Serum gamma-glutamyl transferase and risk of type 2 diabetes in the general Korean population: a Mendelian randomization study

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ABSTRACT

Elevated gamma-glutamyl transferase (GGT) levels are associated with higher risk of type 2 diabetes in observational studies, but the underlying causal relationship is still unclear. Here, we tested a hypothesis that GGT levels have a causal effect on type 2 diabetes risk using Mendelian randomization. Data were collected from 7,640 participants in a South Korean population. In a single instrumental variable (IV) analysis using two stage least squares regression with the rs4820599 in the *GGT1* gene region as an instrument, one unit of GGT levels (IU/L) was associated with 11% higher risk of type 2 diabetes (OR=1.11, 95% CI: 1.04 to 1.19). In a multiple IV analysis using seven genetic variants that have previously been demonstrated to be associated with GGT at a genome-wide level of significance, the corresponding estimate suggested a 2.6% increase in risk (OR=1.026, 95% CI: 1.001 to 1.052). In a two-sample Mendelian randomization analysis using genetic associations with type 2 diabetes taken from a trans-ethnic GWAS study of 110,452 independent samples, the single IV analysis confirmed an association between the rs4820599 and type 2 diabetes risk (P-value=0.04); however, the estimate from the multiple IV analysis was compatible with the null (OR=1.007, 95% CI: 0.993 to 1.022) with considerable heterogeneity between the causal effects estimated using different genetic variants. Overall, there is weak genetic evidence that GGT levels may have a causal role in the development of type 2 diabetes.

INTRODUCTION

According to the World Health Organization (1), the number of people diagnosed with type 2 diabetes has increased consistently in recent decades and has reached 347 million worldwide. Type 2 diabetes is a complex metabolic disease influenced by genetic predisposition and environmental conditions (2). To better understand the pathogenesis of type 2 diabetes, it is important to identify risk factors and their roles in disease development.

A number of studies have reported that an increased level of gamma-glutamyl transferase (GGT) is linked to higher risk of type 2 diabetes (3-8). GGT is an enzyme which catalyzes the transfer of gamma-glutamyl groups from glutathione to another acceptors. Since the liver is the major site for glucose regulation and detoxification after excess alcohol consumption, GGT is often used as a biomarker for the functional state of the liver (9). It has been suggested that the GGT level and type 2 diabetes may be biologically linked through circulating insulin level (10, 11), oxidative stress (12), and non-alcoholic fatty liver disease (13). However, most existing studies on GGT and type 2 diabetes are based on observed associations which do not necessarily imply causation, because associations may be due to confounding factors, reverse causation, or selection bias (14).

The gold standard approach for causal inference is performing a randomized controlled trial, which can be often infeasible. An alternative approach to strengthen causal inference may be applying a technique called Mendelian randomization (MR). MR utilizes a genetic variant as an instrumental variable (IV) or a proxy of an exposure (or a risk factor). Use of a genetic variant (which is randomly assorted at conception, independent of environment) in MR is analogous to the random allocation of subjects in a randomized controlled trial (15, 16).

Assessing the association between the genetic variant and the outcome is analogous to assessing the intention-to-treat effect in a randomized controlled trial, and calculating an IV estimate is analogous to estimating the causal effect of the treatment on the outcome in the setting of full adherence to the treatment assigned, as in an idealized randomized controlled trial. The limitations of MR, including violation of the exclusion criterion or potential genetic pleiotropy, have been discussed in detail elsewhere (15, 16, 17).

Here, we tested a hypothesis that GGT levels have a causal effect on risk of type 2 diabetes using MR. Data were collected from 7,640 participants registered in a two-community-based cohort within the Korean Genome and Epidemiology study (KoGES). Prior to MR, an observational association between measured serum GGT levels and risk of type 2 diabetes was estimated using an ordinary least squares (OLS) regression. This step was designed to replicate previously reported observation-based association using our data. In an MR framework, we considered both single- and multiple- IV analyses. In a single IV analysis, a biologically relevant genetic variant was selected as an instrument to assess unconfounded association between the estimated (i.e. genetically elevated) GGT levels and risk of type 2 diabetes using two stage least squares (2SLS) regression. In multiple IV analyses, genetic variants reported in a published GWAS to be robustly associated with GGT levels were selected as multiple instruments, with which the association between the estimated (or genetically elevated) GGT levels and risk of type 2 diabetes was assessed using three different methods including 2SLS regression, inverse-variance weighted (IVW) regression and MR-Egger regression (51).

RESULTS

General characteristics

General characteristics for a total of 7,640 participants of Korean ancestry are described in Table 1. The mean age was 51.98 (± 8.86) years and nearly half of the participants (3,475 subjects, 45.5%) were male. The proportions of current alcohol drinkers and smokers were 45.4% and 24.1%, respectively. The mean GGT level was 25.93 (± 20.69) IU/L. Participants in the higher quartile group for GGT levels were more likely to be male than female, to live in urban areas, to drink more, to smoke more and to have higher levels of type 2 diabetes risk factors (including higher values in body mass index (BMI), waist circumference (WC), blood pressure, total cholesterol (TC) and triglyceride (TG), and low values in high-density lipoprotein (HDL) cholesterol). GGT levels were shown to be associated with type 2 diabetes risk (as well as its biomarkers including fasting blood glucose (FBG) and glycated hemoglobin (HbA_{1c})) and the risk of related diseases such as hepatitis, hypertension and dyslipidemia. No indication for a U-shaped relationship was observed between GGT levels and type 2 diabetes risk based on the quartile group distribution, justifying the use of a linear model in the following statistical analyses.

Association between GGT levels and type 2 diabetes risk

GGT levels were associated with type 2 diabetes risk in OLS analysis (odds ratio (OR)=1.019, 95% confidence interval (CI): 1.016 to 1.022) (Table 2). This association remained robust after adjustment for covariates including age, sex and residential area (OR=1.021, 95% CI:

1.018 to 1.025) and after further adjustments for potential confounders such as BMI, health behavior and lipid traits (OR=1.017, 95% CI: 1.013 to 1.021). The same pattern of association was observed for log2-transformed GGT levels (Supplementary Table S2).

Estimation of causal relationship using a single instrument

The rs4820599 genetic variant in *GGT1*, was strongly associated with GGT levels (IU/L), validating one IV condition for it to be an instrument (P-value<0.001, F-statistic=16.87 and R-squared (or variance explained)=0.22%) (Supplementary Table S3).

This instrument was directly associated with type 2 diabetes risk, suggesting an underlying causal relationship (OR=1.19 with a risk allele of G, 95% CI: 1.06 to 1.34) (Supplementary Table S4). It appeared this variant was not robustly associated with other type 2 diabetes risk factors in the Korean data, including BMI (P-value=0.94), WC (P-value=0.93), TC (P-value=0.18), TG (P-value=0.56), and HDL cholesterol (P-value=0.52).

The association between rs4820599 and type 2 diabetes has been reported in a published trans-ethnic GWAS with 110,180 participants, replicating the suggestive causal relationship (OR=1.03 with a risk allele of G, 95% CI: 1.00 and 1.06, P-value=0.04) (18).

To quantify the causal effect of GGT levels on type 2 diabetes risk, we performed a single IV analysis using 2SLS regression in the Korean data. One unit of GGT levels (IU/L), estimated using rs4820599, was associated with 11% higher risk of type 2 diabetes (OR=1.11, 95% CI: 1.04 to 1.19) (Table 3).

The same pattern was observed for log2-transformed GGT levels (Supplementary Tables S5

and S6).

Estimation of causal relationship using multiple instruments

In multiple IV analyses, we utilized 7 SNPs having been reported in a large GWAS study of East Asian participants for GGT levels by Kim et al. (19). Each of these SNPs was strongly associated with GGT levels (IU/L) in our data (Supplementary Table S1) and all of them explained 2.1% of variance of GGT levels (IU/L) with an F-statistic of 23.35 under a multivariable linear regression model.

We applied three different methods to quantify causal effects of GGT on type 2 diabetes risk using the Korean data: 2SLS, IVW and MR-Egger. With 2SLS regression, one unit of GGT levels (IU/L), estimated using 7 SNPs, was weakly associated with 2.5% higher risk of type 2 diabetes (OR=1.026, 95% CI: 1.001 to 1.052) (Table 4). With IVW, the effect estimate was almost unchanged (OR=1.024, 95% CI: 1.001 to 1.048). With MR-Egger, the effect estimate was slightly smaller, compatible with the 2SLS and IVW estimates but imprecisely estimated (OR=1.018, 95% CI: 0.950 to 1.090) (Table 4). There was little evidence of directional pleiotropy in the MR-Egger analysis (P-value for intercept=0.80).

We then evaluated causal effects in multiple IV analyses through a two-sample approach to increase statistical power. SNP effects with GGT levels were taken from the Korean study and SNP effects with type 2 diabetes risk were taken from the trans-ethnic GWAS referenced above (18). At this stage, 2SLS regression could not be performed as the method requires individual-level data rather than summarized data. With IVW, the causal effect was essentially null (OR=1.007, 95% CI: 0.993 to 1.022). There was substantial heterogeneity in

the causal estimates based on the 7 SNPs considered individually (Cochan's Q test: P-value=0.005). With MR-Egger, one unit of GGT levels (IU/L) was estimated to lead to 4.6% higher risk of type 2 diabetes (OR=1.046, 95% CI: 1.012 to 1.082) (Table 5). However, the MR-Egger analysis indicated overall directional pleiotropy (P-value=0.025), and the intercept term from the analysis (which, under the MR-Egger assumptions, represent the average pleiotropic effect of a SNP) was OR=0.928, an implausibly extreme value as it is larger than the observed genetic association with the outcome for any of the individual variants. This means that the assumptions necessary for the MR-Egger analysis (in particular, that the pleiotropic effects of SNPs are independent of their association with the risk factor) are unlikely to be satisfied. Visual inspection of the genetic associations with GGT and Type 2 diabetes risk suggested that the 5 variants having the greatest association with GGT had positive causal estimates, whereas the 2 variants having smaller associations with GGT had negative causal estimates (Figure 1; right panel). This suggests that these SNPs have pleiotropic effects on other variables, as noted by some previous studies (19-27).

For instance, the rs12229654 and rs2074365 variants were shown to be associated with both GGT and HDL cholesterol in East Asian populations (19). In several other studies, the rs12539316 variant in the genomic region near *TBL2-BCL7B* had association with TG (19-21) and VLDL (22) as well. Also the rs11066453 variant was reported to be associated with glycemic traits (23), serum creatinine (24) and waist-hip ratio (25); and the rs2393791 with LDL cholesterol, TC (26), and creative protein levels (27). The list of traits associated with these SNPs was searched through www.phenoscanner.medschl.cam.ac.uk (Supplementary Table S8).

The same pattern of causal estimates was observed for log2-transformed GGT levels

(log2(IU/L)) (Supplementary Figures S2 and S3, Supplementary Tables S9 and S10).

DISCUSSION

Here, we used Mendelian randomization to demonstrate that genetically elevated GGT levels were associated with high risk of type 2 diabetes. First, in a single IV analysis utilizing the rs4820599 genetic variant in *GGT1* as an instrument, one unit of GGT levels (IU/L) was associated with 11% higher risk of type 2 diabetes in 7,640 South Korean participants (759 patients and 6,881 controls). This instrument was associated with the risk of type 2 diabetes (P-value=0.04) in a published trans-ethnic GWAS study of 110,180 independent participants, validating our finding on underlying causal relationship. Next, in a multiple IV analysis utilizing 7 independent genetic variants as instruments, one unit of GGT levels (IU/L) was weakly associated with 2.6% higher risk of type 2 diabetes in 7,640 South Korean participants. This multiple IV analysis was also performed under a two-sample approach (combining instrument-exposure associations in 7,640 South Korean participants with instrument-outcome associations in up to 110,452 multi-ethnic participants), where one unit of GGT levels (IU/L) was associated with 0.7% higher risk of type 2 diabetes by the IVW method, although the association was compatible with the null (P-value=0.33). The two-sample estimate from MR-Egger was large and positive, although implausibly so. There was clear heterogeneity in the causal estimates from individual SNPs, with SNPs having greater associations with GGT suggesting a positive causal effect of GGT on type 2 diabetes risk, whereas those variants having smaller associations with GGT suggested negative causal effects. Both single- and multiple- IV analyses provided some evidence of a causal role of elevated GGT levels on the development of type 2 diabetes.

Relevance to previous studies

Observational associations between circulating GGT levels and risk of type 2 diabetes have been reported in several cross-sectional (5, 28, 29) and prospective studies (3, 4, 6-8, 13, 30). For instance, a recent meta-analysis of 24 prospective studies suggested that the risk of type 2 diabetes in the top-third GGT group was 30% higher in comparison with the bottom-third GGT group (31).

There are several biologically plausible mechanisms linking elevated GGT levels and increased diabetes risk. For example, metabolic abnormalities accompanied by elevated GGT such as insulin resistance (32), obesity (33), and hepatic steatosis (34), may be relevant to development of type 2 diabetes. Among these, the relationship between GGT levels and fasting insulin levels has been shown as potentially causal in a previous Mendelian randomization study by Conen et al. (35). This, in combination with the finding of the current study, can strengthen the argument that GGT may play a role in the etiology of type 2 diabetes through insulin level changes.

Instrumental variables for GGT

We utilized the rs4820599 variant of the *GGT1* gene as a single instrument. Biochemically, the *GGT1* gene has a direct functional relevance to GGT levels as it encodes the enzyme that catalyzes the transfer of the glutamyl group of glutathione to various amino acids and dipeptide receptors, and maintains intracellular glutathione levels (36-38). The rs4820599 variant in *GGT1* was associated with circulating GGT levels (P-value ~ 10^{-53}) in a large GWAS study analyzing a total of 42,940 participants in Asian populations which included

8,842 participants from the current study cohort (19). This association remained robust in the current study analyzing a total of 7,640 samples, with F-statistic = 16.87 and R-squared = 0.22%. F-statistic of 10 implies that the bias of the IV estimator is 10% of the bias of the observational estimator, and is often considered to be minimum strength required to avoid weak instrument bias in IV analyses (14, 39).

Another genetic variant in *GGT1*, rs2017869, has been utilized as an instrument for GGT levels in a previous Mendelian randomization study in relation with fasting insulin levels by Conen et al. (35). The rs2017869 variant was in linkage disequilibrium with the rs4820599 variant (r^2=0.5 based on the 1000 Genome Pilot 1 data with CHB + JPT panel). However, a direct comparison between rs4820599 and rs2017869 with regard to the strength as an instrument for GGT levels was not feasible in this study, as only rs4820599 was present in our genotype data. The rs2017869 variant did not associate with type 2 diabetes risk in the trans-ethnic study (P-value=0.64).

Generally it is not straightforward to validate whether the instrument satisfies the no-pleiotropy assumption of the IV analysis (such that the rs4820599 variant in *GGT1* influences type 2 diabetes risk only through GGT level changes), although its violation may result in biases in causal estimates (15). In the Korean data, the rs4820599 variant appeared not associated with risk factors for type 2 diabetes, including BMI, WC, TC, TG and HDL cholesterol, although these null associations might be due to insufficient study power. In previous studies, the *GGT1* gene has been shown to be involved in oxidative stress and pro-inflammatory pathways in both human and cell/animal, which have a pivotal role in the development of diabetes. Increased concentrations of cysteineglycine produced by GGT reaction appeared to generate reactive oxygen species and thus trigger inflammatory

responses (40). This may explain the association of the rs4820599 variant in *GGT1* with chronic pancreatitis (41) and pancreatic carcinogenesis (42), of which the development can be caused by oxidative stress through damaging pancreatic cells and stimulating the inflammatory signaling pathway (41). In addition, inflammatory cytokines such as tumor necrosis factor α have been shown to regulate the expression of GGT through the nuclear factor-κb signaling pathway in a human cell system (43). Based on the aforementioned studies, one can argue that the pro-inflammatory pathway may be involved in a biologically pathogenic link between the *GGT* gene and type 2 diabetes, will contribute to potential pleiotropy of the *GGT1* variant on type 2 diabetes in single IV analysis.

Alternatively, we carried out multiple IV analyses utilizing 7 independent genetic variants as instruments. Most of these variants had little known functional relevance to GGT levels, but all of them showed strong associations with GGT levels in a large GWAS study (19) as well as in the current study. Use of multiple instruments is proposed to interrogate the potential pleiotropy of single instruments, as it is unlikely that multiple independent instruments will have similar pleiotropic effects (14, 16). Although the recently proposed MR-Egger method can improve inferences in some cases, in this example SNPs having different strengths of association with the risk factor did not seem to have the same distribution of pleiotropic effects. However, the MR-Egger analysis did reveal that SNPs having stronger associations with the risk factor did have stronger associations with the disease outcome, as would be expected if GGT were a causal risk factor for Type 2 diabetes.

Strengths and limitations of this study

This is the first study, to our knowledge, which provided evidence for a causal relationship between GGT levels and type 2 diabetes risk using an MR approach. This corroborated previous observational studies, and further broadened our knowledge on causal risk factors for type 2 diabetes.

We acknowledge several limitations of our study. First, use of multiple instruments, selected from the GWAS study that included the current study sample as well as other independent sample (18), may cause an over-fitting bias (such that the IV estimation of exposure-outcome association is biased towards the confounded association) (39, 44). However, these multiple instruments were strongly associated with GGT levels; p-values had magnitudes of 10^{-14}, 10^{-30}, 10^{-58}, 10^{-126}, 10^{-44} and 10^{-53} in Kim et al. and 10^{-5}, 10^{-4}, 10^{-20}, 10^{-31}, 10^{-8} and 10^{-7} in the current study, respectively, as shown in the Supplementary Table 1. (It should be noted that the GGT values were transformed differently in these studies, with inverse square root transformation in Kim et al. and no transformation in the current study. Thus, a direct comparison of effect sizes was not feasible.) The main reason for the smaller p-value in Kim et al. should be its larger sample size and consequently an increased study power (n = up to 28,367 in Kim et al. and 7,640 in the current study). As the instrument-exposure associations were robust across independent cohorts, there is less chance of a selection bias due to over-fitting in the current study.

Secondly, several of the SNPs used in the multiple IV analysis are known to be pleiotropic as shown in the Supplementary Table S8. While some of the SNPs have clear associations with Type 2 diabetes risk, the directions of these associations were not consistent. Also there was clear heterogeneity in the causal estimates calculated using the individual SNPs. This means there is not a consistent picture of causality evidenced by all these SNPs even though the

variants having greater associations with GGT were the ones suggesting a positive causal effect of GGT on Type 2 diabetes risk.

Thirdly, odds ratios in this study differ depending on the methods used. This is because different methods calculate odds ratios differently. The odds ratio in a multivariate logistic model measures an individual effect of a unit increase in the risk factor onto the outcome conditional on covariates included in the model. On the other hand, the odds ratio in Mendelian randomization measures a value close to the population-average effect of a unit increase in the risk factor onto the outcome, marginal across covariates, the effect that one would expect to estimate in an idealized randomized controlled trial (45). Another reason for differences between estimates is that Mendelian randomization estimates reflect the effects of long-term (often life-long) changes in the risk factor, whereas observational estimates reflect the difference in the outcome related to measurement of the risk factor at a single point in time. Mendelian randomization estimates therefore tend to be larger than observational estimates (46). Although the discrepancy in odds ratio estimates by different methods is somewhat expected, the single IV based odds ratio in this study is particularly greater than the other estimates. This may be because of a potential pleiotropic effect of the single IV, rs4820599, given that the odds ratio in a multiple IV analysis overlaps with the odds ratios in multivariate logistic models. Nevertheless, it should be noted that all reported odds ratios suggest the same direction of association between elevated GGT levels and increased type 2 diabetes risk.

Lastly, diagnoses of disease were based on self-reported examinations. However, to minimize the proportion of undiagnosed type 2 diabetes patients and gain more precise classification, we took into account the related phenotype data regarding medication history, FBG levels, or

HbA_{1c} levels.

In conclusion, there is some genetic evidence for causal relationships between elevated GGT levels and increased risk of type 2 diabetes in the general Korean population. Modulation of GGT levels, for example, by diet or pharmacologic intervention, may be worth further investigation to establish if it could be a useful strategy in type 2 diabetes prevention.

MATERIALS AND METHODS

Study population

The study population consisted of subjects who were registered in the Ansung and Ansan cohorts of the KoGES consortium from 2001 to 2003. The KoGES was established to discover new biomarkers and investigate risk factors for chronic diseases such as diabetes, hypertension, and dyslipidemia in individuals aged 39-70 years from the general Korean population. Among 10,038 participants initially recruited, only 7,640 were included in the current study after removing 1,196 due to poor genotyping, 863 due to missing data for key variables (including GGT (n=1), FBG (n=260), blood pressure (n=6), and main SNPs (n=596)), and additional 339 due to outlying GGT levels (more than 2 standard deviations away from the mean (SD)). All participants completed a written consent form and agreed with the Human Subjects Review Committee at the Korea University Ansan Hospital or the Ajou University Medical Center. This study was approved by the Committee on the Institutional Review Board of the Korea University.

General characteristics

Sociodemographic variables included age, sex, residential area, physical activity, smoking status, and alcohol consumption. The residential area was divided into two: rural Ansung and urban Ansan. Age was used as a categorical variable (<50, 50-59 and ≥60). Physical activity was classified according to intensity as follows: sedentary activity for less than 30 minutes

per day, and light, moderate, and intense activity for 30 minutes to more than 5 hours. Participants were grouped into never, previous and current smokers with respect to their smoking status. Similarly, subjects were categorized as never, previous and current drinkers. For drinkers, the amount of alcohol consumption per day was requested. Biochemical variables included BMI, WC, blood pressure, lipid levels, FBG, HbA_{1c}, and GGT. BMI (kg/m^2) was calculated by dividing the weight (kg) by the height squared (m^2), and classified as follows: <18.5, underweight; ≥18.5 and <25, normal; ≥25, obese. WC (cm) was calculated as the average of 3 measurements. Blood pressure was measured in a sitting position and the higher value between the left and right arms was used. The levels of TC (mg/dL), TG (mg/dL), HDL cholesterol (mg/dL), FBG (mg/dL), HbA_{1c} (%, mmol/mol), and GGT (IU/L) were measured in Seoul Clinical Laboratories (Seoul, Republic of Korea) from overnight fasted blood samples.

Definition of diabetes and other diseases

Diagnosis of diseases such as cardiovascular disease (CVD), hepatitis, hypertension, dyslipidemia, and type 2 diabetes was based on self-reported medical history. Subjects who had been diagnosed with type 2 diabetes before or under treatment with drugs or insulin injections, and who had been undiagnosed but having FBG levels higher than 126 mg/dL or HbA_{1c} levels higher than 6.5% were regarded as diabetes cases. Subjects who were diagnosed with myocardial infarction, congestive heart failure, coronary artery disease, peripheral blood vessel disease, and cerebrovascular disease were regarded as CVD cases.

DNA genotyping and imputation

Genomic DNA was extracted from peripheral blood samples of subjects using the QuickGene DNA Whole Blood Kit S with QuickGene-810 equipment (Fujifilm, Tokyo, Japan). Genomic DNA (500 ng) was analyzed using the Affymetrix Genome-Wide SNP Array 5.0 (Affymetrix, Inc., Santa Clara, CA, USA). The Bayesian robust linear modeling with Mahalanobis distance genotyping algorithm was used to calculate the accuracy of genotyping. Detailed information can be found elsewhere (47). Individuals with a high missing genotype call rate, high heterozygosity, gender inconsistencies and any kind of diagnosed cancer were excluded. In addition, SNPs with a high missing genotype call rate (>5%), low minor allele frequency (<0.01), and out of Hardy-Weinberg equilibrium (P value $<1.0x10^{-6}$) were excluded, leaving 352,228 SNPs available for analysis in 8,842 subjects. Additional SNP markers were then imputed from a HapMap reference panel consisting of 3.99 million SNPs (HapMap release 22) in 90 individuals from Japanese (JPT) and Chinese (CHB) populations, by the IMPUTE software (48, 49). After imputation, SNPs with minor allele frequency <0.01 or information score <0.3 were removed due to low quality, leaving a total of 1,804,397 SNPs available for the current study (352,228 SNPs of these were directly genotyped).

Statistical analysis

All statistical analyses were performed using SPSS v.21.0, Stata v.12 and R v.3.1.2. General characteristics of the participants were described as mean ± SD for continuous variables and frequency (%, n) for categorical variables. Differences according to the quartiles of GGT levels were tested by one-way ANOVA for continuous variables and by chi-squared tests for

categorical variables. The distribution of each variable was visually inspected and the TG levels were log-transformed with base 10 to mimic Gaussian distribution. For GGT, both the untransformed values (IU/L) and the log-2 transformed values (log2(IU/L)) were used for further statistical analyses, as the latter would reduce skewness.

Observational association. Observational association between GGT levels and type 2 diabetes risk was assessed under a logistic regression model with OLS estimation. Covariates were considered including age, sex, area, smoking status, alcohol intake, physical activity, BMI, and lipid levels.

Single IV analysis. To assess a causal association in *GGT1* was selected as an instrument for GGT, due to its clear biological relevance (36-38). We first measured association of this instrument with GGT and with type 2 diabetes risk directly using a logistic regression, to quantify the instrument strength and to validate the extent of causal association, respectively. Next, the causal effect of GGT on type 2 diabetes risk was quantified by 2SLS. In the first stage of 2SLS, the GGT value was regressed on this instrument using a linear regression model. In the second stage of 2SLS, the type 2 diabetes risk was regressed on the fitted GGT value obtained from the first stage using logistic regression. In both stages of 2SLS, we considered age, sex and area as covariates.

Replication of single IV analysis. Replication of association between the single instrument and type 2 diabetes was attempted by looking into summary statistics in Mahajan et al., the latest independent GWAS study on type 2 diabetes (18). The samples used in Mahajan et al., from up to 110,452 participants (83,964 controls and 26,488 cases) in multiethnic background (18), are not overlapping with the samples of the current study. Summary statistics from this publication of association between genetic variants and type 2 diabetes risk were available at

http://diagram-consortium.org/.

Selection of multiple instruments. To select multiple instruments, we reviewed SNPs robustly associated with GGT levels in two largest GWAS studies (19, 50). Chambers et al. (50) reported a total of 26 SNPs associated with GGT levels in participants of European and Indian Asian ancestry; and Kim et al. (19) reported a total of 7 SNPs for GGT levels in East Asian populations (Supplementary Table S1). Among these, three loci (*ZNF827, HNF1A* and *GGT1*) were reported in both studies. We first checked data availability; only 8 SNPs out of 26 in Chambers et al. (50) and all 7 SNPs in Kim et al. (19) were present in our genotype data. We then compared ancestry; our study samples had the same East Asian ancestry than those in Kim et al.. Failure of matching on ancestry may result in bias in IV estimation because the random allocation of alleles of IVs can be affected by population structure conditional on ancestry (15). Hence, due to the higher percentage of available SNPs and matching ancestry, the 7 SNPs in Kim et al. (19) were selected as instruments in our multiple IV analyses. These 7 SNPs included the rs4820599 genotype in *GGT1*, a single instrument in the previous single instrument based Mendelian randomization.

Nevertheless, it should be noted that the GWAS study by Kim et al. analyzed a subset of the current study samples (19). Out of a total of 42,940 samples in Kim et al. (19), about 20% (n=8,842) were from the KoGES cohort, the same cohort of the current study. This might result in "over-fitting" problem such that the IV estimation of exposure-outcome association may be biased towards the confounded association, when the instrument-exposure and instrument-outcome associations were estimated from the same cohorts (39, 44).

Multiple IV analysis. For multiple IV analysis, we applied a conventional 2SLS regression, IVW regression and recently proposed MR-Egger regression (51). The 2SLS regression was

carried out as follows: in the first stage, the GGT levels were predicted by 7 SNPs (as well as covariates including age, sex and area) which was then used as a predictor for type 2 diabetes risk in the second stage. Prior to IVW and MR-Egger, the association of each of 7 SNPs on GGT and the association of each of 7 SNPs on type 2 diabetes risk were estimated under a linear regression model adjusting for age, sex and area. We then regressed the coefficients of 7 SNPs on type 2 diabetes risk on the coefficients of 7 SNPs on GGT levels in which the slope estimate was interpreted as the overall causal effect estimate of GGT on type 2 diabetes risk, using IVW and MR-Egger regression models, as shown in Bowden et al. (51). At this stage, the sign of coefficients of 7 SNPs on GGT levels was all positively aligned before regression. Contrary to IVW, MR-Egger allows a non-zero intercept estimate which can indicate an average pleiotropic effect of multiple instruments. IVW and MR-Egger have different model assumptions. IVW is asymptotically the same as 2SLS and performs well under the three IV assumptions (51). On the other hand, the recently proposed MR-Egger performs under relaxed IV assumptions, where the IV assumption on pleiotropy can be substituted to a weaker InSIDE (Instrument Strength Independent of Direct Effect) assumption (that is, there is no correlation between the effects of genetic variants on the exposure and the direct effects of genetic variants on the outcome that are not mediated by the exposure). This implies that MR-Egger provides a robust estimate of the causal effect in comparison to the IVW method, even in the case where there is directional pleiotropy (51). It should be noted that the P-value and the confidence interval of the IVW estimates were calculated using a Z-test in order that these estimates could be directly comparable with the 2SLS estimates.

Multiple IV analysis under a two-sample approach. To increase the power of multiple IV

analysis, we applied a two-sample analysis approach which basically combines the instrument-exposure association estimated in the current study with the instrument-outcome association reported in the published larger study (52, 53). Similarly to our single IV analysis, we looked into findings in a large GWAS for type 2 diabetes by Mahajan et al. (18) to get summary statistics of association between each of 7 instruments and type 2 diabetes (with up to 83,964 controls and 26,488 cases in multiethnic background) (18). Summary statistics from this publication were downloaded from http://diagram-consortium.org/.

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MJS and SYS conceived the study. MJS acquired the data. SYS developed the statistical analysis plan. YSL, YC, SB and SYS analyzed the data. YSL, SYS and MJS prepared the first draft of manuscript. YSL, YC, SB, GDS, CLR, SYS and MJS contributed to the writing of the manuscript. All authors reviewed and agreed on the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Figure legend

Figure 1 Multiple IV analyses: Inverse-variance weighted estimates and MR-Egger estimates of GGT to diabetes risk with 7 SNPs as instruments, in the Korean data (left) and under a two-sample approach (right)

Table 1 Baseline characteristics of the participants according to quartiles of GGT level

	All participants (n=7,640)	GGT (IU/L)				P-value
		Q1 (n=1,727)	Q2 (n=2,188)	Q3 (n=1,824)	Q4 (n=1,901)	
Age (years)	51.98 ± 8.86	51.07 ± 9.05	52.82 ± 8.95	52.39 ± 8.78	51.42 ± 8.54	**<0.001**
Male %, (n)	45.5 (3,475)	10.4 (179)	31.3 (684)	60.3 (1,099)	79.6 (1,513)	**<0.001**
Area %, (n)						
Urban (Ansan)	54.2 (4,144)	49.4 (853)	50.1 (1,097)	58.4 (1,065)	59.4 (1,129)	**<0.001**
Rural (Ansung)	45.8 (3,496)	50.6 (874)	49.9 (1,091)	41.6 (759)	40.6 (772)	
Physical exercise %, (n)						
Lowest	6.2 (470)	4.1 (70)	4.9 (105)	6.5 (118)	9.4 (177)	**<0.001**
Lower middle	37.1 (2,795)	37.2 (632)	37.5 (806)	36.9 (665)	36.7 (692)	
Upper middle	23.3 (1,752)	25.0 (425)	22.4 (483)	24.1 (435)	21.7 (409)	
Highest	33.4 (2,517)	33.6 (570)	35.2 (758)	32.4 (584)	32.1 (605)	
Alcohol drinker %, (n)						
Never	48.1 (3,639)	71.8 (1,223)	59.2 (1,281)	40.2 (729)	21.5 (406)	**<0.001**
Previous	6.5 (494)	4.0 (68)	6.3 (137)	8.7 (157)	7.0 (132)	
Current	45.4 (3,440)	24.2 (413)	34.5 (747)	51.1 (926)	71.6 (1,354)	
Alcohol (g/day)	8.25 ± 19.82	1.16 ± 5.13	3.09 ± 10.14	8.85 ± 18.33	20.05 ± 29.98	**<0.001**
Smoker %, (n)						
Never	60.8 (4,583)	90.3 (1,527)	73.2 (1,576)	49.7 (896)	30.9 (584)	**<0.001**
Previous	15.1 (1,136)	3.8 (64)	11.6 (249)	20.2 (364)	24.3 (459)	
Current	24.1 (1,817)	5.9 (100)	15.3 (329)	30.1 (543)	44.7 (845)	
BMI (kg/m^2)	24.60 ± 3.13	23.75 ± 2.93	24.35 ± 3.12	25.02 ± 3.17	25.28 ± 3.05	**<0.001**
WC (cm)	82.44 ± 8.83	78.74 ± 8.82	81.23 ± 8.86	83.96 ± 8.29	85.75 ± 7.68	**<0.001**
Blood pressure (mmHg)						
Systolic	124.09 ± 18.80	119.89 ± 18.98	123.33 ± 18.96	125.35 ± 18.32	127.56 ± 18.10	**<0.001**
Diastolic	81.43 ± 11.83	77.81 ± 11.85	80.60 ± 11.64	82.39 ± 11.41	84.73 ± 11.41	**<0.001**
TC (mg/dL)	191.59 ± 35.40	177.47 ± 30.25	191.56 ± 34.02	195.29 ± 35.00	200.92 ± 37.66	**<0.001**
HDL (mg/dL)	44.65 ± 9.94	45.74 ± 9.61	45.39 ± 10.06	43.56 ± 9.90	43.84 ± 9.98	**<0.001**
TG (mg/dL)	157.08 ± 95.64	121.49 ± 53.80	139.96 ± 75.28	162.87 ± 89.59	203.54 ± 127.46	**<0.001**
FBG (mg/dL)	87.19 ± 21.38	81.29 ± 13.52	85.27 ± 19.14	88.48 ± 19.91	93.54 ± 28.13	**<0.001**
HbA_{1c} (%)	5.74 ± 0.82	5.54 ± 0.54	5.66 ± 0.69	5.82 ± 0.86	5.94 ± 1.03	**<0.001**
HbA_{1c} (mmol/mol)	39 ± 9	37 ± 6	38 ± 8	40 ± 9	41 ± 11	**<0.001**
GGT (IU/L)	25.93 ± 20.69	9.37 ± 1.33	14.60 ± 1.96	24.37 ± 3.97	55.52 ± 20.59	**<0.001**
Disease diagnosis %, (n)						
Hepatitis	4.2 (318)	3.5 (60)	3.2 (70)	4.6 (83)	5.5 (105)	**0.001**
Hypertension	15.1 (1,151)	9.0 (155)	15.0 (328)	18.1 (331)	17.7 (337)	**<0.001**
Dyslipidemia	2.4 (182)	1.2 (20)	1.6 (34)	3.5 (63)	3.4 (65)	**<0.001**
CVD	3.1 (237)	2.1 (36)	3.1 (67)	3.8 (69)	3.4 (65)	**0.025**
Type 2 diabetes	4.5 (343)	1.9 (33)	2.9 (63)	5.9 (108)	7.3 (139)	**<0.001**

The values were described as mean ± SD for a continuous variable, and as frequency (n) for a categorical variable. Difference among GGT categories was evaluated by one-way ANOVA for continuous variables, and by chi-squared tests for categorical variables.

BMI, Body mass index; WC, Waist circumference; TC, Total cholesterol; HDL, High-density lipoprotein; TG, Triglyceride; FBG, Fasting blood glucose; HbA_{1c}, glycated hemoglobin; GGT, gamma-glutamyl transferase; CVD, Cardio vascular disease.

Table 2 Observational association: Ordinary least squares estimates of GGT to type 2 diabetes risk

		Type 2 diabetes (n=6,881 for control, 759 for case)	
		OR (95% CI) by OLS estimation	P-value
GGT (IU/L)	Unadjusted	1.019 (1.016, 1.022)	**<0.001**
	Model [a]	1.021 (1.018, 1.025)	**<0.001**
	Model [b]	1.020 (1.017, 1.024)	**<0.001**
	Model [c]	1.017 (1.013, 1.021)	**<0.001**

Model [a] was adjusted for age, area, and sex. Model [b] was adjusted for age, area, sex, alcohol use, smoking status, physical activity, and BMI. Model [c] was adjusted for age, area, sex, alcohol use, smoking status, physical activity, BMI, TC, log10-transformed TG, and HDL.

Table 3 Single IV analysis in the Korean data: Two stage least squares estimates of GGT to type 2 diabetes risk with rs4820599_G as an instrument

		Type 2 diabetes (n=6,881 for control, 759 for case)	
		OR (95% CI) by single IV estimation	P-value
GGT (IU/L)	2SLS	1.11 (1.04, 1.19)	**0.003**

2SLS was adjusted for age, area, and sex.

Table 4 Multiple IV analysis in the Korean data: Two stage least squares estimates, inverse-variance weighted estimates and MR-Egger estimates of GGT to type 2 diabetes risk with 7 SNPs as instruments

		Type 2 diabetes (n=6,881 for control, 759 for case)			
		Intercept Estimate (SE, P value)	Slope Estimate (SE, P value)	OR (95% CI) by multiple IV estimation	P-value
GGT (IU/L)	2SLS	-	-	1.026 (1.001, 1.052)	**0.042**
	IVW	Constrained to 0	0.024 (0.012, P=0.044)	1.024 (1.001, 1.048)	**0.044**
	MR-Egger	0.018 (0.066, P=0.798)	0.017 (0.027, P=0.540)	1.018 (0.950, 1.090)	0.540

2SLS was adjusted for age, area, and sex. 7 SNP effects on GGT and type 2 diabetes, used in IVW and MR-Egger, were reported in Supplementary Table S7.

Table 5 Multiple IV analysis under a two sample approach: Inverse-variance weighted estimates and MR-Egger estimates of GGT to type 2 diabetes risk with 7 SNPs as instruments

		Type 2 diabetes (under a two-sample approach*)			
		Intercept Estimate (SE, P value)	Slope Estimate (SE, P value)	OR (95% CI) by multiple IV estimation	P-value
GGT(IU/L)	IVW	Constrained to 0	0.007 (0.007, P=0.332)	1.007 (0.993, 1.022)	0.332
	MR-Egger	-0.075 (0.024, **P=0.025**)	0.045 (0.013, P=0.017)	1.046 (1.012, 1.082)	**0.017**

7 SNP effects on GGT and type 2 diabetes, used in IVW and MR-Egger, were reported in Supplementary Table S7. *SNP effects on GGT were estimated in the Korean data (n=7,640) and SNP effects on type 2 diabetes risk were estimated in a trans-ethnic GWAS (n≤83,964 for control, 26,488 for case).

ABBREVIATIONS

2SLS; 2Stage least square

BMI; Body Mass Index

CI; Confidence interval

CVD; Cardio vascular disease

FBG; Fasting blood glucose

GGT; gamma-glutamyl transferase

GWAS; Genome-wide association study

HbA_{1c}; Glycated hemoglobin

HDL; High-density lipoprotein

IV; Instrument variable

IVW; Inverse-variance weighted

KoGES; Korean Genome and Epidemiology Study

MR; Mendelian randomization

OLS; Ordinary least square

OR; Odds ratio

SD; Standard deviation

SE; Standard error

SNP; Single nucleotide polymorphism

TC; Total cholesterol

TG; Triglyceride

WC; Waist circumference