Association between *KCNQ2*, *TCF4* and *RGS18* polymorphisms and silent brain infarction based on whole-exome sequencing

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Received September 16, 2019; Accepted January 20, 2020

DOI: 10.3892/mmr.2020.10975

Abstract. Silent brain infarction (SBI) is a cerebral infarction identified through brain imaging. In particular, studies have shown that the presence of SBI in elderly patients increases their risk of cognitive dysfunction, impairment and dementia. However, little research has been published on the relevance of SBI to these risks for the Korean population. The association between potassium voltage-gated channel subfamily Q member 2 (*KCNQ2*), transcription factor 4 (*TCF4*) and regulator of G-protein signaling 18 (*RGS18*) genotypes and SBI were investigated using whole-exome sequencing and PCR restriction fragment length polymorphism (RFLP) analysis. The study population included 407 patients with SBI (171 males) and 401 control subjects (172 males). Genotyping was performed using PCR RFLP. Interestingly, *TCF4* rs9957668T>C polymorphisms were associated with SBI prevalence [TT vs. CC: adjusted odds ratio (AOR), 1.815, 95% confidence intervals (CI), 1.202-2.740; TT vs. TC+CC: AOR, 1.492, 95% CI, 1.066-2.088; TT+TC vs. CC: AOR, 1.454, 95% CI, 1.045-2.203]. The combination of *KCNQ2* rs73146513A>G and *TCF4* rs9957668T>C genotypes was associated with increasing SBI prevalence (AG/CC: AOR, 3.719, 95% CI, 1.766-7.833; AA/CC: AOR, 3.201, 95% CI, 1.387-7.387). The present study showed that *TCF4* rs9957668T>C polymorphisms may be risk factors for SBI. Therefore, the *TCF4* rs9957668T>C polymorphism may serve as a biomarker for increased risk of SBI in the Korean population.

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Abbreviations: AOR, adjusted odds ratio; aPTT, activated partial thromboplastin time; DBP, diastolic blood pressure; HWE, Hardy-Weinberg equilibrium; *ITF-2*, immunoglobulin transcription factor 2; *KCNQ2*, potassium voltage-gated channel subfamily Q member 2; MAF, minor allele frequency; MDR, multifactorial demention reduction; MRI, magnetic resonance imaging; NGS, next-generation sequencing; PLT, platelet; PT, prothrombin time; *RGS18*, regulator of G-protein signaling 18; SBI, silent brain infarction; SBP, systolic blood pressure; *TCF4*, transcription factor 4; WES, whole exome sequencing

Key words: silent brain infarction, prevalence, risk factors, case-control studies, single nucleotide polymorphism

Introduction

Silent brain infarction (SBI) is a cerebral infarction that is verified clinically by brain imaging (1). The occurrence of SBIs is influenced by multiple genetic and environmental factors (2). In an aging society, as in the case of Japan, medical prevention of SBI is important for preventing vascular dementia (3). SBI, a risk factor for stroke, is more frequently found with the advent of modern MRI technology, and the most direct consequence of SBI is a symptomatic stroke (4). For this reason, researchers have worked to demonstrate the relevance of SBI to stroke through comparative studies (5-10). As SBI damages the brain without causing identifiable symptoms, the risk of subsequent transient ischemic attacks and major strokes increases (11). SBI detection could provide more information concerning ischemic tolerance because it yields conclusive results for imaging (12). However, few detailed studies on SBI have been performed to date, opening a timely and necessary opportunity for SBI research.

Next-generation sequencing (NGS) technologies, such as whole-genome sequencing and whole-exome sequencing (WES), are useful for detecting and discovering new variants that can account for a number of heritable diseases and disorders (13-15). In particular, WES focuses on the coding region (i.e., the exons) of the genome, which corresponds to approximately 2.5% of the human genome, and identifies rare or common variants associated with a disorder or phenotype (16). Using WES, variants that were associated with SBI

risk were selected based on the following criteria: Fisher's exact test (P<0.05), Hardy-Weinberg equilibrium (HWE) >0.05 and minor allele frequency (MAF) >0.01 (Table SI). Based on these analyses, four single nucleotide polymorphisms (SNPs) were investigated, including potassium voltage-gated channel subfamily Q member 2 (*KCNQ2* rs73146513 A>G), transcription factor 4 (*TCF4* rs9957668 T>C) and regulator of G-protein signaling 18 (*RGS18* rs4329489 A>G and rs4454527 A>G).

KCNQ2 encodes a transmembrane potassium channel that is a member of the acetylcholinergic pathway (17). Pathogenic mutations of *KCNQ2* are associated with epilepsy (17) and pre-eclampsia (18), suggesting that *KCNQ2* is important in neuronal signaling and artery development. Furthermore, previous findings demonstrated that *KCNQ2* expression and function were associated with pathways involving neurotransmitters, and cerebral arterial development and maintenance (19-22). *TCF4* encodes a transcription factor that is also known as immunoglobulin transcription factor 2 (*ITF-2*) (23). *ITF-2* binds to the immunoglobulin enhancer μ-E5/κ-E2 motif to initiate transcription of its target genes (23). *TCF4* is primarily involved in the neurological development of the fetus during pregnancy and initiates neural differentiation by binding to DNA (24). *TCF4* gene variants have been reported to be associated with Pitt-Hopkins syndrome (25) and epileptic encephalopathy (26). Finally, *RGS18* is involved in the G-protein signaling pathway and is a member of the regulator of the G-protein signaling family (27). *RGS18* hydrolyzes G protein and thereby plays an important role in the cell signaling pathway (27,28). Genetic variants of *RGS18* occur in metabolic disorders and are associated with numerous diseases (29). Specifically, previous fjndings have demonstrated an associated between *RGS18* variants and platelet aggregation, hemostasis and thrombosis (29,30).

In the present study, four polymorphisms were investigated, namely *KCNQ2* rs73146513 A>G, *TCF4* rs9957668 T>C, *RGS18* rs4329489 A>G and *RGS18* rs4454527 A>G, based on WES data and their association with the risk of SBI. All four SNPs were located in non-coding regions and no functional differences due to these polymorphisms have been reported. Despite these reports, in the present study NGS analysis revealed that these four SNPs had MAF >0.01, genotype frequencies >0.05 and HWE P>0.05. Importantly, the MAFs were significantly enriched in patients with SBI with a P-value from Fisher's exact test of <0.05. Therefore, *TCF4* was selected as the subject of the case-control study. The correlations of each polymorphism were investigated, alone and in combination, with SBI in a Korean population. The present results suggested the possibility of a biomarker for SBI through these key polymorphisms in the Korean population.

Materials and methods

Ethics statement. All study protocols of participants were reviewed and approved by The Institutional Review Board of CHA Bundang Medical Center and followed the recommendations of The Declaration of Helsinki. Study subjects were recruited by the CHA Bundang Medical Center from the South Korean provinces of Seoul and Gyeonggi-do between June 2000 and December 2010. The Institutional Review Board of CHA Bundang Medical Center approved this genetic study in June 2000 and informed consent was obtained from the study participants.

Study population. SBI was diagnosed using the following criteria (2,31): i) Spot areas with a diameter of ≥3 mm in the area supplied by deep penetrating arteries; ii) the same spot areas showing high intensity in T2 and FLAIR (fluid attenuated inversion recovery) images and low intensity in T1 images; iii) absence of neurological symptoms corresponding to MRI lesions; and iv) no history of clinical stroke, including transient ischemic attack. Subjects were also required to be descendants of Koreans living in Seoul or Kyeonggi-do. Patients with SBI had no signs or symptoms of neurological disorders and no clinical history of stroke, including transient ischemic attacks. Patients with a history of stroke or cardiovascular disease were excluded from this study.

Over the same period, 401 control subjects (172 males, 229 females; age range, 20-97 years; mean ± SD, 63.10±11.36) were selected from patients who had visited our hospitals for biochemical tests, electrocardiograms and brain MRIs, and only those without a previous history of myocardial infarction or cerebral vascular disease were included. Control subjects were matched to the SBI group by age and sex. Baseline demographic data and a history of risk factors were obtained from each group. As with the SBI group, subjects with known stroke or cardiovascular disease were excluded. Among the initial 891 participants evaluated, 83 were excluded, leaving 401 controls and 407 cases.

Hypertension was diagnosed using high baseline blood pressure readings (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg) when one or more antihypertensive medications were being taken or prescribed. Diabetes mellitus was defined as current high fasting plasma glucose levels (≥126 mg/dl) or current treatment with oral hypoglycemic or insulin. Hyperlipidemia was defined as a high serum total cholesterol level (≥240 mg/dl) in the fasting state or a history of antihyperlipidemic treatment. A complete description of the study was provided. Written consent was obtained from all the subjects regarding the provision of information.

WES. WES was performed on samples from 20 control subjects and 20 patients with SBI, they were selected from each total group (controls: 401 individuals and SBI: 407 individuals), considering the sex ratio and mean age of the population. In control subjects, 20 individuals were selected who had no family history of myocardial infarction or cerebral vascular disease. A total of 20 patients with SBI applied the same criteria as the selection criteria of the entire sample group (2,31).

WES was conducted by Macrogen, Inc. The libraries were sequenced on an Illumina HiSeq 2000/2500/4000 instrument, and the analysis was performed using Burrows-Wheeler Alignment Tool (version 0.7.12; bio-bwa.sourceforge.net), Picard (version 1.130; broadinstitute.github.io/picard), Genome Analysis Toolkit (version 3.4.0; gatk.broadinstitute.org/hc/en-us/articles/360035530852-How-should-I-cite-GATK-in-my-own-publications) and SnpEff (version 4.1g) software (32). The annotation databases included dbSNP (142), 100 Genome (Phase 3) (33), ClinVar (05/2015) and ESP (ESP6500SI_V2). The accession number for the data was PRJNA601005. The resulting WES data were used to select

Table I. Baseline characteristics between patients with SBI and control subjects.

Characteristics	Controls (n=401)	Patients with SBI (n=407)	P-value[a]
Male (%)	172 (42.9)	171 (42.0)	0.856
Age (years, mean ± SD)	63.10±11.36	63.88±11.63	0.334
Smoking (%)	135 (33.7)	50 (12.3)	0.049
Hypertension (%)	166 (41.4)	196 (48.2)	0.024
Diabetes mellitus (%)	53 (13.2)	55 (13.5)	0.597
Hyperlipidemia (%)	93 (23.2)	100 (24.6)	0.526
BMI (kg/m^2, mean ± SD)	24.29±3.24	24.52±4.42	0.512
HDL-C (mg/dl, mean ± SD)	46.45±13.79	47.30±17.14	0.609
LDL-C (mg/dl, mean ± SD)	118.74±42.71	122.61±32.04	0.333
Homocysteine (μmol/l, mean ± SD)	10.10±4.23	11.13±5.61	0.004
Folate (nmol/l, mean ± SD)	8.85±7.98	9.05±5.70	0.691
Vitamin B12 (pg/ml, mean ± SD)	743.45±672.27	466.20±586.38	<0.0001
Total cholesterol (mg/dl, mean ± SD)	193.28±37.64	205.28±41.90	<0.0001
Triglyceride (mg/dl, mean ± SD)	146.63±90.33	161.36±118.52	0.053
PLT (103/μl, mean ± SD)	242.57±67.25	237.70±69.19	0.319
PT (sec, mean ± SD)	11.78±0.79	12.18±1.48	0.0001
aPTT (sec, mean ± SD)	33.24±18.61	33.13±7.43	0.923
Fibrinogen (mg/dl, mean ± SD)	400.22±120.39	395.21±123.61	0.735
Antithrombin (%, mean ± SD)	94.24±44.19	97.05±17.83	0.493
BUN (mg/dl, mean ± SD)	15.90±5.03	16.20±6.17	0.452
Uric acid (mg/dl, mean ± SD)	4.70±1.46	4.66±1.58	0.762
SBP (mmHg, mean ± SD)	132.01±17.04	140.32±22.52	<0.0001
DBP (mmHg, mean ± SD)	80.31±11.43	83.68±13.87	0.0003
HbA1c (%, mean ± SD)	6.58±4.35	6.25±1.26	0.370
FBS (mg/dl, mean ± SD)	114.27±36.14	114.52±47.22	0.934

[a]P-values were calculated by two-sided t-test for continuous variables and χ^2 test for categorical variables. SBI, silent brain infarction; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PLT, platelet count; PT, prothrombin time; aPTT, activated partial thromboplastin time; BUN, blood urea nitrogen; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; FBS, fasting blood sugar.

SNPs for additional study based on the following criteria: significant Fisher's exact test (P<0.05), HWE >0.05 and MAF >0.01 (Table SI). SNPs found only in control or patient groups were excluded. Of the genes that met these criteria, genes that had been previously implicated in brain diseases were selected for additional study. Genes associated with the platelet formation pathway were also included for additional analysis. To ensure high-quality and consistent experimental results, only genes that met all the criteria were investigated further in this study.

Genotyping. Genomic DNA was extracted from leukocytes using a G-DEX™ Genomic DNA Extraction kit (Intron Biotechnology, Inc.). Genetic polymorphisms were determined by PCR restriction fragment length polymorphism (RFLP) analysis. The PCR primers and PCR conditions for *KCNQ2*, *TCF4* and *RGS18* polymorphisms are described below.

The sequences of the *KCNQ2* rs73146513 A>G primers were: Forward, 5'-CGG TCA CAG TTC CAG ACA CA-3' and reverse, 5'-TGG CCC TGC TTG TCT TTC CT-3'. PCR conditions included an initial denaturation at 95˚C for 15 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 61˚C for 30 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 5 min. The 321 bp PCR product was then digested with 5U *Dde*I and yielded the following fragments: GG, 321 bp; GA, 321, 256 and 65 bp; and AA, 256 and 65 bp.

The sequences of the *TCF4* rs9957668 T>C primers were: Forward, 5'-TAA ACC AAG GCC AAG TCT CCC-3' and reverse, 5'-GGC CCC TTA AAA GAA AGG CCT-3'. PCR conditions included an initial denaturation at 95˚C for 5 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 63˚C for 30 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 5 min. The PCR product was digested with *Nsi*I and yielded the following fragments: TT, 356 and 297 bp; TC, 636, 356 and 297bp; and CC, 636 bp.

The sequences of the *RGS18* rs4329489 A>G primers were: Forward, 5'-TGT TAT CTG TGC CCT TTA ACC-3' and reverse, 5'-ATG ATT CAC CCC ATT TCA CTG-3'. PCR conditions included an initial denaturation at 95˚C for 15 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 5 min. The 335 bp PCR product was digested

Table II. Genotype frequency of *KCNQ2*, *TCF4* and *RGS18* gene polymorphisms between patients with SBI and control subjects.

A, *KCNQ2* rs73146513A>G

Genotype	Controls (n=401)	Patients with SBI (n=407)	COR (95% CI)	P-value[b]	FDR-P	AOR (95% CI)[a]	P-value[b]	FDR-P
AA	113 (28.2)	113 (27.8)	1.000 (reference)	1.000 (reference)				
AG	204 (50.9)	201 (49.4)	0.985 (0.712-1.364)	0.929	0.929	1.025 (0.735-1.431)	0.883	0.883
GG	84 (20.9)	93 (22.9)	1.107 (0.747-1.641)	0.612	0.612	1.126 (0.745-1.702)	0.573	0.573
Dominant (AA vs. AG+GG)			1.021 (0.751-1.388)	0.895	0.895	1.049 (0.765-1.438)	0.766	0.766
Recessive (AA+AG vs. GG)			1.118 (0.801-1.561)	0.513	0.513	1.100 (0.779-1.554)	0.587	0.587
HWE P	0.648	0.842						

B, *TCF4* rs9957668T>C

Genotype	Controls (n=401)	Patients with SBI (n=407)	COR (95% CI)	P-value[b]	FDR-P	AOR (95% CI)[a]	P-value[b]	FDR-P
TT	109 (27.2)	84 (20.6)	1.000 (reference)	1.000 (reference)				
TC	207 (51.6)	207 (50.9)	1.298 (0.920-1.830)	0.137	0.274	1.353 (0.949-1.930)	0.095	0.300
CC	85 (21.2)	116 (28.5)	1.771 (1.188-2.640)	0.005	0.020	1.815 (1.202-2.740)	0.005	0.020
Dominant (TT vs. TC+CC)			1.435 (1.037-1.988)	0.030	0.120	1.492 (1.066-2.088)	0.020	0.080
Recessive (TT+TC vs. CC)			1.482 (1.074-2.045)	0.017	0.068	1.454 (1.045-2.023)	0.026	0.104
HWE P	0.469	0.635						

C, *RGS18* rs4329489A>G

Genotype	Controls (n=401)	Patients with SBI (n=407)	COR (95% CI)	P-value[b]	FDR-P	AOR (95% CI)[a]	P-value[b]	FDR-P
AA	249 (62.1)	278 (68.3)	1.000 (reference)	1.000 (reference)				
AG	138 (34.4)	121 (29.7)	0.785 (0.583-1.058)	0.112	0.274	0.799 (0.589-1.084)	0.150	0.300
GG	14 (3.5)	8 (2.0)	0.512 (0.211-1.241)	0.138	0.276	0.450 (0.177-1.142)	0.093	0.186
Dominant (AA vs. AG+GG)			0.760 (0.569-1.016)	0.064	0.128	0.770 (0.571-1.036)	0.084	0.168
Recessive (AA+AG vs. GG)			0.554 (0.230-1.336)	0.189	0.378	0.502 (0.199-1.267)	0.144	0.288
HWE P	0.333	0.211						

Table II. Continued.

D, *RGS18* rs4454527A>G

Genotype	Controls) (n=401	Patients with SBI (n=407)	COR (95% CI)	P-value[b]	FDR-P	AOR (95% CI)[a]	P-value[b]	FDR-P
AA	233 (58.1)	217 (53.3)	1.000 (reference)			1.000 (reference)		
AG	148 (36.9)	163 (40.0)	1.183 (0.886-1.579)	0.256	0.341	1.196 (0.888-1.609)	0.239	0.319
GG	20 (5.0)	27 (6.6)	1.450 (0.790-2.660)	0.231	0.308	1.383 (0.738-2.592)	0.312	0.416
Dominant (AA vs. AG+GG)			1.214 (0.920-1.604)	0.171	0.228	1.223 (0.920-1.627)	0.167	0.223
Recessive (AA+AG vs. GG)			1.354 (0.746-2.455)	0.319	0.425	1.285 (0.694-2.379)	0.426	0.568
HWE P	0.571	0.626						

[a]Adjusted by age, sex, hypertension, diabetes mellitus and hyperlipidemia; [b]P-values were calculated by logistic regression analysis. SBI, silent brain infarction; KCNQ2, potassium voltage-gated channel subfamily Q member 2; TCF4, transcription factor 4; RGS18, regulator of G-protein signaling 18; COR, crude odds ratio; AOR, adjusted odds ratio; HWE, Hardy-Weinberg equilibrium; 95% CI, 95% confidence interval; FDR, false discovery rate.

with 5U *Hpy*CH4V and yielded the following fragments: AA, 335 bp; AG, 335, 175 and 160 bp; and GG, 175 and 160 bp.

Finally, the sequences of the *RGS18* rs4454527 A>G primers were: Forward, 5'-GAT TGT CGG TGA GCA AAA GG-3' and reverse, 5'-CGG GTG TCT TCA TGA AAC TC-3'. PCR conditions included an initial denaturation at 95˚C for 15 min; 35 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 5 min. The PCR product was digested by 5U *Nde*I and yielded the following fragments: AA, 265 bp; AG, 265, 232 and 33 bp; and GG, 232 and 33 bp.

To validate RFLP findings, 10% of the PCR assays for each polymorphism were randomly selected, repeated and subjected to DNA sequencing. Sequencing was performed with an ABI 3730xl DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The concordance of the quality control samples was 100%.

Statistical analysis. To estimate the relative risk of the *KCNQ2*, *TCF4* and *RGS18* genotypes for SBI, the odds ratio (OR) and 95% confidence intervals (CIs) were calculated using Fisher's exact test. Case and control subjects were compared using two-sided t-tests for continuous variables and the Chi-square test was used for categorical variables. The adjusted ORs (AORs) for *KCNQ2*, *TCF4* and *RGS18* polymorphisms were determined from logistic regression analyses used to adjust for possible confusion, including age, sex, hypertension, diabetes mellitus and hyperlipidemia. $P<0.05$ was considered statistically significant for all the tests. To solve the multiple comparison problem, post-hoc analyses were performed using false discovery rates. Analyses were performed using GraphPad Prism 4.0 (GraphPad Software, Inc.) and MedCalc version 12.7.1.0 (MedCalc Software bvba; http://www.medcalc.org; 2013). Haplotypes were evaluated using HAPSTAT software version 3.0 (version 3.0; by maximizing the likelihood that properly accounts for phase uncertainly and study design) (34). Genetic interaction analysis was carried out using the multidimensional reduction (MDR) software package (v.2.0), available from www.epistasis.org.

Results

Clinical characteristics. Various clinical characteristics, including homocysteine, folate, vitamin B12, total cholesterol, platelet (PLT), prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen levels of patients with SBI and controls are summarized in Table I. The patients with SBI had significantly increased of levels of hypertension, homocysteine, total cholesterol, PT, systolic blood pressure (SBP) and diastolic blood pressure (DBP). Patients with SBI also had decreased levels of smoking frequency and vitamin B12 levels.

Genotype frequencies. The genotype frequencies of *KCNQ2*, *TCF4* and *RGS18* polymorphisms were then compared between patients with SBI and control subjects. AORs were calculated using multivariate logistic regression analyses with respect to age, sex and incidence of hypertension, diabetes mellitus and hyperlipidemia (Table II). The frequencies of *KCNQ2*, *TCF4* and *RGS18* genotypes in patients with SBI and control subjects were consistent with the expected frequencies under HWE

Table III. Allele combinations for the *KCNQ2*, *TCF4* and *RGS18* gene polymorphisms in patients with SBI and control subjects by MDR.

A, *KCNQ2* rs73146513A>G/*TCF4* rs9957668T>C/*RGS18* rs4329489A>G/*RGS18* rs4454527A>G

Allele combination	SBI controls (2n=802)	Patients with SBI (2n=814)	OR (95% CI)	P-value[a]	FDR-P
A-T-A-A	497 (62.0)	465 (57.1)	1.000 (reference)		
A-T-A-G	15 (1.9)	22 (2.7)	1.568 (0.803-3.059)	0.240	0.649
A-T-G-A	13 (1.6)	6 (0.7)	1.315 (0.626-2.765)	0.573	0.758
A-T-G-G	5 (0.7)	7 (0.9)	1.496 (0.472-4.749)	0.569	0.758
A-C-A-A	59 (7.4)	90 (11.1)	1.630 (1.147-2.318)	0.006	0.090
A-C-A-G	11 (1.3)	14 (1.7)	1.360 (0.611-3.027)	0.545	0.758
A-C-G-A	10 (1.3)	5 (0.6)	0.534 (0.181-1.576)	0.303	0.649
A-C-G-G	5 (0.6)	11 (1.4)	2.351 (0.811-6.821)	0.132	0.649
G-T-A-A	54 (6.8)	76 (9.3)	1.504 (1.038-2.180)	0.032	0.240
G-T-A-G	14 (1.7)	7 (0.9)	0.534 (0.214-1.336)	0.192	0.649
G-T-G-A	8 (1.0)	7 (0.9)	0.935 (0.336-2.600)	1.000	1.000
G-T-G-G	6 (0.8)	4 (0.5)	0.713 (0.200-2.542)	0.754	0.870
G-C-A-A	47 (5.8)	46 (5.6)	1.046 (0.683-1.601)	0.914	0.979
G-C-A-G	22 (2.7)	25 (3.1)	1.215 (0.675-2.184)	0.552	0.758
G-C-G-A	19 (2.4)	11 (1.4)	0.619 (0.291-1.314)	0.266	0.649
G-C-G-G	16 (2.0)	18 (2.2)	1.202 (0.606-2.386)	0.606	0.758

B, *TCF4* rs9957668T>C/*RGS18* rs4329489A>G/*RGS18* rs4454527A>G

Allele combination	SBI controls (2n=802)	Patients with SBI (2n=814)	OR (95% CI)	P-value[a]	FDR-P
T-A-A	548 (68.3)	540 (66.3)	1.000 (reference)		
T-A-G	31 (3.9)	30 (3.7)	0.982 (0.586-1.645)	1.000	1.000
T-G-A	23 (2.8)	14 (1.7)	0.618 (0.315-1.213)	0.182	0.425
T-G-G	12 (1.5)	11 (1.4)	0.930 (0.407-2.127)	1.000	1.000
C-A-A	108 (13.5)	137 (16.8)	1.287 (0.974-1.701)	0.077	0.270
C-A-G	31 (3.9)	39 (4.8)	1.277 (0.785-2.077)	0.388	0.543
C-G-A	29 (3.6)	15 (1.8)	0.525 (0.278-0.990)	0.046	0.270
C-G-G	20 (2.5)	28 (3.5)	1.421 (0.791-2.553)	0.302	0.529

C, *TCF4* rs9957668T>C/*RGS18* rs4329489A>G

Allele combination	SBI controls (2n=802)	Patients with SBI (2n=814)	OR (95% CI)	P-value[a]	FDR-P
T-A	578 (72.1)	568 (69.8)	1.000 (reference)		
T-G	35 (4.4)	26 (3.2)	0.756 (0.449-1.272)	0.297	0.446
C-A	141 (17.5)	177 (21.8)	1.277 (0.995-1.640)	0.057	0.171
C-G	48 (6.0)	42 (5.2)	0.890 (0.579-1.369)	0.662	0.662

[a]P-values were calculated by Fisher's exact test. KCNQ2, potassium voltage-gated channel subfamily Q member 2; TCF4, transcription factor 4; RGS18, regulator of G-protein signaling 18; SBI, silent brain infarction; OR, odds ratio; 95% CI, 95% confidence interval; FDR, false discovery rate; MDR, multifactor dimensionality reduction.

(P>0.05). It was shown that only the *TCF4* rs9957668 T>C polymorphism correlated significantly with SBI prevalence (TT vs. CC: AOR, 1.815, 95% CI, 1.202-2.740; TT vs. TC+CC: AOR, 1.492, 95% CI, 1.066-2.088; TT+TC vs. CC: AOR, 1.454, 95% CI, 1.045-2.023).

To determine whether combinations of the selected variants were associated with SBI risk, genotype combination frequencies of *KCNQ2* rs73146513 A>G, *TCF4* rs9957668 T>C, *RGS18* rs4329489 A>G and *RGS18* rs4454527 A>G were analyzed (Table III). It was found that several genotype combinations were significantly associated with SBI when the SBI group was compared with the control group. Specifically, these combinations included *KCNQ2* rs73146513 and *TCF4* rs9957668 genotype combinations [AA-TC (AOR, 2.662,

Table IV. Combined genotype analysis for the *KCNQ2*, *TCF4* and *RGS18* gene polymorphisms between patients with SBI and control subjects.

A, *KCNQ2* rs73146513A>G/*TCF4* rs9957668T>C

Genotype	SBI controls (n=401)	Patients with SBI (n=407)	AOR (95% CI)[a]	P-value[b]
AA-TT	35 (8.7)	19 (4.7)	1.000 (reference)	
AA-TC	52 (13.0)	60 (14.7)	2.662 (1.274-5.561)	0.009
AA-CC	26 (6.5)	34 (8.4)	3.201 (1.387-7.387)	0.006
AG-CC	41 (10.2)	68 (16.7)	3.719 (1.766-7.833)	0.001
GG-TC	41 (10.2)	50 (12.3)	3.193 (1.405-7.252)	0.006

B, KCNQ2 rs73146513A>G/*RGS18* rs4454527A>G

Genotype	SBI controls (n=401)	Patients with SBI (n=407)	AOR (95% CI)[a]	P-value[b]
AA-AA	70 (17.5)	51 (12.5)	1.000 (reference)	
AA-AG	36 (9.0)	54 (13.3)	2.174 (1.201-3.935)	0.010
GG-AA	43 (10.7)	57 (14.0)	1.784 (1.013-3.140)	0.045

C, *TCF4* rs9957668T>C/*RGS18* rs4329489A>G

Genotype	SBI controls (n=401)	Patients with SBI (n=407)	AOR (95% CI)[a]	P-value[b]
TT-AA	67 (16.7)	55 (13.5)	1.000 (reference)	
CC-AA	54 (13.5)	80 (19.7)	1.731 (1.036-2.890)	0.036

D, *RGS18* rs4329489A>G/*RGS18* rs4454527A>G

Genotype	SBI controls (n=401)	Patients with SBI (n=407)	AOR (95% CI)[a]	P-value[b]
AA-AA	151 (37.7)	170 (41.8)	1.000 (reference)	
AG-AA	76 (19.0)	42 (10.3)	0.504 (0.321-0.791)	0.003

Data with P-values >0.05 were excluded, and excluded data are presented in Tables SII and SIII. [a]Adjusted by age, sex, hypertension, diabetes mellitus and hyperlipidemia; [b]P-values were calculated by logistic regression analysis. KCNQ2, potassium voltage-gated channel subfamily Q member 2; TCF4, transcription factor 4; RGS18, regulator of G protein signaling 18; SBI, silent brain infarction; AOR, adjusted odds ratio; 95% CI, 95% confidence interval.

95% CI, 1.274-5.561, P=0.009), AA-CC (AOR, 3.201, 95% CI, 1.387-7.387, P=0.006), AG-CC (AOR, 3.719, 95% CI, 1.766-7.833, P=0.001) and GG-TC (AOR, 3.193, 95% CI, 1.405-7.252, P=0.006)]; *KCNQ2* rs73146513 and *RGS18* rs4454527 genotype combinations [AA-AG (AOR, 2.174, 95% CI, 1.201-3.935, P=0.010) and GG-AA (AOR, 1.784, 95% CI, 1.013-3.140, P=0.045)]; and a *TCF4* rs9957668 and *RGS18* rs4329489 genotype combination [CC-AA (AOR, 1.731, 95% CI, 1.036-2.890, P=0.036)]. In contrast, a genotype combination of *RGS18* rs4329489 and *RGS18* rs4454527 [AG-AA (AOR, 0.504, 95% CI, 0.321-0.791, P=0.003)] was associated with lower SBI prevalence (Table IV). Furthermore, allele combination analyses were conducted to compare patients with SBI and control subjects using the MDR method (Table SII). All data for genotype combination frequencies of *KCNQ2* rs73146513 A>G, *TCF4* rs9957668 T>C, *RGS18* rs4329489 A>G and *RGS18* rs4454527 A>G are detailed in Tables SIII and SIV.

t-tests were performed between *KCNQ2*/*TCF4* genotype combinations and clinical parameters (Table IV). The comparisons included only genotype combinations that were statistically significant in the analysis detailed in Table IV. Several combined genotypes of *KCNQ2* rs73146513 and *TCF4* rs9957668, namely, AA-TC, AA-CC, AG-CC and GG-TC, were associated with increased SBI prevalence. In this analysis, the *KCNQ2* and *TCF4* AA-CC genotype combination correlated with a significant difference in PT and uric acid levels (PT, 12.10±1.38, P=0.043; uric acid, 4.41±1.20, P=0.044), and the GG-TC combination correlated with a significant difference in PT and fasting blood sugar (FBS) levels (PT, 12.14±1.00, P=0.004; FBS, 105.27±23.80, P=0.046). In addition, the association of various clinical parameters and genotypes in patients with SBI, as well as the risk of disease with each SNP under various conditions were analyzed by stratification analyses. These analyses are detailed in Tables SV-SXI but did not yield any significant associations.

Table V. Clinical variables in patients with SBI and controls stratified *KCNQ2* rs73146513/*TCF4* rs9957668 combined genotypes status by t-test.

	Homocysteine (mmol/l)		Folate (mg/ml)		Vitamin B12 (pg/ml)		Total cholesterol (mg/dl)	
Genotype	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
AA/TT	10.16±3.77	0.291	7.83±4.29	0.559	556.08±300.05	0.630	199.00±41.50	0.961
AA/TC	10.85±4.00		8.50±7.50		514.62±570.02		199.35±43.23	
AA/CC	11.13±4.76	0.234	9.14±6.36	0.218	528.05±477.72	0.720	201.07±43.57	0.802
AG/CC	10.81±5.83	0.459	10.16±11.02	0.148	664.48±1035.54	0.470	205.45±39.04	0.343
GG/TC	10.24±2.93	0.879	8.35±5.19	0.546	583.28±552.28	0.748	198.79±37.57	0.976
	Triglyceride (mg/dl)		PLT (103/μl)		BMI (kg/m^2)		SBP (mmHg)	
Genotype	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
AA/TT	165.48±119.12	0.449	241.07±45.37	0.989	24.58±3.27	0.346	139.10±18.24	0.312
AA/TC	151.29±107.25		241.20±61.46		23.97±2.88		135.61±20.74	
AA/CC	174.33±161.39	0.749	241.58±57.09	0.959	24.85±8.89	0.873	136.51±20.61	0.495
AG/CC	150.35±82.54	0.357	236.89±54.16	0.629	25.02±3.21	0.530	135.64±19.74	0.303
GG/TC	156.47±109.07	0.650	249.95±78.63	0.451	24.25±3.37	0.661	136.11±20.46	0.394
	DBP (mmHg)		HbA1c (%)		PT (sec)		aPTT (sec)	
Genotype	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
AA/TT	84.92±13.36	0.102	6.36±1.59	0.583	11.56±0.79	0.133	40.51±43.33	0.148
AA/TC	81.31±12.43		6.15±1.22		11.80±0.77		32.70±10.34	
AA/CC	82.46±13.56	0.347	6.21±1.24	0.769	12.10±1.38	0.043	31.40±5.00	0.185
AG/CC	80.66±12.51	0.058	6.34±1.08	0.958	11.83±0.77	0.092	33.65±8.53	0.202
GG/TC	81.64±14.63	0.195	5.90±0.79	0.196	12.14±1.00	0.004	33.79±10.99	0.251
	Fibrinogen (mg/dl)		Antithrombin (%)		BUN (mg/dl)		Uric acid (mg/dl)	
Genotype	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]
AA/TT	386.34±163.41	0.931	98.93±29.11	0.723	17.03±8.14	0.506	5.01±1.78	0.367
AA/TC	389.56±120.74		105.70±81.91		16.30±5.58		4.76±1.59	
AA/CC	357.49±100.88	0.518	91.36±16.18	0.347	16.11±3.94	0.443	4.41±1.20	0.044
AG/CC	414.18±154.00	0.540	93.87±15.06	0.436	16.13±5.20	0.398	4.69±1.50	0.237
GG/TC	412.93±103.36	0.481	90.21±13.07	0.156	15.81±7.61	0.373	4.69±1.55	0.269
	HDL-cholesterol (mg/dl)		LDL-cholesterol (mg/dl)		FBS (mg/dl)			
Genotype	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]	Mean ± SD	P-value[a]		
AA/TT	50.01±12.46	0.320	124.21±37.72	0.889	123.04±74.43	0.153		
AA/TC	46.95±13.52		122.74±48.48		111.07±30.00			
AA/CC	51.40±24.30	0.792	124.00±29.92	0.984	114.07±33.14	0.424		
AG/CC	49.36±27.16	0.903	126.56±41.81	0.805	115.22±40.07	0.404		
GG/TC	47.61±12.29	0.435	113.31±28.98	0.192	105.27±23.80	0.046		

[a]P-values were calculated by two-sided t-test. SBI, silent brain infarction; KCNQ2, potassium voltage-gated channel subfamily Q member 2; TCF4, transcription factor 4; PLT, platelet count; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; PT, prothrombin time; aPTT, activated partial thromboplastin time; BUN, blood urea nitrogen; HDL, high density lipoprotein; LDL, low density lipoprotein; FBS, fasting blood sugar.

Discussion

To date, the risk factors for SBI have been poorly defined. There are reports that SBIs occur more frequently in women than in men (35); however, this hypothesis is not clearly established and few data exist to support the claim. Typical risk factors, such as diabetes and smoking, are also not clearly correlated with SBI risk (35). In fact, the only patient characteristic that has been conclusively classified as an independent risk factor for SBI is hypertension (36). The present study provides data that support classifying diabetes as a risk factor for SBI. Moreover, the SBI risk factors reported here are similar to those reported for symptomatic strokes (37). Identification of genetic risk factors would further the clinical ability to detect and address SBI occurrence prior to the onset of more serious neurological events.

To address the genetic risk of SBI, previously reported genetic variants were analyzed by NGS analysis and selected variants of interest based on MAF and genotype frequencies in SBI and control subjects. A variant set of three genes were constructed (Table SI) and confirmed these three genes in a large sample of patients with SBI and control subjects. Through validation, false positives and errors were eliminated, and the remaining four SNPs were analyzed. In the present study, the associations between *KCNQ2* rs73146513 A>G, *TCF4* rs9957668 T>C, *RGS18* rs4329489 A>G and *RGS18* rs4454527 A>G polymorphisms and SBI prevalence were examined. It was found that *TCF4* rs9957668 T>C genotypes were strongly associated with SBI susceptibility. In particular, the *TCF4* rs9957668 T>C CC genotype was found to be approximately 7% higher in patients with SBI than in control subjects. The C allele in *TCF4* rs9957668 T>C had an AOR of 1.325 after adjusting for several SBI risk factors, which included age, sex, hypertension, diabetes mellitus and hyperlipidemia. This result indicated that the risk of SBI was increased approximately 1.3 times in patients with this polymorphism. Therefore, *TCF4* rs9957668 T>C may be a specific polymorphism that indicates susceptibility to SBI. Based on these data, it is suggested that the *TCF4* rs9957668 T>C genotype may contribute to the occurrence of SBI and should be considered during diagnosis of the disease.

In addition, the *TCF4* rs9957668 T>C polymorphism may be an important diagnostic indicator of psychosis in patients, because SBI increases the risk of stroke and dementia (37). In the WES results (Table SI), the OR of *TCF4* rs9957668 T>C was 6.261, indicating high sensitivity to SBI. However, as the number of samples increased, the OR of *TCF4* rs9957668 T>C decreased approximately 1.6 times. This phenomenon raises some important questions concerning methodologies that are based on WES results and the importance of providing answers to these questions in articles reporting such data. In fact, it was confirmed that, although the OR of *TCF4* rs9957668 T>C changed, the overall outcome remained consistent. Thus, these results can be reported with confidence in our hypothesis based on WES results.

In addition to single variant effects, the present study also investigated the effect of SNP combinations on the prevalence of SBI. As *TCF4* rs9957668 was independently associated with SBI risk, several genotype combinations were analyzed and significant associations were found. In these combinatorial analyses, the ORs were low and the probability of statistical significance was also reduced due to the small sample size. Nevertheless, several genotype combinations of *TCF4* rs9957668 and *KCNQ2* rs73146513 increased the risk of SBI. Naturally, *TCF4* rs9957668 is likely to play a substantial role in these associations, but it is important to note that the combination of *KCNQ2* and *TCF4* increased the association with SBI by ~2-3 times compared to *TCF4* rs9957668 alone. Such analyses may be a useful basis for predicting disease risk due to genetic variation.

A number of previous studies have linked *TCF4* to intelligence, schizophrenia and endothelial dystrophy (38-40). However, evidence regarding its association with cerebrovascular diseases, such as stroke or SBI, is lacking. Therefore, it must be acknowledged that there are some limitations of the present study. First, it needs to be confirmed whether the susceptibility to SBI that is associated with the *TCF4* rs9957668 T>C polymorphism is specific to Koreans or applies to other ethnic groups as well. If similar results are found in other races, this raises the possibility that *TCF4* rs9957668 T>C could be a more general biomarker for SBI. Second, it remains to be clarified whether there is a correlation between schizophrenia and SBI. If the pathogenesis of schizophrenia is associated with the occurrence of SBI, this represents a new research direction for genes known to be related to this disease. One report out of Japan suggests that there has been an increase in the proportion of SBI and cerebral infarction in patients with psychosis relative to controls (37). Based on this report, it was postulated that an association between SBI and patients with psychosis could be identified. Third, the number of patients in this study with the *TCF4* rs9957668 T>C polymorphism was very low, thereby limiting the ability to implicate it as a biological indicator of SBI. Generally, smaller study populations tend to have an increased error rate because statistical power is limited. Thus, it is important to have a large, representative cohort of patients to support the results found. Finally, SBI control studies of SNPs other than rs9957668 T>C polymorphism in the *TCF4* gene are required to confirm specificity. Studies of other polymorphisms in the same gene could play a crucial role in interpretation of this and other results. If results from multiple studies conflict with each other, it will influence confidence in the results reported here.

It is acknowledged that these limitations may affect confidence in the present results. Therefore, the aim of future studies is to address these limitations, allowing clearer conclusions to be drawn. If future studies indicate that the *TCF4* pathway is critical in the pathogenesis of SBI, it may suggest that modulating *TCF4* gene expression and/or activity could facilitate prevention or early treatment of SBI. Finally, more epidemiological studies are needed to clarify the causal relationship between *TCF4* polymorphisms and the prevalence of SBI, and meta-analyses of heterogeneous populations should be conducted.

In conclusion, an association between the prevalence of SBI with the *KCNQ2* rs73146513 A>G, *TCF4* rs9957668 T>C, *RGS18* rs4329489 A>G and *RGS18* rs4454527 A>G polymorphisms has been demonstrated in a Korean population. These results suggested that *TCF4* polymorphism may contribute to SBI and be used as a potential biomarker to evaluate SBI risk.

Acknowledgements

Not applicable.

Funding

The present study was partially supported by National Research Foundation of Korea Grants funded by the Korean Government (grant nos. NRF-2016R1D1A1B03930141 and NRF-2017R1D1A1B03030110) and by the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare (grant no. HI18C19990200), Republic of Korea.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the NCBI SRA repository (www.ncbi.nlm.nih.gov/sra/PRJNA601005).

Authors' contributions

NKK and OJK were involved in study conception and design, and gave the final approval of the version to be published. JOK, HWK, HSP, JK and JHS were involved in data acquisition and analysis. JOK, KOL, HSP, DO and NKK interpreted the data for the work. JOK and HWK drafted the work. JOK and KOL revised the manuscript.

Ethics approval and consent to participate

All study protocols of participants were reviewed and approved by The Institutional Review Board of CHA Bundang Medical Center and followed the recommendations of the Declaration of Helsinki. The Institutional Review Board of CHA Bundang Medical Center approved this genetic study in June 2000 and informed consent was obtained from the study participants.

Patient consent for publication

Written consent was obtained from all subjects regarding the provision of information.

Competing interests

The authors declare that they have no competing interests.

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