Biochemical and Biophysical Research Communications 456 (2015) 631–636

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Genetic variants of vitamin D receptor and susceptibility to ischemic stroke

Puttachandra Prabhakar [a], Vijaya Majumdar [a], Girish Baburao Kulkarni [b], Rita Christopher [a,*]

[a] Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, Karnataka 560029, India
[b] Department of Neurology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, Karnataka 560029, India

ARTICLE INFO

Article history:
Received 24 November 2014
Available online 8 December 2014

Keywords:
Vitamin D
VDR polymorphism
Ischemic stroke

ABSTRACT

Vitamin D receptor (*VDR*) is a potential candidate for cardiovascular disease. To date the genetic association of *VDR* with ischemic stroke has not been explored. In the present study we aimed to evaluate the association between *VDR* gene variants and ischemic stroke in Asian Indian population.

Overall, 557 subjects were investigated that included 313 ischemic stroke patients and 244 control subjects. Four single nucleotide polymorphisms of the *VDR* gene termed as Fok I, Apa I, Taq I and Bsm I were genotyped by using PCR-RFLP method. The genotype distribution of Bsm I polymorphism was found to deviate from the Hardy–Weinberg equilibrium in control subjects, and hence excluded from the study. Apa I and Taq I polymorphisms were not found to be associated with ischemic stroke. However, presence of ff genotype of Fok I was found to confer 2.97-fold risk of ischemic stroke (95% CI = 1.16–7.63, $P = 0.02$) as compared to FF genotype. This association was found to be independent of various demographic and important biochemical covariates including age, gender, smoking, alcohol intake, BMI, and serum glucose, lipid profile, insulin and HOMA-IR, 25-hydroxyvitamin D and plasma NOx levels [OR = 2.27, 95% CI = 1.25–4.09, $P = 0.01$]. However, adjustment for lipid metabolites attenuated the genetic association [OR = 1.68, 95% CI = 0.75–3.78, $P = 0.21$]. Fok I polymorphism was also found to be associated with total cholesterol levels; ff genotype carriers were found to have significantly higher cholesterol levels (203.56 ± 30.50 mg/dl) as compared to FF carriers (177.38 ± 47.90 mg/dl) ($P = 0.04$). On stratification by gender the genetic association between Fok I polymorphism and ischemic stroke remained significant in females only (OR = 2.28, 95% CI = 1.15–4.53, $P = 0.02$). This genetic association was also found to attenuate on adjustment with lipid variables.

In the present study we could associate the only known functional polymorphism of *VDR* i.e., Fok I, with ischemic stroke in a gender specific manner. Adjustment with lipid variables was found to attenuate this association indicating that impaired lipid metabolism may be the underlying mechanism of action of this polymorphism which leads to an increase in the risk of ischemic stroke. Further larger scale validations in other population are warranted in other population.

1. Introduction

Stroke is the second leading cause of death and disability across the world. Ischemic stroke constitutes 80% of total strokes [1]. India, the second most populous country in the world, is facing a stroke epidemic [2]. Etiologically, stroke is a complex and heterogeneous disorder with a strong genetic component [3]. Genetic dissection of this complex disorder has targeted many association studies involving potential candidate genes such as apolipoprotein E (*APOE*), methylenetetrahydrofolate reductase (*MTHFR*), endothelial nitric oxide synthase (*ENOS*), factor V Leiden, cytochrome P450 4F2 (*CYP4F2*), beta-fibrinogen and phosphodiesterase 4D (*PDE4D*) [4]. The selection of these genes was driven by the underlying pathways related to stroke pathophysiology such as coagulation, lipid metabolism, renin–angiotensin–aldosterone system, endothelial dysfunction, inflammation and abnormalities in homocysteine metabolism [4]. However, there are several more functional candidate genes whose contribution to stroke risk needs to be assessed. Vitamin D receptor (*VDR*) is one of potential candidates for cardiovascular disorders [5], but its role in the pathogenesis of stroke remains unexplored. VDR is a nuclear receptor, a phosphoprotein and the main effector of all the biological actions of vitamin D [6]. Hence, it is a crucial component of the vitamin D endocrine system. After activation by its ligand, this nuclear receptor localizes to the regulatory

* Corresponding author. Fax: +91 80 26564830.
E-mail addresses: rita@nimhans.kar.nic.in, rita.nimhans@yahoo.com (R. Christopher).

http://dx.doi.org/10.1016/j.bbrc.2014.12.007
0006-291X/

regions of target genes and binds to their vitamin D-responsive element (VDREs) thereby modulating their transcriptional output [6]. A wide range of vasoprotective effects of vitamin D and *VDR* activation have been documented including slowing down of atherosclerosis, promotion of endothelial function and suppression of the renin-angiotensin-aldosterone (RAA) system [7–12]. Parallely, expression of VDR has been reported to be ubiquitous: the highest being in the small intestine, colon, kidney, bone and skin, along with other tissues and cell types like the vascular system, endocrine organs, immune system, brain and muscle, which point to a possible involvement of the receptor in a plethora of physiological aspects [13]. Moreover, this endocrine system has also been reported to have neuroprotective effects [14]. These lines of evidence indicate a strong candidature of *VDR* in the biology of ischemic stroke.

The chromosomal location of *VDR* gene is 12q13.1 [15]. The gene spans a stretch of 75 kb containing 11 exons [16,17]. More than sixty *VDR* polymorphisms have been detected so far. However, most of these, except one, are anonymous without any known functional effect. Four RFLPs of *VDR* gene named Fok I, Apa I, Bsm I and Taq I, have been thoroughly studied for their effects on various disease outcomes like cancer, diabetes and CAD [18]. Some studies have compared the genotypic distribution of *VDR* polymorphisms across various world populations. Interestingly, the frequency and distribution of *VDR* polymorphisms in India has been found to be substantially different from other populations and ethnic groups [19,20]. The Fok I polymorphism, associated with disruption of the restriction site in exon 2, is the only polymorphism of the *VDR* with functional impact; it results in loss of a transcription start site [21]. Bsm I and Apa I sites are located in intron 8 of the *VDR* gene, whereas the synonymous Taq I site is present in its 9th exon [21].

To date no study has been reported on the genetic association between *VDR* polymorphisms and ischemic stroke. Hence, we undertook the present study to test the association of four genetic variants of *VDR* with ischemic stroke in our population.

2. Materials and methods

The study was approved by institutional ethical committee and informed consent was obtained from all study participants. Patients aged between 23 and 85 years who presented at the neurological service of the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India, a tertiary care centre for neurological disorders were recruited for the study. Diagnosis of ischemic stroke was confirmed by neuro-imaging studies (CT/MRI). Patients diagnosed with haemorrhagic stroke, stroke secondary to neuro-infection, malignancy or other terminal illnesses were excluded. Control samples were collected from age and gender-matched healthy subjects having no prior history of cerebrovascular disorders. Clinical and demographic data of the study participants were recorded using the standard procedure. Demographic and anthropometric analyses included the following variables: age, gender, smoking status, alcohol intake, height, weight and blood pressure levels. Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. Serum levels of total cholesterol, high-density lipoprotein-cholesterol (HDL–C), triglycerides, and glucose were determined using auto analyzer (Olympus AU640, Munich, Germany). Fasting serum insulin was measured by ELISA with commercial kits [Human Insulin specific, Calbiotech, Inc., San Diego, 113 CA]. Plasma nitric oxide concentrations were measured as NOx (nitrite plus nitrate). Nicotinamide adenine dinucleotide phosphate (NADPH)-nitrate reductase (EC 1.6.6.2)-mediated enzymatic conversion of nitrate was combined along with addition of glucose-6-phosphate enzymatic conversion as described by Verdon et al. [22]. Insulin resistance was assessed using the homeostasis model assessment (HOMA-IR) originally described by Mathew et al. [23]. HOMA-IR was calculated using the following formula:

$$\text{HOMA} - \text{IR}\ (\text{mmol/L} \times \mu\text{U/ml}) = \text{fasting glucose}\ (\text{mmol/L}) \times \text{fasting insulin}\ (\mu\text{U/ml})/22.5$$

2.1. DNA isolation and PCR

Genomic DNA from human peripheral blood samples were isolated by the standard phenol chloroform method [24]. DNA was quantified by using NanoDrop 2000 (Thermo Fisher Scientific, MA, USA).

2.2. Taq I and Bsm I multiplex PCR and restriction digestion

Sample DNA was amplified using Taq I and Bsm I multiplex PCR (Taq I – forward primer 5′-TCAGCAGTCATAGAGGGGTG-3′; reverse primer 5′-AGCACAAGGGGCGTTAGCTT-3′ and Bsm I – forward primer 5′ GCATCGTCTCCCCAGGTAT-3′, reverse primer 5′-CAGTTCACGCAAGAGCAGAG-3′) with 2U of Taq DNA Polymerase (Solis Biodyne, Tartu), and supplied buffer under the following conditions: 94 °C for 6 min, followed by 35 cycles of 94 °C for 30 s, 60.0 °C for 30 s, and 72 °C for 40 s, followed by a 5-min at 72 °C final extension. PCR product for Taq I (364 bp) and Bsm I (335 bp) were then digested with Taq I and Bsm I restriction endonucleases at 65 °C for 4 h and were electrophoretically separated on a 2.5% agarose gel. Taq I digestion gives restriction fragments of 364 bps for 'TT' genotype, 201 and 163 bps for 'tt' genotype and 364, 201 and 163 bps for 'Tt' genotype. Bsm I digestion gives products of size 335 bps for 'BB', 298, 37 bps for 'bb' and 335, 298, 37 bps for 'Bb' genotype.

2.3. Apa I and Fok I PCR and restriction digestion

Multiplexed PCR amplification of Fok I (rs10735810) and Apa I (rs11168271) polymorphisms was done using the oligonucleotide primers (Sigma Aldrich, Bangalore, India); Fok I – forward primer 5′-GGCCTGCTTGCTGTTCTTAC-3′, reverse primer 5′-TGCTTCTTCTCCCTCCCTTT-3′ and Apa I – forward primer 5′-ATAGAGAAGAAGGCACAGGAG-3′, reverse primer 5′-AGGTCTGGATCCTAAATGCA-3′, using the following conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 25 s, 58.2 °C for 25 s, and 72 °C for 25 s, followed by a 5-min at 72 °C. PCR product for Fok I (221 bp) and Apa I (185 bp) were analyzed in 2.0% agarose gel and documented using Bio-Rad Gel Doc system and Quantity one software version 4.3.1 (Bio-Rad Laboratories, Inc., CA 94547 USA). Then the multiplex PCR products were digested independently using Fok I restriction endonucleases at 37 °C for 4 h and Apa I restriction endonucleases at 25 °C for 4 h, and were electrophoretically separated on a 2.5% agarose gel. Fok I digestion gives a band of 221bps for 'FF', and for 'ff' it produces a product size of 185 and 36 bps for 'Ff', and 221, 185 and 36 bps for 'Ff' genotypes. Apa I digestion gives a product size of 150, 35 bps for 'aa' genotype, 185 bps for 'AA' and 185, 150 and 35 bps for 'Aa' genotype.

2.4. Statistical analysis

Statistical analysis was performed with IBM SPSS statistics 17.0 (IBM Corporation, NY, USA) and GraphPad Prism v.5.0.1 (Graph Pad Software, La Jolla, CA, USA). Differences in baseline characteristics between patients and healthy controls were assessed by the chi-squared test for categorical variables and the *t*-test for continuous parameters. Genotypic frequencies of polymorphisms were tested for Hardy–Weinberg equilibrium by Chi-squared analysis (HWE).

The frequency distribution of the alleles and genotypes were compared between patient and control groups by chi-squared test. Logistic regression analysis was used to analyze the association between genetic polymorphisms and ischemic stroke. *P* values of <0.05 were considered to be statistically significant.

3. Results

The study group comprised of 313 patients diagnosed with ischemic stroke (236 males and 77 females) and 244 healthy control subjects (170 males and 74 females) (Table 1). Distribution of age between patients (48.5 ± 15.6 years) and controls (46.4 ± 13.4 years) was not significantly different ($P = 0.109$) (Table 1). Risk factors such as smoking, alcohol use and hypertension were found to be more prevalent in stroke patients compared to controls (Table 1). Amongst continuous variables, systolic blood pressure (SBP), diastolic blood pressure (DBP), blood glucose, total cholesterol and low density lipoprotein (LDL) cholesterol were found to be elevated in patients compared to controls (Table 1).

3.1. Genotyping results for Bsm I

Bsm I polymorphism was detected in controls as well as patients. However, the polymorphism was not found to be in Hardy–Weinberg equilibrium in control subjects (*P* value = 0.008, $\chi^2 = 7.07$), hence excluded from further statistical analyses.

3.2. Genotyping results for Taq I

Taq I polymorphism was genotyped in 283 stroke patients and 231 controls, polymorphism was in Hardy–Weinberg equilibrium in controls (*P* value = 0.25, $\chi^2 = 0.13$) as well as patients (*P* value = 0.97, $\chi^2 < 0.001$). The polymorphism was not found to be associated with any of the studied demographic or biochemical parameters in stroke cases (Table 2). The genotypic distribution of the polymorphism was also not found to be significantly different between patients and controls ($\chi^2 = 1.03$, $P = 0.599$) (Table 3).

Table 1
Clinical and demographic variables in ischemic stroke patients and controls.

Variable	Controls (*n* = 244)	Stroke cases (*n* = 313)	*P* value
Age, years	46.4 ± 13.4	48.5 ± 15.6	0.109
Sex, male/female	170/74	236/77	0.132
Smoking (%)	30 (12.9)	92 (31.5)	<0.0001
Alcohol consumption (%)	37 (16.0)	73 (25)	0.013
Hypertension (%)	66 (28.6)	147 (50.4)	<0.0001
Diabetes (%)	37 (16.0)	27 (9.2)	0.022
BMI (kg/m^2)	24.6 ± 2.2	26.1 ± 4.1	<0.0001
SBP (mmHg)	126.1 ± 12.9	134.3 ± 20.1	<0.0001
DBP (mmHg)	83.4 ± 9.7	85.3 ± 10.6	0.047
Glucose (mg/dl)	97.8 ± 43.2	116.3 ± 59.3	<0.0001
Cholesterol (mg/dl)	178.5 ± 45.8	190.6 ± 40.9	0.004
TG (mg/dl)	152.1 ± 85.9	161.9 ± 110.9	0.354
HDL (mg/dl)	40.7 ± 8.9	38.8 ± 15.5	0.123
LDL (mg/dl)	105.2 ± 38.3	119.6 ± 38.1	0.005
VLDL (mg/dl)	32.3 ± 18.9	32.6 ± 24.3	0.952
Plasma NOx (μmol/L)	32.73 ± 22.3	30.24 ± 22.10	0.041
Insulin (μUI/ml)	14.6 ± 1.21	15.1 ± 1.06	0.721
HOMA-IR	3.4 ± 0.27	4.5 ± 0.33	0.012

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HOMA-IR, homeostatic model assessment for insulin resistance; *P* value < 0.05 is statistically significant.
Continuous data were tested using two-tailed Student's *t*-test/Mann–Whitney test, and categorical data were tested using a v2 test or Fisher's exact test for difference between patient and control groups.

Table 2
Distribution of cardiovascular risk factors in *VDR* polymorphisms in ischemic stroke patients.

Variables	Fok I						Apa I						Taq I					
	FF (n = 148)	Ff (n = 124)	ff (n = 20)	*P* value	*Ff vs. FF	*ff vs. FF	AA (n = 100)	Aa (n = 138)	aa (n = 54)	*P* value	*Aa vs. AA	*aa vs. AA	TT (n = 127)	Tt (n = 125)	tt (n = 31)	*P* value	*Tt vs. TT	*tt vs. TT
Age (years)	47.79 ± 15.11	48.24 ± 15.60	53 ± 19.14	0.376	0.992	0.227	49.24 ± 14.31	48.08 ± 16.56	48.48 ± 14.60	0.832	0.583	0.825	49.31 ± 15.79	48.65 ± 16.21	51.31 ± 13.53	0.573	0.741	0.407
BMI (kg/m^2)	24.49 ± 1.96	24.63 ± 2.33	25.29 ± 2.86	0.366	0.821	0.360	24.47 ± 2.39	24.59 ± 1.95	24.92 ± 2.33	0.494	0.608	0.219	24.62 ± 2.46	24.46 ± 1.98	24.32 ± 1.84	0.833	0.573	0.778
SBP (mm Hg)	135.87 ± 20.76	133.01 ± 18.51	131.76 ± 22.15	0.473	0.360	0.270	132.96 ± 18.87	135.25 ± 20.64	134.04 ± 20.32	0.690	0.423	0.951	135.89 ± 20.23	133.25 ± 19.33	129.17 ± 15.58	0.339	0.443	0.151
Glucose (mg/dl)	113.28 ± 55.99	123.62 ± 69.22	111.59 ± 37.62	0.423	0.236	0.470	114.88 ± 56.23	122.13 ± 67.19	108.20 ± 52.84	0.555	0.847	0.409	117.49 ± 57.90	118.32 ± 62.61	112.61 ± 56.03	0.775	0.996	0.487
Chol (mg/dl)	177.38 ± 47.90	178.17 ± 42.29	203.56 ± 30.50	0.141	0.756	0.048	178.15 ± 38.47	181.34 ± 47.79	173.69 ± 49.61	0.559	0.739	0.390	175.75 ± 51.33	178.45 ± 39.93	166.78 ± 43.21	0.592	0.567	0.575
TG (mg/dl)	167.70 ± 130.62	158.59 ± 88.67	148.33 ± 76.91	0.841	0.643	0.652	167.30 ± 152.35	161.91 ± 81.88	159.54 ± 99.72	0.755	0.624	0.812	154.87 ± 73.45	156.40 ± 86.46	210.11 ± 99.78	0.915	0.862	0.782
HDL–C (mg/dl)	39.03 ± 18.17	37.42 ± 11.57	48.25 ± 9.95	0.389	0.678	0.064	38.30 ± 10.35	40.07 ± 19.65	34.15 ± 7.26	0.309	0.893	0.275	35.73 ± 11.19	40.96 ± 19.83	37.19 ± 10.29	0.383	0.174	0.653
LDL–C (mg/dl)	107.65 ± 42.90	98.99 ± 41.55	137.67 ± 27.32	0.198	0.398	0.171	107.26 ± 42.07	103.72 ± 41.56	103.25 ± 47.09	0.881	0.718	0.594	107.35 ± 47.13	98.45 ± 40.42	104.94 ± 43.21	0.587	0.313	0.887
Insulin (μU/ml)	16.47 ± 23.55	13.79 ± 7.38	13.23 ± 4.88	0.763	0.505	0.654	15.85 ± 19.73	16.03 ± 19.90	12.60 ± 5.63	0.719	0.847	0.637	13.70 ± 10.25	17.30 ± 24.65	13.44 ± 6.52	0.393	0.171	0.589
HOMA-IR	4.80 ± 6.97	4.29 ± 4.01	3.86 ± 2.85	0.734	0.431	0.792	4.31 ± 4.57	5.19 ± 7.21	3.37 ± 2.21	0.607	0.845	0.395	4.31 ± 5.88	4.89 ± 5.95	4.08 ± 4.22	0.514	0.368	0.701
Nitric Oxide (μmol/l)	31.62 ± 22.64	28.99 ± 22.12	33.75 ± 22.72	0.477	0.268	0.594	30.11 ± 22.84	30.99 ± 22.92	30.36 ± 22.33	0.835	0.590	0.903	28.39 ± 19.09	31.92 ± 24.18	32.53 ± 21.48	0.580	0.424	0.396

Chol: Cholesterol, TG: Triglycerides, *P* value < 0.05 is statistically significant.
* *P* value for genotype comparisons.

3.3. Genotyping results for Apa I

The genotypic distribution of Apa I polymorphism was found to be in Hardy–Weinberg equilibrium in both controls (P value = 0.35, $\chi^2 = 0.87$) as well as patient samples (P value = 0.59, $\chi^2 = 0.27$). None of the biochemical covariates were found to be associated with the polymorphism (Table 2). There was no significant variation in the genotype frequency distribution between controls ($n = 221$) and cases ($n = 292$) for Apa I polymorphism (Table 3). Logistic regression analysis did not indicate any association between the polymorphism and ischemic stroke when analyzed under various models of covariate adjustment (Table 4).

3.4. Genotyping results for Fok I

Fok I polymorphism could be genotyped in 292 ischemic stroke patients and 219 control subjects. The genotypic distribution of the Fok I polymorphism was in Hardy–Weinberg equilibrium in both controls (P value = 0.12, $\chi^2 = 2.45$) as well as patients (P value = 0.38, $\chi^2 = 0.77$) ff vs. FF; OR = 2.97 (95% CI = 1.16–7.63) (Table 3). The polymorphism was found to be significantly associated with serum total cholesterol levels; mean values of cholesterol were comparatively higher for ff carriers (203.56 ± 30.50 mg/dl) as compared to FF carriers (177.38 ± 47.90 mg/dl) (Table 2). A significant difference was found in distribution of genotype frequencies of the polymorphism between patients and controls (Table 3) ($P = 0.027$, $\chi^2 = 7.19$). When analyzed by logistic regression ff genotype was found be confer 2.27-fold risk (95% CI = 1.25–4.09) of ischemic stroke after adjustment for various covariates such as age, sex, smoking, alcohol consumption, BMI, SBP, cholesterol and triglyceride levels, glucose, plasma insulin, HOMA-IR and plasma NOx levels. However, blood lipid levels (cholesterol and TG) were found to abrogate this genetic association (Table 4) (OR = 1.68, 95% CI = 0.75–3.78, $P = 0.21$). When stratified by gender, females were found to have significant difference in genotypic distribution of the polymorphism between patients and controls ($P = 0.013$, $\chi^2 = 8.73$) but in males the distribution was not statistically significant ($P = 0.372$, $\chi^2 = 1.98$). In females the presence of f allele was associated with significant risk of ischemic stroke. On univariate analysis, Ff genotype was found to be associated with 2.24-fold risk (95% CI = 1.09–4.61, $P = 0.029$) as compared to FF genotype (Table 3). Similarly, ff genotype conferred 2.28-fold risk 95% CI = 1.15–4.53, $P = 0.018$. On adjustment of various covariates such as age, sex, smoking, alcohol consumption, BMI, SBP, cholesterol and triglyceride levels, glucose, plasma insulin, HOMA-IR, and plasma NOx levels there was an increase in risk associated with ff genotype (OR = 5.48, 95% CI = 1.33–22.49, $P = 0.02$) as well as the Ff genotype (OR = 3.40, 95% CI = 0.95–12.21, $P = 0.06$) as compared to FF genotype. However, these associations were abrogated on additional adjustment with lipid metabolites (Table 4).

4. Discussion

In the present study we aimed to evaluate the association of *VDR* variants with susceptibility to ischemic stroke. We found a significant difference in genotypic distribution of Fok I polymorphism and ischemic stroke ($\chi^2 = 7.19$, $P = 0.027$). Compared to FF, ff genotype was found to confer 2.97-fold (95% CI = 1.16–7.63, p

Table 3
Genotype distribution of *VDR* polymorphisms and risk association with ischemic stroke.

	Genotype		Case $n = 313$	Control $n = 244$	χ^2 value	P value (Chi-square)	Odds ratio (95% CI), P
			$n = 283$	$n = 231$			
Taq I	Overall	TT	127 (44.88)	107 (45.34)	1.03	0.599	1 (ref)
		Tt	125 (44.17)	97 (41.10)			0.97 (0.69–1.34), 0.89
		tt	31 (10.95)	32 (13.56)			0.93 (0.71–1.21), 0.60
			$n = 213$	$n = 159$			
	Males	TT	95 (44.60)	66 (41.51)	1.46	0.481	1 (ref)
		Tt	97 (45.54)	71 (44.65)			0.95 (0.61–1.47), 0.82
		tt	21 (9.86)	22 (13.84)			0.81 (0.51–1.14), 0.23
			$n = 70$	$n = 72$			
	Females	TT	32 (45.71)	36 (0.50)	0.281	0.869	1 (ref)
		Tt	28 (40.00)	26 (36.11)			1.21 (0.59–2.48), 0.60
		tt	10 (14.29)	10 (13.89)			1.06 (0.64–1.75), 0.82
			$n = 292$	$n = 221$			
Apa I	Overall	AA	100 (34.2)	73 (33.03)	0.436	0.804	1 (ref)
		Aa	138 (47.3)	102 (46.15)			1.04 (0.72–1.49), 0.83
		aa	54 (18.5)	46 (20.81)			0.97 (0.77–1.23), 0.83
			$n = 223$	$n = 150$			
	Males	AA	73 (32.73)	51 (34.00)	0.522	0.770	1 (ref)
		Aa	109 (48.89)	68 (45.33)			1.12 (0.70–1.79), 0.64
		aa	41 (18.38)	31 (20.67)			0.92 (0.51–1.66), 0.79
			$n = 69$	$n = 71$			
	Females	AA	27 (39.13)	22 (30.99)	1.022	0.600	1 (ref)
		Aa	29 (42.03)	34 (47.88)			0.69 (0.33–1.47), 0.34
		aa	13 (18.84)	15 (21.13)			0.71 (0.28–1.79), 0.46
			$n = 292$	$n = 219$			
Fok I	Overall	FF	148 (50.7)	132 (58.8)	7.19	0.027	1 (ref)
		Ff	124 (42.5)	81 (36.9)			1.36 (0.95–1.97), 0.09
		ff	20 (6.8)	6 (4.3)			2.97 (1.16–7.63), 0.02
			$n = 222$	$n = 147$			
	Males	FF	118 (53.15)	85 (57.82)	1.98	0.372	1 (ref)
		Ff	94 (42.34)	59 (40.14)			1.15 (0.75–1.76), 0.53
		ff	10 (4.51)	3 (2.04)			1.55 (0.80–3.00), 0.19
			$n = 70$	$n = 72$			
	Females	FF	30 (42.86)	47 (65.28)	8.73	0.013	1 (ref)
		Ff	30 (42.86)	22 (30.55)			2.24 (1.09–4.61), 0.03
		ff	10 (14.28)	3 (4.17)			2.28 (1.15–4.53), 0.02

$P < 0.05$ is statistically significant.

Table 4
Odds ratios of genetic association of Fok I polymorphism with ischemic stroke differentially modeled by adjustment of covariates.

		Model I	Model II	Model III	Model IV	Model V
Overall	FF	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	Ff vs. FF	1.01 (0.99–1.02), 0.09	1.52 (0.99–2.34), 0.05	1.54 (0.99–2.37), 0.05	1.69 (1.08–2.65), 0.02	1.25 (0.71–2.20), 0.44
	ff vs. FF	1.89 (1.17–3.07), 0.01	1.98 (1.15–3.41), 0.01	2.05 (1.19–3.54), 0.01	2.27 (1.25–4.09), 0.01	1.68 (0.75–3.78), 0.21
Males	FF	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	Ff vs. FF	1.23 (0.79–1.90), 0.36	1.28 (0.79–2.07), 0.32	1.31 (0.77–2.22), 0.31	1.53 (0.89–2.66), 0.13	1.21 (0.64–2.31), 0.56
	ff vs. FF	1.59 (0.81–3.10), 0.17	1.80 (0.87–3.69), 0.12	1.97 (0.93–4.20), 0.08	1.91 (0.89–4.09), 0.09	1.21 (0.47–3.08), 0.69
Females	FF	1 (ref)	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	Ff vs. FF	2.50 (1.15–5.44), 0.02	4.58 (1.50–14.03), 0.01	4.29 (1.30–14.18), 0.02	3.40 (0.95–12.21), 0.06	3.45 (0.50–24.10), 0.21
	ff vs. FF	3.22 (1.45–7.19), 0.004	4.18 (1.37–12.71), 0.01	3.86 (1.23–12.14), 0.02	5.48 (1.33–22.49), 0.02	4.99 (0.56–44.85), 0.15

Model I: adjusted for age and gender; Model II: further adjusted for BMI, smoking, alcohol consumption, SBP levels; Model III: additionally adjusted for blood glucose, plasma insulin and HOMA-IR; Model IV: additionally adjusted for plasma NOx levels; Model V: additionally adjusted for lipid parameters, total cholesterol and triglycerides.
P value < 0.05 is statistically significant.

value = 0.02) risk of ischemic stroke. We did not observe any significant association between other studied *VDR* polymorphisms (Taq I and Apa I) and ischemic stroke. Our results support the previously reported functional consequences associated with the f allele substitution of the Fok I polymorphism; presence of the allele in the 5′-promoter region of the *VDR* leads to reduction in the potency of the protein as a transcription activator [21]. Our results also support of role of decreased levels of vitamin D in the pathophysiology of ischemic stroke [25–27].

Based on its expression in vascular tissue and its various vasoprotective effects, several possible mechanisms might explain the genetic association between *VDR* and ischemic stroke. For example, *in vivo* deletion of *VDR* results in development of atherosclerotic lesions accompanied by increase in adhesion molecules and proinflammatory cytokines in the aorta and cholesterol influx in macrophages [7]. *VDR* also regulates the expression of renin, the key gene of the renin–angiotensin system (RAS) [8]. RAS plays a central role in the regulation of blood pressure, volume and electrolyte homeostasis. Inappropriate activation of the RAS may lead to hypertension which is one of the major and well-established risk factors for stroke. Mice lacking *VDR* have elevated production of renin and angiotensin II which leads to hypertension, cardiac hypertrophy, and increased water intake [8]. Mechanistically *VDR* suppresses renin expression by binding to the transcription factor cAMP-response element-binding protein (CREB) [12]. *VDR* has also been found to control systemic vascular tone by regulating the expression of the key NO synthesizing enzyme, endothelial NO synthase [8]. Decreased bioavailability of the vasodilator nitric oxide and consequent endothelial dysfunction are salient features for disturbed vascular homeostasis common to the pathogenesis of ischemic stroke [28]. The available literature suggests a potential impact of vitamin D on insulin sensitivity and glucose homeostasis [29]. Clinical reports also indicate a possible association between *VDR* polymorphisms and insulin resistance/secretion [30]. Insulin resistance could be a major underlying mechanism for accelerated atherosclerosis and inflammation common to stroke pathophysiology [31]. In order to explore all these possible mechanistic links between *VDR* variants and ischemic stroke pathophysiology, we checked the impact of *VDR* polymorphisms on important parameters like blood pressure, plasma NOx levels and insulin resistance; however, we could not find any link. Genetic association between Fok I polymorphism and ischemic stroke was also found to be independent of these covariates. When adjusted for these factors, the strength of association between Fok I polymorphism and ischemic stroke was not found to change significantly (OR = 2.27, 95% CI = 1.25–4.09, *P* = 0.01).

Interestingly, on adjustment with lipid metabolites the association between Fok I polymorphism and ischemic stroke was found to attenuate. This indicates that the association of the polymorphism with ischemic stroke could be partially mediated by impaired lipid metabolism. Additionally, Fok I variant was also found to be related to cholesterol levels in ischemic stroke patients. Dyslipidemia is a well known risk factor for stroke [32]. Our results indicate that *VDR* polymorphism might influence the risk of stroke through impaired lipid metabolism. This speculation is supported by the reported impact of *VDR* activation on increased expression of human cholesterol 7α-hydroxylase which in turn leads to reduction in cholesterol levels [33].

We could also observe an influence of gender on the genetic association between *VDR* polymorphism and ischemic stroke. Gender stratification indicated female-specific genetic influence of *VDR* Fok I polymorphism on the risk of ischemic stroke along with the lipid-related attenuation of the association. Cross-talks between estrogen and vitamin D endocrine system are well known. Estrogen has been shown to modulate VDR activity; estrogen up-regulates VDR expression in the duodenal mucosa and concurrently increases the responsiveness to endogenous 1,25(OH)2D [34]. Moreover, vasoprotective effects of estrogen including lowering of cholesterol levels have been reported before [35]. Hence, it could be hypothesized that the interplay between *VDR* polymorphisms, estrogen levels and lipid metabolites could underlie the gender differentiation of stroke etiology.

The present study is limited by its small sample size. However the strength of this study is enhanced by the inclusion of important biochemical covariates like lipid metabolites, plasma NOx levels and indices of insulin resistance which could play an important role in modulating the genetic association between *VDR* polymorphisms and ischemic stroke. This is the first study to evaluate and report genetic association between *VDR* Fok I polymorphism and risk of ischemic stroke, which could be partly explained by influence of this variant on cholesterol levels. Larger sample examination across various ethnic groups will further clarify the role of *VDR* polymorphisms in the etiology of ischemic stroke.

Source of funding

This study was funded by the Indian Council of Medical Research (ICMR), New Delhi, India. (Grant Ref No: 5/4-5/18/Neuro/2010-NCD-I).

Disclosures

None.

References

[1] D. Della-Morte, F. Guadagni, R. Palmirotta, G. Testa, V. Caso, M. Paciaroni, P. Abete, F. Rengo, P. Ferroni, R.L. Sacco, T. Rundek, Genetics of ischemic stroke, stroke-related risk factors, stroke precursors and treatments, Pharmacogenomics 13 (2012) 595–613.

[2] P.M. Dalai, A. Shenoy, Stroke epidemic in India – time to prioritize prevention strategies, J. Assoc. Physicians India 61 (2013) 693–695.
[3] L.M. Brass, J.L. Isaacsohn, K.R. Merikangas, C.D. Robinette, A study of twins and stroke, Stroke 23 (1992) 221–223.
[4] A. Kumar, R. Sagar, P. Kumar, J.K. Sahu, A. Grover, A.K. Srivastava, S. Vivekanandhan, K. Prasad, Identification of genetic contribution to ischemic stroke by screening of single nucleotide polymorphisms in stroke patients by using a case control study design, BMC Neurol. 13 (2013) 136.
[5] E. Gonzalez-Parra, J. Rojas-Rivera, J. Tuñón, M. Praga, A. Ortiz, J. Egido, Vitamin D receptor activation and cardiovascular disease, Nephrol. Dial. Transplant. 27 (2012) iv17–iv21.
[6] M.R. Haussler, C.A. Haussler, P.W. Jurutka, P.D. Thompson, J.C. Hsieh, L.S. Remus, S.H. Selznick, G.K. Whitfield, The vitamin D hormone and its nuclear receptor: molecular actions and disease states, J. Endocrinol. 154 (Suppl.) (1997) S57–73.
[7] O. Andrukhova, S. Slavic, U. Zeitz, S.C. Riesen, M.S. Heppelmann, T.D. Ambrisko, M. Markovic, W.M. Kuebler, R.G. Erben, Vitamin D is a regulator of endothelial nitric oxide synthase and arterial stiffness in mice, Mol. Endocrinol. 28 (2014) 53–64.
[8] J. Kong, G. Qiao, Z. Zhang, S.Q. Liu, Y.C. Li, Targeted vitamin D receptor expression in juxtaglomerular cells suppresses renin expression independent of parathyroid hormone and calcium, Kidney Int. 74 (2008) 1577–1581.
[9] Y.C. Li, J. Kong, M. Wei, Z.F. Chen, S.Q. Liu, L.P. Cao, 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin–angiotensin system, J. Clin. Invest. 110 (2002) 229–238.
[10] F.L. Szeto, C.A. Reardon, D. Yoon, Y. Wang, K.E. Wong, Y. Chen, J. Kong, S.Q. Liu, R. Thadhani, G.S. Getz, Y.C. Li, Vitamin D receptor signaling inhibits atherosclerosis in mice, Mol. Endocrinol. 26 (2012) 1091–1101.
[11] W. Yuan, W. Pan, J. Kong, W. Zheng, F.L. Szeto, K.E. Wong, R. Cohen, A. Klopot, Z. Zhang, Y.C. Li, 1,25-Dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter, J. Biol. Chem. 282 (2007) 29821–29830.
[12] C. Zhou, F. Lu, K. Cao, D. Xu, D. Goltzman, D. Miao, Calcium-independent and 1,25(OH)2D3-dependent regulation of the renin-angiotensin system in 1alpha-hydroxylase knockout mice, Kidney Int. 74 (2008) 170–179.
[13] N.M. Maalouf, The noncalciotropic actions of vitamin D: recent clinical developments, Curr. Opin. Nephrol. Hypertens. 17 (2008) 408–415.
[14] D. Gezen-Ak, E. Dursun, S. Yilmazer, Vitamin D inquiry in hippocampal neurons: consequences of vitamin D-VDR pathway disruption on calcium channel and the vitamin D requirement, Neurol. Sci. 34 (2013) 1453–1458.
[15] S.E. Taymans, S. Pack, E. Pak, Z. Orban, J. Barsony, Z. Zhuang, C.A. Stratakis, The human vitamin D receptor gene (VDR) is localized to region 12cen-q12 by fluorescent in situ hybridization and radiation hybrid mapping: genetic and physical VDR map, J. Bone. Miner. Res. 14 (1999) 1163–1166.
[16] L.A. Crofts, M.S. Hancock, N.A. Morrison, J.A. Eisman, Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 10529–10534.
[17] K. Miyamoto, R.A. Kesterson, H. Yamamoto, Y. Taketani, E. Nishiwaki, S. Tatsumi, Y. Inoue, K. Morita, E. Takeda, J.W. Pike, Structural organization of the human vitamin D receptor chromosomal gene and its promoter, Mol. Endocrinol. 11 (1997) 1165–1179.
[18] J.M. Valdivielso, E. Fernandez, Vitamin D receptor polymorphisms and diseases, Clin. Chim. Acta. 371 (2006) 1–12.
[19] A.A. Bhanushali, N. Lajpal, S.S. Kulkarni, S.S. Chavan, S.S. Bagadi, B.R. Das, Frequency of Fok I and Taq I polymorphism of vitamin D receptor gene in Indian population and its association with 25-hydroxyvitamin D levels, Indian J. Hum. Genet. 15 (2009) 108–113.
[20] N. Swapna, U.M. Vamsi, G. Usha, T. Padma, Risk conferred by Fok I polymorphism of vitamin D receptor (VDR) gene for essential hypertension, Indian J. Hum. Genet. 17 (2011) 201–206.
[21] A.G. Uitterlinden, Y. Fang, J.B. Van Meurs, H.A. Pols, J.P. Van Leeuwen, Genetics and biology of vitamin D receptor polymorphisms, Gene 338 (2004) 143–156.
[22] C.P. Verdon, B.A. Burton, R.L. Prior, Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite, Anal. Biochem. 224 (1995) 502–508.
[23] D.R. Matthews, J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, R.C. Turner, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man, Diabetologia 28 (1985) 412–419.
[24] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989.
[25] P. Brondum-Jacobsen, B.G. Nordestgaard, P. Schnohr, M. Benn, 25-Hydroxyvitamin D and symptomatic ischemic stroke: an original study and meta-analysis, Ann. Neurol. 73 (2013) 38–47.
[26] J.R. Chaudhuri, K.R. Mridula, S. Alladi, A. Anamika, M. Umamahesh, B. Balaraju, A. Swath, V.S. Bandaru, Serum 25-hydroxyvitamin D deficiency in ischemic stroke and subtypes in Indian patients, J. Stroke 16 (2014) 44–50.
[27] Q. Sun, A. Pan, F.B. Hu, J.E. Manson, K.M. Rexrode, 25-Hydroxyvitamin D levels and the risk of stroke: a prospective study and meta-analysis, Stroke 43 (2012) 1470–1477.
[28] F. Cosentino, S. Rubattu, C. Savoia, V. Venturelli, E. Pagannonne, M. Volpe, Endothelial dysfunction and stroke, J. Cardiovasc. Pharmacol. 38 (Suppl. 2) (2001) S75–78.
[29] J.A. Alvarez, A. Ashraf, Role of vitamin D in insulin secretion and insulin sensitivity for glucose homeostasis, Int. J. Endocrinol. 2010 (2010) 351385.
[30] R. Jain, P.R. von Hurst, W. Stonehouse, D.R. Love, C.M. Higgins, J. Coad, Association of vitamin D receptor gene polymorphisms with insulin resistance and response to vitamin D, Metabolism 61 (2012) 293–301.
[31] C.F. Semenkovich, Insulin resistance and atherosclerosis, J. Clin. Invest. 116 (2006) 1813–1822.
[32] K. Tziomalos, V.G. Athyros, A. Karagiannis, D.P. Mikhailidis, Dyslipidemia as a risk factor for ischemic stroke, Curr. Top. Med. Chem. 9 (2009) 1291–1297.
[33] E.C. Chow, L. Magomedova, H.P. Quach, R. Patel, M.R. Durk, J. Fan, H.J. Maeng, K. Irondi, S. Anakk, D.D. Moore, C.L. Cummins, K.S. Pang, Vitamin D receptor activation down-regulates the small heterodimer partner and increases CYP7A1 to lower cholesterol, Gastroenterology 146 (2014) 1048–1059.
[34] Y. Liel, S. Shany, P. Smirnoff, B. Schwartz, Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa 1, Endocrinology 140 (1999) 280–285.
[35] V. Guetta, R.O. Cannon, Cardiovascular effects of estrogen and lipid-lowering therapies in postmenopausal women, Circulation 93 (1996) 1928–1937.