S2). The dominant model showed no association between SNPs and the three T2DM-related parameters. In total, the GEE model showed that three SNPs were associated with either of the following pairs: (i) prevalence of T2DM and FPG, and (ii) FPG and HbA_{1c} (Fig. 1).

In our longitudinal EWASs, rs6414624 of the EvC ciliary complex subunit 1 gene (*EVC*), rs78338345 of the golgi associated, gamma adaptin ear containing, ARF binding protein 3 gene (*GGA3*), and rs11558471 of the solute carrier family 30 member 8 gene (*SLC30A8*) were regarded as genetic variants that confer susceptibility to T2DM. Of these candidate SNPs, the association of rs11558471 in *SLC30A8* with T2DM-related phenotypes has been shown by previous studies [16–19]. The remaining two SNPs have not been reported, according to the GWAS Central, GWAS Catalogue, and DisGeNET databases. For novel candidate SNPs, no homozygote with minor alleles was affected by T2DM, and the minor alleles significantly decreased both FPG and HbA_{1c} values in the longitudinal data (Table 2). These results suggest that minor alleles of two candidate SNPs are protective against T2DM.

In the recessive model, two SNPs [rs10490775 of the protein tyrosine phosphatase, receptor type G gene (*PTPRG*) and rs61739510 of the glycosyltransferase 6 domain containing 1 gene (*GLT6D1*)] showed a significant ($P < 2.26 \times 10^{-7}$) association with the prevalence of T2DM (Supplementary Table S1). These SNPs were also related to the

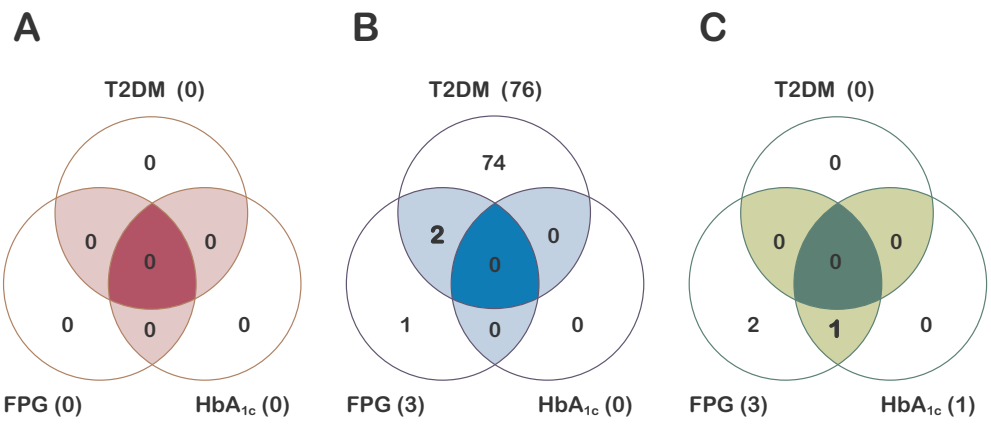

Fig. 1. SNPs showing significant relations to type 2 diabetes mellitus in the (A) dominant, (B) recessive, and (C) additive genetic models. Relations were tested using the generalized estimating equation (GEE) model with adjustments for age, gender, BMI, and smoking status. Candidate SNPs were determined when at least two out of three GEE analyses commonly showed significant relations. The three GEE analyses independently tested the association between SNPs and three parameters; prevalence of type 2 diabetes mellitus (T2DM), fasting plasma glucose (FPG) level, and blood glycosylated hemoglobin (HbA_{1c}). Based on Bonferroni's correction, *P*-values of $< 2.26 \times 10^{-7}$ for the three genetic models were considered statistically significant.

Table 2
T2DM-associated SNPs detected by the generalized estimating equation with adjustments for age, gender, BMI, and smoking status, using 24,579 SNPs ($P < 2.26 \times 10^{-7}$).

RefSNP ID	Location[a]	Gene	Genotype	MAF	All subjects[b]	Subjects with T2DM[b]	Controls[b]	*P*-value (T2DM)	FPG (mmol/l)[c]	*P*-value (FPG)	HbA_{1c} (%)[c]	*P*-value (HbA_{1c})
rs10490775[d]	3: 62,051,050	*PTPRG*	GG	0.062	25,166 (88.2%)	3156 (89.7%)	22,010 (88.0%)	0.353[e]	5.6 ± 0.01	0.021[e]	5.7 ± 0.00	0.514[e]
			GA		3236 (11.3%)	364 (10.3%)	2872 (11.5%)	**< 2.0×10^{-16}**[f]	5.6 ± 0.02	**3.3×10^{-7}**[f]	5.7 ± 0.01	0.097[f]
			AA		118 (0.4%)	0 (0.0%)	118 (0.5%)	0.481[g]	5.1 ± 0.04	0.069[g]	5.5 ± 0.03	0.658[g]
rs6414624	4: 5,741,785	*EVC*	CC	0.469	24,192 (84.8%)	3019 (85.8%)	21,173 (84.7%)	0.096[e]	5.6 ± 0.01	0.347[e]	5.7 ± 0.00	0.837[e]
			CT		4153 (14.6%)	501 (14.2%)	3652 (14.6%)	**< 2.0×10^{-16}** [f]	5.6 ± 0.02	**9.1×10^{-11}**[f]	5.7 ± 0.01	0.001[f]
			TT		172 (0.6%)	0 (0.0%)	172 (0.7%)	0.263[g]	5.3 ± 0.03	0.706[g]	5.6 ± 0.02	0.530[g]
rs61739510[d]	9: 135,624,282	*GLT6D1*	CC	0.085	25,423 (89.1%)	3162 (89.8%)	22,261 (89.0%)	0.105[e]	5.6 ± 0.01	0.692[e]	5.7 ± 0.00	0.116[e]
			CT		2984 (10.5%)	358 (10.2%)	2626 (10.5%)	**< 2.0×10^{-16}**[f]	5.6 ± 0.02	**5.8×10^{-7}**[f]	5.7 ± 0.01	0.078[f]
			TT		113 (0.4%)	0 (0.0%)	113 (0.5%)	0.202[g]	5.3 ± 0.04	0.934[g]	5.6 ± 0.04	0.167[g]
rs78338345	17: 75,242,428	*GGA3*	CC	0.446	22,343 (78.4%)	2802 (79.8%)	19,541 (78.2%)	0.745[e]	5.6 ± 0.01	0.065[e]	5.7 ± 0.01	0.738[e]
			CG		5794 (20.3%)	711 (20.2%)	5083 (20.3%)	**< 2.0×10^{-16}**[f]	5.6 ± 0.01	**4.3×10^{-9}**[f]	5.7 ± 0.01	0.009[f]
			GG		376 (1.3%)	0 (0.0%)	376 (1.5%)	0.637[g]	5.3 ± 0.02	0.251[g]	5.6 ± 0.02	0.811[g]

[a] Location on NCBI build GRCh38.
[b] Values indicate the numbers of measurements taken, with the percentages in parentheses.
[c] Quantitative values are means and stand errors.
[d] SNP showing borderline significance with T2DM-related parameters.
[e] Additive model, AA < AB < BB (A, major allele; B, minor allele).
[f] Recessive model, AA + AB vs. BB.
[g] Dominant model, AA vs. AB + BB. A statistically significant *P*-value corrected using Bonferroni's method ($P < 2.26 \times 10^{-7}$) is shown in bold. MAF, minor allele frequency. T2DM, type 2 diabetes mellitus. FPG, fasting plasma glucose. HbA_{1c}, blood glycosylated hemoglobin.

FPG level with borderline significance ($P = 3.3 \times 10^{-7}$ to 5.8×10^{-7}) in our longitudinal EWASs. In the additive model, two SNPs (rs3802177 and rs13266634) of *SLC30A8* were significantly ($P < 2.26 \times 10^{-7}$) associated with the FPG levels (Supplementary Table S2), but these were related to HbA_{1c} content with borderline significance ($P = 2.5 \times 10^{-7}$ to 4.5×10^{-7}). The two SNPs of *SLC30A8* were in LD with rs11558471 ($r^2 = 0.86$ to 0.95; $D' = 0.99$ to 1.00; $P < 2.26 \times 10^{-7}$).

2.3. Relation of candidate SNPs to T2DM-related phenotypes among subjects with T2DM or controls

We examined the relations of the four candidate SNPs including SNPs with borderline significance (rs6414624 of *EVC*, rs78338345 of *GGA3*, rs10490775 of *PTPRG*, and rs61739510 of *GLT6D1*) to FPG levels and HbA_{1c} content by the GEE model in subjects with T2DM and controls separately (748 cases and 5225 controls for FPG levels, and 598 cases and 3930 controls for HbA_{1c} content). The results are shown in Supplementary Table S3. Given that the number of subjects became smaller in this analysis, *P*-values were larger than those in longitudinal EWASs for all individuals. There was no significant difference between *P*-values for subjects with T2DM and controls ($P = 0.7$ to 1.0, Wilcoxon signed-rank test). Therefore, the effects of SNPs on T2DM-related phenotypes were similar in both groups.

2.4. Genetic variants with high frequency in East Asia

We surveyed allele frequencies of novel candidate SNPs detected in the present study, based on information from the 1000 Genomes Project and ExAC (Exome Aggregation Consortium) Browser databases (Table 3 and Supplementary Table S4). The survey revealed that the frequency of a minor allele at rs78338345 of *GGA3* was extremely low outside of East Asia. This result suggests that the MAF of *GGA3* has increased within East Asian populations after the split of East Asian and non-East Asian human lineages.

To examine whether the minor allele in *GGA3* spread across East Asia in recent evolutionary time, we conducted linkage disequilibrium (LD) and extended haplotype homozygosity (EHH) analyses for the GGA3 genomic region using datasets from the 1000 Genomes database. The LD between SNPs across the genomic region in East Asians appeared to be stronger than in non-East Asian populations (Supplementary Fig. S1). In the EHH analysis, high EHH values (> 0.9) of the minor allele in East Asians extended approximately 13 kb, while the other populations showed low EHH values outside the core SNP rs78338345 (Supplementary Fig. S2).

2.5. Significance levels of newly identified SNPs in previous GWASs and previously identified SNPs in the present study

We examined three T2DM-associated SNPs identified in our longitudinal EWASs, using information on *P*-values in datasets of meta-analysis studies [10,11,20–22] from DIAGRAM Consortium (Supplementary Table S5). Among the SNPs, rs11558471 of *SLC30A8* was significantly ($P = 1.6 \times 10^{-18}$ to 6.4×10^{-8}) related to T2DM. Two newly identified SNPs of *EVC* and *GGA3* did not show the association ($P = 0.970$ to 0.127). This discrepancy might be attributable to the difference in ethnic groups examined.

We have examined relations of 212 genetic variants at 32 previously identified loci for T2DM in our EWASs (Supplementary Table S6). Of the 32 loci, 28 (125 genetic variants) were related ($P < 0.05$) to T2DM-related phenotypes in either of three genetic models, including 20 loci (64 genetic variants) with *P*-value of < 0.0001. Three SNPs of *GCKR* were significantly ($P < 2.0 \times 10^{-16}$) associated with HbA_{1c} content in the dominant model and FPG level in the recessive model. The rs200626286 of *TCF7L2* showed significant relations ($P < 2.0 \times 10^{-16}$) to all T2DM-related phenotypes in dominant and

Table 3
Allele frequencies of T2DM-associated single nucleotide polymorphisms in human populations.

RefSNP ID	East Asian							South Asian[a]	European[a]	African[a]
	All	JP-Inabe	JPT[a]	CDX[a]	CHB[a]	CHS[a]	KHV[a]			
rs10490775[b]	G: 0.939 (12,249)	G: 0.940 (11,319)	G: 0.957 (199)	G: 0.909 (169)	G: 0.908 (187)	G: 0.919 (193)	G: 0.919 (182)	G: 0.764 (747)	G: 0.895 (900)	G: 0.870 (1150)
PTPRG	A: 0.061 (794)	A: 0.060 (725)	A: 0.043 (9)	A: 0.091 (17)	A: 0.092 (19)	A: 0.081 (17)	A: 0.081 (16)	A: 0.236 (231)	A: 0.105 (106)	A: 0.130 (172)
rs6414624	C: 0.918 (11,974)	C: 0.919 (11,062)	C: 0.918 (191)	C: 0.892 (166)	C: 0.903 (186)	C: 0.924 (194)	C: 0.884 (175)	C: 0.831 (813)	C: 0.793 (798)	C: 0.502 (663)
EVC	T: 0.082 (1076)	T: 0.081 (980)	T: 0.082 (17)	T: 0.108 (20)	T: 0.097 (20)	T: 0.076 (16)	T: 0.116 (23)	T: 0.169 (165)	T: 0.207 (208)	T: 0.498 (659)
rs61739510[b]	C: 0.943 (12,309)	C: 0.944 (11,369)	C: 0.957 (199)	C: 0.930 (173)	C: 0.913 (188)	C: 0.919 (193)	C: 0.944 (187)	C: 0.948 (927)	C: 0.952 (958)	C: 0.996 (1317)
GLT6D1	T: 0.057 (743)	T: 0.056 (675)	T: 0.043 (9)	T: 0.070 (13)	T: 0.087 (18)	T: 0.081 (17)	T: 0.056 (11)	T: 0.052 (51)	T: 0.048 (48)	T: 0.004 (5)
rs78338345	C: 0.888 (11,586)	C: 0.886 (10,670)	C: 0.861 (179)	C: 0.973 (181)	C: 0.869 (179)	C: 0.914 (192)	C: 0.934 (185)	C: 0.998 (976)	C: 1.000 (1006)	C: 1.000 (1322)
GGA3	G: 0.112 (1464)	G: 0.114 (1372)	G: 0.139 (29)	G: 0.027 (5)	G: 0.131 (27)	G: 0.086 (18)	G: 0.066 (13)	G: 0.002 (2)		

[a] Allele frequency obtained from 1000 Genomes Project database.
[b] SNP showing borderline significance with T2DM-related parameters. Values indicate the percentages of allele, with the observed numbers in parentheses. JP-Inabe is Japanese in Inabe City, Mie, Japan; JPT is Japanese in Tokyo, Japan; CDX is Chinese Dai in Xishuangbanna, China; CHB is Han Chinese in Beijing, China; CHS is Southern Han Chinese; KHV is Kinh in Ho Chi Minh City, Vietnam. T2DM, type 2 diabetes mellitus.

recessive models, and rs138649767 of *TCF7L2* was significantly related to the prevalence of T2DM ($P = 2.4 \times 10^{-11}$), FPG level ($P = 1.1 \times 10^{-14}$), and HbA_{1c} content ($P < 2.0 \times 10^{-16}$) in the additive model.

3. Discussion

Our longitudinal EWASs of the Inabe cohort showed that three SNPs were significantly associated with T2DM-related parameters, according to the GEE models. Of the three SNPs, rs11558471 in *SLC30A8* is known to be associated with T2DM [16–19]. The relation of the remaining two SNPs in *EVC* and *GGA3* to T2DM has not been reported previously according to the three databases. Hence, we focused on these two novel susceptibility loci in the present study.

3.1. rs6414624 of EVC at 4p16.2

EVC is single-pass type I transmembrane protein involved in endochondral growth and skeletal development. This protein is expressed in various tissues and organs including the pancreas, according to The Human Protein Atlas. *EVC* has been implicated in Ellis-van Creveld syndrome [23]. Given that type 1 diabetes mellitus may be associated with this syndrome [24] and that the frequency of patients with T2DM is high in families with type 1 diabetes mellitus [25,26], *EVC* might affect insulin secretion, although the functional relevance of *EVC* to the pathogenesis of T2DM is unknown. In the Inabe cohort, values of both FPG and HbA_{1c} in subjects with the minor allele at rs6414624 were significantly lower than those in subjects with the major allele, suggesting that the minor allele at the SNP is protective against T2DM.

3.2. rs78338345 of GGA3 at 17q25.1

The rs78338345 C to G transversion changes an amino acid residue at position 147 of GGA3 (Q147E, though there are splice variants). According to the Ensembl human database (http://www.ensembl.org/Homo_sapiens/), the missense mutation may disrupt protein function (by PolyPhen prediction; score = 0.621–1.000). GGA3 protein is ubiquitously expressed across various tissues and organs, including the pancreas (The Human Protein Atlas). This protein is involved in the intracellular trafficking of lysosomal enzymes by IGF2R protein [27]. *IGF2R* was shown to be related to T2DM [28,29]. The rs78338345 [C/G (Q147E)] of *GGA3* may thus influence the development of T2DM. The functional relevance of this gene to the pathogenesis of T2DM, however, remains unclear.

The frequency of the minor allele in the candidate SNP was highest in East Asian populations, whereas the frequencies in other ethnic groups were extremely low. The EHH analysis showed the high EHH values of the minor allele 'G' across a 13 kb genomic region in East Asians. Although this length is not strong evidence of recent positive selection [30], the EHH value outside the candidate SNP was low for non-East Asian populations. The result suggests that the MAF of rs78338345 has increased in East Asia in recent evolutionary time. One might hypothesize that the minor allele has some advantages in East Asian environments, and the frequency has recently increased after the divergence of the East Asian and non-East Asian ancestral populations.

3.3. Study limitations

There were some limitations to the present study: (i) the longitudinal EWAS was conducted only in a local Japanese population. Replication of the longitudinal EWAS in other Japanese populations or other ethnic groups is required to clarify the relations of the identified SNPs to T2DM; (ii) the functional relevance of the candidate SNPs to the pathogenesis of T2DM remains unclear. Further functional analysis is required to clarify the results of this study; (iii) the follow-up period of annual health check-ups varied from 1 to 11 years among individuals;

(iv) although participants did not have medical treatment in the health care center, the effects of medical treatment in other clinics or hospitals on the data cannot be ruled out; (v) given that the EWAS is a focus genotyping method that covers exonic regions, other genomic regions were not examined.

4. Conclusion

Our longitudinal EWASs for the Inabe cohort identified three SNPs that confer susceptibility to T2DM. Of the SNPs, two of *EVC* and *GGA3* were novel susceptibility loci, and minor alleles in these SNPs may be protective against T2DM. Furthermore, the frequency of the minor allele of the candidate SNP in *GGA3* may have increased in East Asian populations after the split of the East Asian and non-East Asian lineages.

5. Materials and methods

5.1. Ethics statement

The study protocol complied with the Declaration of Helsinki and was approved by the Committees on the Ethics of Human Research of Mie University Graduate School of Medicine and Inabe General Hospital. Written informed consent was obtained from all subjects prior to enrolment in the study.

5.2. Study subjects

The 6022 community-dwelling individuals in Inabe City, Mie Prefecture, Japan, were recruited from individuals who visited the health care center of Inabe General Hospital for an annual health check-up and followed-up yearly. These individuals were registered between March 2010 and September 2012, and medical examination data were obtained from April 2003 to March 2014 (11 years). Methods for the recruitment of subjects and for the collection and storage of medical examination data and genomic DNA samples have been described in detail previously [31].

T2DM was defined as either FPG level of ≥ 6.93 mmol/L (126 mg/dL), HbA_{1c} values of ≥ 6.5%, or use of antidiabetic medications, according to WHO criteria [32,33]. Individuals with type 1 diabetes mellitus, maturity-onset of the young, diabetes associated with mitochondrial diseases or single-gene disorders, pancreatic disease, or other metabolic or endocrinological diseases were excluded from this study. In addition, subjects taking medications that may cause secondary diabetes were excluded. The control subjects had an FPG level of < 6.05 mmol/L (110 mg/dL), HbA_{1c} content of < 6.2%, and no history of diabetes mellitus or of having taken antidiabetic medication.

In the longitudinal EWAS for the prevalence of T2DM, 6022 subjects (5267 controls and 755 subjects with T2DM) were examined. In addition, the FPG levels of 5983 subjects (3319 males and 2664 females) were examined in the EWAS. In the EWAS for HbA_{1c} content, 4540 subjects (2349 males and 2191 females) were examined. Each subject had one set of health data for each year of attendance at the medical check-up. Therefore, all participants had undergone 1–11 medical examinations, and the average followed-up period was five years. The distributions of the number of visits per participant for T2DM-related phenotypes are shown in Supplementary Fig. S3.

5.3. Longitudinal EWAS

A longitudinal EWAS for the Inabe cohort was performed using Illumina's exome arrays and longitudinal data from medical examinations. The genotype dataset was generated using Infinium HumanExome-12 ver 1.2 BeadChip and Infinium Exome-24 ver 1.0 BeadChip (Illumina, San Diego, CA, USA). These arrays include ~244,000 putative functional exonic variants selected from > 12,000 individual exome and whole-genome sequences across diverse ethnic populations, including European, African, Chinese, and Hispanic individuals [34]. We examined the population stratification in the Inabe cohort by principal component analysis with the EIGENSTRAT method [35], using JMP Genomics version 6.0 (SAS Institute, Cary, NC, USA). Four outliers were removed from further analyses. Genotyping data of 6022 individuals were converted into numeric data with the dominant, recessive, and additive models, using JMP Genomics, and then monomorphic sites among subjects were excluded. The dominant and recessive models were defined as "0, AA; 1, AB + BB" and "0, AA + AB; 1, BB" (A, major allele; B, minor allele), respectively, whereas the additive model was defined as "0, AA; 1, AB; 2, BB." The following SNPs were also discarded; SNPs whose genotype distributions significantly deviated from Hardy–Weinberg equilibrium ($P < 0.001$) in controls, SNPs on mitochondrial DNA or sex chromosomes, and SNPs with MAF of < 0.05. A total of 24,579 SNPs that passed quality control was subjected to further analysis. The marked decrease in the number of SNPs after quality control was mainly attributable to removal of non-polymorphic SNPs (179,865–199,701 SNPs) and SNPs with MAF of < 0.05 (13,549–33,383 SNPs) contained in human exome arrays we used. Since the exome arrays were not specifically designed for the Japanese population, a large number of monomorphic sites were observed. Distributions of SNPs before or after quality controls were quite similar, indicating that coverage of the exome was similar before or after quality control. Rearrangement of Inabe longitudinal data was conducted using R software version 3.32 [36] via RStudio version 1.0.136 [37] and Perl script. The mRNA and protein expression encoded by genes with candidate SNPs were evaluated according to The Human Protein Atlas (http://www.proteinatlas.org/).

Quantile-quantile plots for *P*-values of allele frequencies in the longitudinal EWASs are shown in Supplementary Fig. S4. The genomic inflation factor (λ) of *P*-values was 1.03 for the prevalence of T2DM, 1.10 for FPG, and 1.08 for HbA_{1c} in the dominant model (Supplementary Fig. S4a). In the additive model, the λ was 1.05 for the prevalence of T2DM, 1.12 for FPG, and 1.11 for HbA_{1c} (Supplementary Fig. S4b). In the recessive model, the λ was 1.07 for the prevalence of T2DM, 1.13 for FPG, and 1.12 for HbA_{1c} (Supplementary Fig. S4c).

5.4. Statistical analyses

The association of three T2DM-related parameters (prevalence of T2DM, FPG level, and HbA_{1c} content) was assessed by the GEE model [14,15] with adjustments for age, gender, BMI, and smoking status, using the R package 'geepack' [38]. Since the prevalence of T2DM is repeated categorical data (case or control), a binomial distribution was applied for assessing the correlation between the repeated categorical outcomes and SNPs in the GEE method. A Gaussian (normal) distribution was selected for the family with an identity link in the GEE for FPG level and HbA_{1c} content, because the repeated measurements are continuous data. The statistical significance of the association was $P < 2.26 \times 10^{-7}$ (0.05/24,579 SNPs × 9) for the three inheritance models after Bonferroni's correction to compensate for multiple comparison of genotypes with the T2DM-related parameters. Relations of candidate SNPs identified in our longitudinal EWASs were tested, using information on *P*-values in datasets of meta-analysis studies [10,11,20–22] from DIAGRAM Consortium (http://www.diagram-consortium.org/).

5.5. Estimates of evolutionary events

The LD among SNPs was estimated in Haploview version 4.2 program [39]. Perl and R scripts were written to convert genotype data used in the present study into suitable formats to the program. Information regarding allele frequencies of target SNPs within human populations was obtained from 1000 Genomes Project (http://www.internationalgenome.org/, [40]) and ExAC Browser (http://exac.broadinstitute.org/, [41]). To infer the recent expansion of alleles at

candidate SNPs in modern humans, we estimated the EHH [42] using the R package 'rehh 2.0' [43,44]. The categories of four ethnic groups (East Asian, South Asian, European, and African populations) are listed at the following URL: http://www.internationalgenome.org/data-portal/population.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2017.12.010.

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Conflict of interest statement

The authors declare no competing financial interests.

Authors' contributions

Y. Yasukochi contributed to analysis and interpretation of the data, and to drafting of the manuscript. J. Sakuma and I. Takeuchi contributed to analysis and interpretation of the data as well as revision of the manuscript. K. Kato, M. Oguri, T. Fujimaki, and H. Horibe each contributed to acquisition of the data and revision of the manuscript. Y. Yamada contributed to conception and design of the study, and to acquisition, analysis, and interpretation of the data.

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