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MYH9 gene polymorphisms may be associated with cerebrovascular blood flow in patients with type 2 diabetes

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ABSTRACT. Genetic factors play an important role in type 2 diabetes (T2D) complications. Alteration of cerebrovascular blood flow (CBF) is a direct result of cerebrovascular diseases. However, few studies have reported the role of genetics on CBF in patients with T2D. We investigated whether single-nucleotide polymorphisms (SNPs) in metabolic disease genes are associated with CBF in patients with T2D. CBF velocities of CBF were measured in 337 Han Chinese patients with T2D using transcranial Doppler sonography, with 54 cerebrovascular blood flow parameters documented. Fifty-two SNPs from 31 metabolic disease candidate genes were genotyped in patients. Quantitative allelic association and haplotype analyses were performed for candidate gene SNPs and CBF phenotypes. Spearman correlation was used to determine the relationship between CBF parameters and basic clinical characteristics, particularly, body mass index, lipids, fibrinogen, and GHbA1c. *MYH9* gene SNPs (rs875726 and rs735853) may be associated with the peak velocity of the right-middle cerebral

artery. SNPs rs875726, rs2009930, and rs375246 of the *MYH9* gene may be associated with the mean velocity of the right-anterior and posterior cerebral artery. The haplotype G-C-A (rs2239782-rs3752462-rs2269532) of *MYH9* may be associated with CBF. *MYH9* gene polymorphisms may be associated with multiple CBF phenotypes in Chinese patients with T2D. However, whether *MYH9* is a candidate gene for cerebrovascular diseases in Chinese patients with T2D remains unknown.

Key words: Association study; Cerebrovascular blood flow; GHbA1c; *MYH9*

INTRODUCTION

The brain is one of the most perfused organs in the body, receiving 15-20% of the total cardiac output. The high oxidative metabolic status of the brain relies on both high cardiac output and relatively constant cerebrovascular blood flow (CBF). CBF must be precise, as both hyperemia and ischemia can damage brain tissue or cause death of brain cells. The Global Burden of Disease Study predicted that cerebrovascular disease will be the second leading cause of death from 1990-2020 (American Diabetes Association, 1997). In patients with type 2 diabetes (T2D), the risk of stroke is increased 2- to 3-fold, while the risk of death is increased 2-fold compared to patients without diabetes (Almdal et al., 2004). Diabetes is an independent risk factor for death from stroke (Tuomilehto et al., 1996), and it can increase the risk of cerebrovascular disease through its effects on blood flow regulation (Novak et al., 2006) and even drive cerebrovascular remodeling (Harris et al., 2005), directly altering CBF.

Preliminary studies showed that genetic factors play an important role in cerebrovascular diseases. The C allele of codon 29 of the translated sequence of transforming growth factor-beta1 gene is a susceptibility allele for cerebral infarction in Japanese patients with T2D (Katakami et al., 2011). The 1908T allele of the *LMNA* gene is generally associated with cerebral vascular disease, but it is not related to age, hypertension, total cholesterol, or triglycerides (Liang et al., 2005). However, the global phenotype of CBF in patients with T2D has not been thoroughly examined. In particular, whether T2D-related single-nucleotide polymorphisms (SNPs) contribute equally to the alteration of CBF remains unknown. Importantly, intensive glucose control does not affect the incidence of stroke in patients with T2D (Marso et al., 2010). Thus, we investigated whether T2D candidate genes are also associated with CBF.

MATERIAL AND METHODS

Patients

A cohort of 337 hospitalized Chinese patients with T2D was recruited from the Metabolic Diseases Hospital of Tianjin Medical University. T2D was diagnosed according to criteria of the American Diabetes Association (1997). This study was conducted according to the Declaration of Helsinki and was approved by the Committee on Studies Involving Human Beings at Tianjin Medical University. All subjects provided written informed consent prior to this study. Clinical data were recorded using Filemaker Pro 11, including age, gender, body

mass index (BMI), T2D duration, total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL), GHbA1c, and plasma fibrinogen (FIB). Mean age of patients was 59.2 ± 11.1 years, BMI was 26.24 ± 3.48 kg/m^2, and T2D duration was 11.0 ± 7.24 years. Among these patients, 201 (59.6%) individuals had a family history of T2D (Table 1).

Table 1. Clinical characteristics of 337 subjects (189 males, 148 females).

	N	Minimum	Maximum	Mean	SD
Age (years)	337	27.0	84.0	59.2	11.1
BMI (kg/m^2)	334	15.8	39.92	26.24	3.48
DM duration (years)	336	0.02	36.00	11.00	7.24
TG (mM)	319	0.53	21.29	2.33	2.45
TC (mM)	320	2.71	11.92	5.56	1.34
HDL (mM)	322	0.80	3.20	1.48	0.34
LDL (mM)	321	1.12	7.92	3.43	1.06
FIB (g/L)	323	1.00	8.40	3.63	1.07
GHbA1c [% (mM)]	310	5.0 (31)	16.3 (154)	8.2 (66)	1.8 (-4)
AI	320	0.90	6.73	2.84	0.84

SD = standard deviation; BMI = body mass index; DM = diabetes mellitus; TG = triglycerides; TC = total cholesterol; HDL = high-density lipoproteins; LDL = low-density lipoproteins; FIB = fibrinogen; GHbA1c = glycosylated hemoglobin A1c; AI = atherogenic index = (TC - HDL) / HDL.

Transcranial Doppler sonography (TCD) measurements

CBF velocities were measured using noninvasive TCD through temporal and ocular windows. We collected 54 phenotypes from the TCD tests, including peak, mean, and minimum velocities of blood flow in the binary (anterior, middle, and posterior) cerebral arteries, vertebral arteries, and basilar arteries. In addition, the pulsatility index (PI), resistance index (RI), and systolic and diastolic ratio (S:D) were also tested.

Molecular genotyping

Thirty-one genes associated with diabetes, diabetic nephropathy, obesity, insulin resistance, and hyperuricemia were selected based on previous studies (Price et al., 2008; Wang et al., 2011). Fifty-two SNPs in candidate genes with minor allele frequencies of >0.1 were examined. The *MYH9* and *ABI2* gene regions were well covered ($r^2 > 0.8$). Genomic DNA was extracted from peripheral blood using the high-salt method. SNPs were genotyped using the Sequenom MassARRY iPLEX^{TM} platform (Sequenom, San Diego, CA, USA), which is based on matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF MS) technology, at the Beijing Genome Institute. Hardy-Weinberg equilibrium (HWE) was tested using the PLINK (version 1.07) program (Purcell et al., 2007) (http://pngu.mgh.harvard.edu/purcell/plink/) (Table 2).

Statistical analysis

Outliers (±4 SD from the mean) were deleted prior to analysis. To screen for the influence of potential non-genetic factors on CBF, Spearman correlation was performed for 54 parameters of CBF and age, gender, T2D duration, lipid profiles [TC, TG, HDL, low-density

lipids (LDL), very low-density lipids (VLDL)], FIB, and GHbA1c (**Table S1**). Linear regressions were performed for each CBF parameter against age within gender, and standardized residuals were saved such that the mean = 0 and SD = 1 for each parameter for quantitative association studies. Associations between genotypes (52 SNPs) and the 54 quantitative CBF parameters, as well as haplotype association analyses, were performed using the PLINK software (version 1.07) (2007). General statistical analyses were performed using the SPSS software (SPSS Inc., Chicago, IL, USA).

Table 2. Candidate SNPs and HWE examined in this study.

CHR	SNP	Gene	HWE-P	MAF	CHR	SNP	Gene	HWE-P	MAF
2	rs16867321	*UBE2E3*	0.496	0.285	8	rs13266634	*SLC30A8*	0.653	0.265
2	rs11675251	*ABI2*	0.590	0.380	9	rs10811661	*CDKN2A/B*	0.837	0.211
2	rs3731652	*ABI2*	0.320	0.490	10	rs7923837	*HHEX*	0.348	0.409
2	rs62183937	*ABI2*	0.346	0.250	10	rs7903146	*TCF7L2*	0.111	0.221
2	rs1376877	*ABI2*	0.501	0.373	12	rs7312112	*IGF1*	0.542	0.479
2	rs11677793	*SPAG16*	0.477	0.273	12	rs11067076	*TBX5*	1.000	0.189
2	rs7578326	*IRS1*	0.324	0.298	12	rs11067083	*TBX5*	0.614	0.193
3	rs2929402	*PCAF*	1.000	0.431	13	rs371276	*SLITRK5*	0.330	0.270
3	rs4402960	*IGF2BP2*	0.456	0.353	13	rs409762	*SLITRK5*	0.334	0.269
4	rs13129697	*SLC2A9*	0.919	0.422	14	rs11624704	*NRXN3*	0.241	0.119
4	rs1014290	*SLC2A9*	0.596	0.296	15	rs12102171	*SMAD3*	0.814	0.263
4	rs6856526	*LPHN3*	0.553	0.137	16	rs17818920	*FTO*	0.374	0.266
4	rs2231142	*ABCG2*	0.812	0.137	20	rs4814615	*PCSK2*	0.129	0.275
6	rs10946398	*CDKAL1*	0.612	0.427	22	rs2106294	*LIMK2*	0.678	0.156
6	rs7756992	*CDKAL1*	0.538	0.428	22	rs5749286	*SFI1*	0.294	0.264
6	rs881858	*VEGFA*	0.861	0.363	22	rs5753669	*SFI1*	0.086	0.265
6	rs9395706	*PKHD1*	0.831	0.268	22	rs2295251	*SFI1*	0.600	0.413
6	rs722208	*ESR1*	0.760	0.417	22	rs875726	*MYH9*	0.779	0.300
7	rs1581498	*IL-6*	0.447	0.390	22	rs2009930	*MYH9*	0.577	0.300
7	rs768403	*GBX1*	1.000	0.466	22	rs2239783	*MYH9*	0.096	0.390
7	rs386956	*NUB1*	0.258	0.400	22	rs3752462	*MYH9*	0.397	0.310
7	rs7805834	*NUB1*	0.742	0.139	22	rs2269532	*MYH9*	0.746	0.230
7	rs446886	*NUB1*	0.763	0.321	22	rs2071731	*MYH9*	0.745	0.220
8	rs1526167	*TOX*	0.613	0.460	22	rs739097	*MYH9*	0.861	0.160
8	rs2726557	*TOX*	0.102	0.490	22	rs735853	*MYH9*	0.603	0.242
8	rs11777927	*TOX*	0.819	0.380	22	rs738409	*PNPLA3*	0.504	0.246

CHR = chromosome; SNP = single-nucleotide polymorphism; HWE-P = P value of Hardy-Weinberg equilibrium test; MAF = minor allele frequency.

RESULTS

High GHbA1c level is weakly correlated with CBF in patients with T2D

Of the 337 Chinese T2D subjects, 12.5% (42/337) had histories of cerebrovascular disease. Lipid profiles (TC, LDL, HDL, and VLDL) and FIB levels of patients did not significantly differ from the international standard healthy values. TG (2.33 ± 2.45 mM) was increased (<1.7 mM), while the GHbA1c level (8.2 ± 1.8%; 66 ± 4 mM) on the day of hospitalization was significantly abnormal at 3.5-5.5% (3.0-6.2 mM). In this study, CBF was strongly correlated with age and diabetes mellitus (DM) duration (P = 0.00), and was moderately correlated with gender, BMI, FIB, and TG (P < 0.05). However, few lipid profiles were correlated (P = 0.05) with any of the 54 TCD phenotypes. GHbA1c was correlated with only 1 CBF measurement [right-posterior cerebral artery (RPCA), peak velocity, r_s = 0.18, P = 0.00] (**Table S1**).

Polymorphisms of *MYH9* may be associated with CBF

To determine whether T2D-related genes are associated with CBF in patients with T2D, we performed quantitative association studies for candidate gene SNPs. Interestingly, we found that 8 SNPs of *MYH9* were generally associated with CBF parameters, including 2 *MYH9* SNPs (rs875726, rs735853) with a peak velocity of the right-middle cerebral artery (P = 0.0044 and 0.0019) and 3 SNPs (rs875726, rs2009930, and rs375246) with mean velocity of the RPCA and the right-anterior cerebral artery (P = 0.003, 0.0037, and 0.0087, respectively). Haplotype analysis of *MYH9* candidate SNPs revealed that the G-C-A haplotype of rs2239782, rs3752462, and rs2269532 was generally associated with extensive TCD phenotypes of the cerebral arteries (Table 3), particularly with the minimum velocity of RPCA (P = 0.0003).

Table 3. Association analysis among the CBF phenotypes and candidate genes.

CBF	SNP	Gene	P	CBF	SNP	Gene	P
LACA-Peak	rs1581498	*IL-6*	0.046	RACA-PI	rs3746876	*KCNJ15*	0.004
LACA-Mini	rs5753669	*SFI1*	0.044	RACA-RI	rs6856526	*LPHN3*	0.004
LACA-Mean	-	-	NA	RACA-S:D	rs3746876	*KCNJ15*	0.011
LACA-PI	rs2106294	*LIMK2*	0.022	RMCA-Peak	rs735853	***MYH9***	0.002
LACA-RI	rs2106294	*LIMK2*	0.007	RMCA-Mini	-	-	NA
LMCA-S:D	rs13129697	*SLC2A9*	0.029	RMCA-Mean	rs7903146	*TCF7L2*	0.021
LMCA-Peak	rs7903146	*TCF7L2*	0.029	RMCA-PI	rs13266634	*SLC30A8*	0.021
LMCA-Mini	rs3746876	*KCNJ15*	0.031	RMCA-RI	-	-	NA
LMCA-Mean	rs446886	*NUB1*	0.010	RMCA-S:D	-	-	NA
LMCA-PI	rs738409	*PNPLA3*	0.002	RPCA-Peak	rs735853	***MYH9***	0.007
LMCA-RI	rs738409	*PNPLA3*	0.017	RPCA-Mini	rs875726	***MYH9***	0.039
LMCA-S:D	rs738409	*PNPLA3*	0.013	RPCA-Mean	rs875726	***MYH9***	0.011
LPCA-Peak	rs2239782	***MYH9***	0.016	RPCA-PI	rs738409	*PNPLA3*	0.006
LPCA-Mini	rs7903146	*TCF7L2*	0.017	RPCA-RI	rs738409	*PNPLA3*	0.022
LPCA-Mean	rs446886	*NUB1*	0.032	RPCA-S:D	rs738409	*PNPLA3*	0.008
LPCA-PI	rs446886	*NUB1*	0.015	RV-Peak	rs875726	***MYH9***	0.002
LPCA-RI	rs2269532	***MYH9***	0.021	RV-Mini	rs722208	*ESR1*	0.029
LPCA-S:D	rs11777927	*TOX*	0.012	RV-Mean	rs875726	***MYH9***	0.010
LV-Peak	rs11675251	*ABI2*	0.006	RV-PI	rs768403	*GBX1*	0.019
LV-Mini	rs1581498	*IL-6*	0.050	RV-RI	rs768403	*GBX1*	0.025
LV-Mean	rs1581498	*IL-6*	0.022	RV-S:D	rs16867321	*UBE2E3*	0.011
LV-PI	rs2295251	*SFI1*	0.020	BA-Peak	rs2295251	*SFI1*	0.005
LV-RI	rs11675251	*ABI2*	0.003	BA-Mini	rs1581498	*IL-6*	0.004
LV-S:D	rs17782313	*MC4R*	0.012	BA-Mean	rs2295251	*SFI1*	0.006
RACA-Peak	rs875726	***MYH9***	0.007	BA-PI	rs62183937	*ABI2*	0.025
RACA-Mini	rs875726	***MYH9***	0.002	BA-RI	rs62183937	*ABI2*	0.018
RACA-Mean	rs875726	***MYH9***	0.003	BA-S:D	rs3746876	*KCNJ15*	0.044

CBF = cerebrovascular blood flow; SNP = single-nucleotide polymorphism; LACA = left-anterior cerebral artery; LMCA = left-middle cerebral artery; LPCA = left-posterior cerebral artery; RACA = right-anterior cerebral artery; RMCA = right-middle cerebral artery; RPCA = right-posterior cerebral artery; LV = left vertebral artery; RV = right vertebral artery; BA = basilar artery; Peak = peak flow velocity; PI = pulsatility index; RI = resistance index; S:D = systolic and diastolic ratio.

Susceptibility genes of T2D were not equally associated with CBF

Our results showed that several previously reported T2D-related gene SNPs, including rs7578326 near the *IRS1* gene (Rung et al., 2009), rs10811661 of *CDKN2A/B*, rs10946398 and rs775699 of *CDKAL1*, and rs4402960 of *IGF2BP2* (Duesing et al., 2008; Omori et al., 2008), were not associated with CBF in our subjects. Moreover, rs17818920 of the *FTO* gene, which is associated with extreme obesity, and rs371276 and rs409762 of the neurite-modulation gene *SLITRK5* showed no association with the CBF phenotypes.

DISCUSSION

Cerebrovascular disease is one of the main complications of T2DM. Typically, CBF is determined by a number of factors such as blood viscosity, blood vessel dilation, and cerebral perfusion pressure. However, studies have begun to focus on factors contributing to CBF maintenance or regulation under high glucose levels. Diabetes is a genetic predisposition disease, and the genetic background of CBF in patients with diabetes has not been well established. This is the first genetic investigation of CBF in patients with T2D.

In this study, TCD was selected as a noninvasive method for mass screening of CBF. Adjusted parameter values representing the global CBF conditions of patients with T2D were used for correlation and quantitative allelic association studies. We confirmed that CBF was correlated to DM duration, age, and BMI (**Table S1**). However, CBF was not globally correlated with the high level of GHbA1c. GHbA1c is used to measure the amount of glucose bound to red blood cells, which indicates a patient's blood glucose control over the past 3 months. Interestingly, a recent study indicated that the relationship between GHbA1c and macrovascular complications was not as significant as that of GHbA1c with microvascular complications and demonstrated no correlation with stroke (Penno et al., 2013). However, studies suggest that a higher GHbA1c level may be a serological marker to evaluate the severity and prognosis of acute brainstem infarctions (Li et al., 2012). In addition, an elevated GHbA1c level (>7.5%) was associated with an increased risk of all-cause mortality and a lower revascularization rate in elderly patients with new-onset diabetes (Twito et al., 2013). In our study, the mean GHbA1c level was 8.2% (10.5 mM). Although we did not identify a globally significant correlation between GHbA1c and CBF, we cannot rule out the influence of elevated GHbA1c on cerebral vascular system. The cerebral microvascular complications of T2D should be further examined to illustrate the potential effect of GHbA1c on cerebral vascular.

In this study, quantitative allelic association and haplotype analysis revealed that SNPs of the *MYH9* gene may be associated with CBF in patients with T2D. Previous studies indicated that mutations in the *MYH9* gene were responsible for various platelet disorders including hereditary macrothrombocytopenia (Dong et al., 2005), May-Hegglin syndrome, Fechtner syndrome, Sebastian syndrome, Epstein syndrome (Heath et al., 2001), Alport syndrome, and progressive sensorineural deafness (Brodie et al., 1992). Genome-wide admixture scanning revealed a highly significant association between *MYH9* polymorphisms and non-diabetic end-stage renal diseases in African Americans (Kao et al., 2008). In addition, a recent study suggested that SNP rs375246 is an independent predictor of reduced glomerular filtration rate in the Spanish RENASTUR cohort population (Tavira et al., 2013). In this study, 8 SNPs of the *MYH9* gene were strongly associated with CBF phenotypes, and the *MYH9* G-C-A haplotype of SNPs rs2239782-rs3752462-rs2269532 were associated with most of the CBF traits, particularly the minimum blood flow velocity of RPCA (Table 4). Some of these syndromes may overlap. For example, molecular genetic analysis showed that structural changes in the myosin gene had large effects on platelets, leukocytes, and megakaryocytes. The *MYH9* gene polymorphisms may have some effects on the composition of blood and partly alter the blood flow velocities in patients with T2D. However, additional studies are needed to determine whether and how these SNPs influence gene expression and protein structure.

Although none of the results revealed a genome-wide association ($P < 1 \times 10^{-7}$) or significant association after Bonferroni correction, it was unusual to observe multiple *MYH9* associations with independent CBF phenotypes. *MYH9* polymorphisms were associated with

nearly all of the 54 CBF phenotypes, including the 5 most significant associations in 9 cerebral arteries (Table 4). Rather than increasing sample size, detailed phenotyping can also provide self-replication for association studies.

Table 4. G-C-A *MYH9* haplotype (rs2239782-rs3752462-rs2269532) was associated with cerebral CBF phenotypes.

Phenotype	P value
Left-posterior cerebral artery	
Peak	0.037
Mean	0.032
Right-anterior cerebral artery	
Peak	0.012
Mini	0.010
Mean	0.010
Right middle cerebral artery	
Peak	0.007
Mini	0.040
Mean	0.018
Right-posterior cerebral artery	
Peak	0.010
Mini	0.0003
Mean	0.001
PI	0.014
RI	0.022
Right vertebral artery	
Peak	0.026

Peak = peak flow velocity; Mean = mean flow velocity; Mini = minimum flow velocity; PI = pulsatility index; RI = resistance index.

In addition to the *MYH9* association, various SNPs were likely associated with middle cerebral artery (MCA) blood flow velocities. Because MCA is the most common site of cerebrovascular diseases, stenosis of the MCA increased the risk of vascular disease mortality in patients with T2D (Thomas et al., 2008). In our study, rs7903146 of *TCF7L2* (Palmer et al., 2011) and rs735853 of *MYH9* (Freedman et al., 2009; Cooke et al., 2012) were likely associated with right middle cerebral artery (RMCA) peak blood flow velocity (P = 0.0085, and P = 0.0019, respectively); rs12102171 of *SMAD3*, rs3746876 of *KCNJ15* (Okamoto et al., 2010), rs5753669 of *SFI1* (McDonough et al., 2011), and rs13266634 of *SLC30A8* (Hu et al., 2009) with RMCA S:D (P = 0.018, 0.011, 0.014, and 0.014, respectively); SNP rs446886 of *NUB1* with the left middle cerebral artery (LMCA) mean blood flow velocity (P = 0.01); SNP rs738409 of *PNPLA3* with LMCA PI (P = 0.0019); and SNP rs13129697 of *SLC2A9* with LMCA S:D (P = 0.029). Many of these associations were moderate; however, several candidate gene SNPs were associated with multiple TCD phenotypes, suggesting weak but detectable contributions to the genetic relative risks of cerebrovascular diseases. In addition, SNP rs1581498 near the gene for interleukin-6 may be associated with a large spectrum of TCD phenotypes. In this study, the SNP rs1014290 of the *SLC2A9* gene was not associated with any TCD phenotype, although an association with T2D in the Han Chinese has been reported (Liu et al., 2011) (Table 3).

The genetic background of CBF is clearly polygenic and was not treated as a binary disease in our study. Because changes in blood vessel structure, hemodynamics, and blood pressure are important for CBF, each of these factors may contribute to the overall genetic

background. This study revealed some of these contributions and illustrated the association between metabolic disease candidate genes and CBF in patients with T2D.

In conclusion, we performed association and haplotype analyses for 52 SNPs from 31 candidate genes of metabolic diseases and found that SNPs in the *MYH9* gene may be associated with CBF in Chinese patients with T2D. However, these findings require further assessment and validation within a large cohort.

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Supplementary material

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