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Gene variations of ROCKs and risk of ischaemic stroke: the Women's Genome Health Study

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Abstract

Recent animal and human studies have demonstrated the importance of the ROCK (RhoA/Rho-associated kinase) pathway in IsST (ischaemic stroke). Whether the genetic variation within ROCK-associated genes modulates the risk of IsST remains elusive. The association between 66 tSNPs [tagging SNPs (single nucleotide polymorphisms)] of three ROCK-associated genes [*ROCK1*, *ROCK2* and *ARHGEF10* (Rho guanine-nucleotide-exchange factor 10)] and the incidence of IsST was investigated in 23 294 Caucasian female participants of the prospective WGHS (Women's Genome Health Study). All were free of known cancer and cardiovascular disease at baseline. During a 15-year follow-up period, 323 participants developed their first ever IsST. Multivariable Cox regression analysis was performed to investigate the relationship between genotypes and risk of IsST assuming an additive genetic model. Haplotype-block analysis was also performed. A total of ten tSNPs were associated with the risk of IsST (three in *ARHGEF10* and seven in *ROCK1*; $P < 0.050$). Further investigation using the haplotype-block analysis revealed a similar significant association of pre-specified haplotypes of *ROCK1* with the risk of IsST ($P = 0.005$). If corroborated in other large prospective studies, the findings of the present study suggest that genetic variation within the ROCK-associated pathway gene loci examined, and in particular *ROCK1* gene variation, may influence the risk of IsST.

Keywords

genetic epidemiology; ischaemic stroke; RhoA/Rho-associated kinase (ROCK); single nucleotide polymorphism (SNP)

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INTRODUCTION

Stroke is the third most common cause of death in the United States and a leading cause of long-term disability [1]. Twin and family studies have suggested that susceptibility to stroke has a significant genetic component [2–4]. Although GWASs (genome-wide association studies) [5,6] and case-control association studies [7,8] have uncovered several susceptibility genes, the genetic basis remains largely unknown.

Many lines of evidence suggest that the ROCK (RhoA/Rho-associated kinase) pathway is involved in the pathogenesis of IsST (ischaemic stroke) [9]. RhoA is a member of the small GTP-binding proteins and acts as a molecular ‘on–off’ switch in multiple signalling pathways [10,11]. RhoA is activated by GEFs (guanine-nucleotide-exchange factors), which catalyse the exchange of GDP by GTP. ROCKs are the main downstream target of RhoA [12,13] and regulate diverse cellular functions [14], including actin cytoskeletal organization [15], cell adhesion/proliferation/migration [16], smooth muscle contraction [17], gene transcription [18,19] and platelet activation [20]. Two isoforms of ROCKs have been identified, ROCK1 and ROCK2 [21], with an overall 65% homology in amino acid sequence and a 92% homology in their kinase domains. Abnormal activation of ROCKs has been linked to many disease processes, including atherosclerosis formation [22], hypertension [23], vascular inflammation [24], and cerebral and coronary vasospasm [25,26].

Elevated ROCK activity leads to atherosclerosis formation via activating inflammatory process and smooth muscle proliferation. In ROCK1$^{+/-}$ haploinsufficient mice, reduced neointima formation, decreased levels of pro-inflammatory adhesion molecule expression and reduced leucocyte infiltration were observed following vascular injury [27]. In an apolipoprotein E-knockout mice model with accelerated atherosclerosis, atherosclerosis was reduced by a ROCK inhibitor (Y-27632) [28]. The elevated ROCK activity observed in hypertension and cerebral vasospasm probably contributes to increased smooth muscle contraction [23,26]. In addition, ROCKs might regulate vascular tone indirectly through inhibition of eNOS (endothelial nitric oxide synthase) expression and activity [29,30]. Furthermore, alteration of RhoA/ROCK signalling has been shown to lead to endothelial dysfunction in subjects with diabetes [31] and to aortic stiffness in aging subjects and those that smoke [32]. Treatment with a ROCK inhibitor (fasudil) improves endothelial function in human subjects with coronary artery disease [29]. Evidence from clinical studies has shown that leucocyte ROCK activity was elevated in patients with acute IsST [33]. Inhibition of ROCK activity by statins probably contributes to the non-cholesterol or ‘pleiotropic’ effects of statins in preventing IsST [34–36] and in inhibiting venous thromboembolic events [37].

However, the genetic contribution of *ROCK1* and *ROCK2* towards the risk of IsST has not been reported. In the present study, we used a candidate gene approach to investigate the potential association of *ROCK1* and *ROCK2* tSNPs [tagging SNPs (single nucleotide polymorphisms)] with the risk of IsST in participants drawn from the WGHS (Women’s Genome Health Study). In addition, on the basis of a previous study which showed that a

functional SNP of RhoA GEF encoded by *ARHGEF10* (Rho GEF 10) was a susceptibility gene in the Japanese population [8], we also investigated the genetic variation in *ARHGEF10* in the Caucasian population of the present study.

MATERIALS AND METHODS

Study design

Details of the design of the present study have been described previously [38]. In brief, participants in the WGHS, a genetic sub-study of the Women's Health Study [39,40], included initially healthy North American women aged 45 years or older with no previous history of cardiovascular disease, cancer or other major chronic illnesses. A baseline blood sample was collected during the enrolment phase of the Women's Health Study between 1992 and 1995. Study participants, who gave an informed consent for blood-based analyses related to risks of incident chronic diseases, were followed up for incident events that were adjudicated by an end points committee using standardized criteria and a full medical record review [39,40]. The present investigation included 23 294 participants of European ancestry of the WGHS. During a 15-year follow-up period, 323 cases of newly diagnosed IsST were identified. DNA extracted from the baseline WGHS blood samples underwent tSNP ($r^2 \approx 0.80$) genotyping using the genome-wide Illumina Infinium II Human HAP300 panel that was designed with an LD (linkage disequilibrium) tagging strategy to capture common variation among Caucasians, as described previously [41,42]. The Brigham and Women's Hospital Institutional Review Board for Human Subjects Research approved the study protocol.

Statistical analysis

Genotype frequencies were compared with values predicted by the Hardy–Weinberg equilibrium using the χ^2 test with one degree of freedom. HRs (hazard ratios) associated with each of the tSNPs were calculated separately by Cox regression analysis adjusting for age and smoking status, and further adjusting for BMI (body mass index), randomized treatment assignment, history of diabetes, hypertension and hyperlipidaemia, and current hormone use, assuming an additive model for genetic effects.

Haplotype estimation and inference were determined by the expectation-maximization algorithm. Haplotype blocks were defined using the software Haploview v4.1 [43]. In addition, the relationship between haplotypes and risk of IsST was evaluated by a referent (wild-homozygous) haplotype-based Cox regression analysis, adjusting for the same potential confounders/risk factors used in the single SNP analysis. All analyses were carried out using SAS v9.1 package (SAS Institute) or R software. A two-tailed (uncorrected/unadjusted for multiple testing) P value of 0.05 was considered as a statistically significant result. Genotyping call rates were >99% per SNP.

RESULTS

The baseline characteristics of the 23 294 initially healthy Caucasian women are shown in Table 1. Of the 66 SNPs evaluated, ten were not in Hardy–Weinberg equilibrium with an uncorrected/unadjusted $P < 0.05$ (Table 2). Results from the multivariable Cox regression

analysis showed evidence for differential associations of ten SNPs (three for *ARHGEF10* and seven for *ROCK1*) with the risk of IsST ($P_{\text{unadjusted}}$<0.050; Table 2). Figure 1 shows the LD pattern of the tSNPs of *ROCK1* in the sample population of the present study. The haplotype distribution (defined by Haploview v4.1) is shown in Table 3. Only one Haploview-defined haplotype block of *ROCK1* (encompassing rs2127958 and rs1481280) was identified. Results from the haplotype-based analysis again showed an association of pre-specified Haploview-defined haplotype, carrying the minor alleles at both polymorphic sites of *ROCK1* with the risk of IsST (Table 3; $P_{\text{unadjusted}}$ = 0.0049). All of the SNPs evaluated were in agreement of the proportionality of hazard assumption.

DISCUSSION

Abnormal activation of ROCKs has been shown to play an important role in the pathogenesis of IsST. The results from the present study revealed that seven (out of eight) of the tSNPs evaluated in *ROCK1* were associated significantly with the risk of IsST. The positive tSNPs are located at intervals of 104 924 bases apart. A total of four tSNPs are located within introns, one tSNP is located in the 5′-UTR and two tSNPs are located in the 5′ promoter region. The functional SNPs need to be identified further. In contrast, none of the tSNPs in *ROCK2* were associated with the risk of IsST.

The specific functions of ROCK1 and ROCK2 remain unclear due to the lack of specific inhibitors that distinguish ROCK1 from ROCK2, as well as from other serine/threonine kinases such as PKA (protein kinase A) and PKC (protein kinase C) [44]. Because homozygous ROCK1$^{-/-}$ [45] and ROCK2$^{-/-}$ [46] knockout mice are lethal, a genetic approach using conditional or halploinsufficient ROCK1- and ROCK2-knockout mice provides a good opportunity to ascertain the function. Previous studies have shown that ROCK1$^{+/-}$, but not ROCK2$^{+/-}$, halploinsufficient mice have reduced neointima formation following vascular injury [27]. In addition, deficiency of ROCK1 in bone-marrow-derived cells protects against atherosclerosis [47]. Consistently, results from the present study suggest that genetic variations in *ROCK1*, but not *ROCK2*, are associated with the risk of IsST. These findings warrant further investigation of the specific role of ROCK1 and ROCK2 in IsST, and may provide an insight into the development of specific ROCK inhibitors to prevent IsST.

The present study also revealed that three tSNPs from *ARHGEF10* are associated with the risk of IsST. *ARHGEF10* encodes a GEF. *In vitro* small GTPase activity assays showed that a gene product of *ARHGEF10* activated RhoA [8]. In this Japanese study, a SNP (rs4376531) affected *ARHGEF10* transcriptional activity via regulating the binding affinity of Sp1 and was found to be associated with the risk of IsST [8]. The present study suggests that there are different susceptible *ARHGEF10* SNPs in the Caucasian population.

The strengths of the present study are the overall sample size, the biological relevance of the polymorphisms considered, the prospective design and the complete long-term follow up. We also chose, on an *a priori* basis, to present all our data simultaneously rather than focusing on any one specific finding. Nonetheless, some potential limitations of the present study require discussion, including generalization and potential bias. We only examined

Caucasian middle-aged and older women with a distinct socioeconomic status (health professionals), and our findings may not represent other populations with diverse ethnicity or socioeconomic backgrounds. Cautious interpretation of the findings of the present study (uncorrected/unadjusted for multiple testing may lead to chance findings) should be exercised. Furthermore, the U-shaped haplotypic relationship, as shown in Table 3, could be due partly to a phenomenon previously termed/described as heterosis with hybrids/heterozygotes displaying altered levels of growth, survival or fitness relative to their parental (homozygous) states, although the exact molecular bases for this phenomenon remain elusive [48]. Moreover, an alternative explanation is that the observed U-shaped relationship could be due to the rarity of the heterozygous haplotype, compared with the homozygous haplotype, with a wide 95% CI (confidence interval). Hence, taken altogether, confirmation of the findings of the present study is required.

In the present study, we had the ability to detect, on the basis of the sample size, assuming 80% power, at an α of 0.05, an HR of greater than 1.30 if the minor allele frequency was 0.50 and of greater than 2.80 if the minor allele frequency was 0.01, assuming a univariable-additive model. Thus we cannot rule out a low-to-modest risk of IsST associated with the tSNPs tested. Furthermore, the present investigation (decided *a priori*) did not examine IsST subtypes with the genetic loci evaluated; thus further subtype-specific investigation is needed.

In conclusion, the findings of the present study warrant further investigation into the involvement of the ROCK-associated pathway genes tested in the pathogenesis of IsST. More importantly, the findings of the present study require confirmation/replication in future large prospective studies.

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Abbreviations

ARHGEF10	Rho GEF 10
BMI	body mass index
CI	confidence interval
GEF	guanine-nucleotide-exchange factor
HR	hazard ratio
IsST	ischaemic stroke
LD	linkage disequilibrium
ROCK	RhoA/Rho-associated kinase
tSNP	tagging single nucleotide polymorphism
WGHS	Women’s Genome Health Study

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CLINICAL PERSPECTIVES

- Recent animal and human studies have demonstrated the relevance of the ROCK pathway in the pathogenesis of IsST. Whether the genetic variation within the ROCK-associated genes modulates IsST risk remains elusive.
- In the present large prospective study, we observed an association of *ROCK1* gene variation with the risk of IsST.
- In a clinical situation, the findings of the present study highlight the potential prognostic utility of ROCK-associated gene variation in the prediction of the risk of IsST.

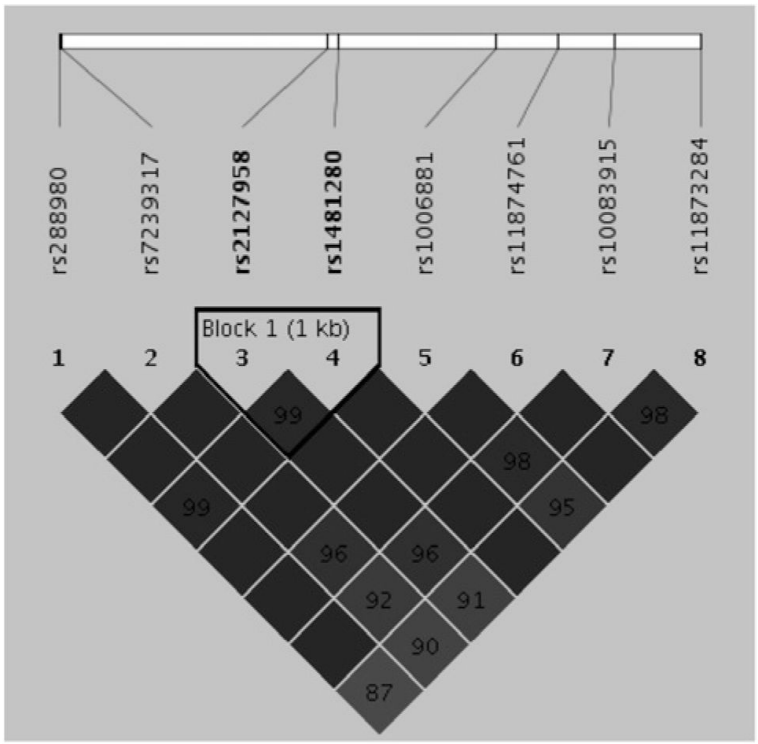

Figure 1. LD pattern of the *ROCK1* locus, generated by Haploview v4.1 using the default D'/LOD determination of the SNPs tested

D' is the value of D prime between the two loci. LOD is the log of the likelihood odds ratio, a measure of confidence in the value of D'.

Table 1
Baseline characteristics of the study population

Results are the medians and interquartile range for continuous variables and percentages for categorical variables.

Variable	Value
n	23 294
Age (years)	52.90 (48.92–59.01)
BMI (kg/m^2)	24.89 (22.46–28.32)
Smoking status	
Current (%)	11.64
Past (%)	37.45
Never (%)	50.91
History of diabetes (%)	2.52
History of hyperlipidaemia ≥240 mg/dl (%)	29.76
History of hypertension ≥140/90 mmHg (%)	24.61
Aspirin use (%)	49.87
β-Carotene use (%)	49.81
Vitamin E use (%)	50.08
Current hormone use (%)	43.86

Table 2

Cox regression analysis of the incidence of IsST

Results are adjusted for age, BMI, current smoking, treatment assignment, history of diabetes, hypertension and hyperlipidaemia, and current hormone use. HWE, Hardy–Weinberg equilibrium; MA, minor allele; MAF, minor allele frequency.

Gene	Chromosome	Position	MA	MAF	dbSNP SNP_ID	HR	Lower 95% CI	Upper 95% CI	$P_{uncorrected}$	HWE
ROCK2	2p24	11218182	A	0.4874	rs2271622	0.963	0.829	1.119	0.6198	0.5460
		11229674	G	0.4432	rs3732103	0.988	0.850	1.149	0.8769	0.8630
		11237110	A	0.4811	rs921322	0.966	0.831	1.121	0.6470	0.4143
		11238327	A	0.4809	rs8996	0.980	0.843	1.140	0.7968	0.0005
		11244205	A	0.4818	rs6753921	0.979	0.844	1.136	0.7803	0.3315
		11276570	C	0.4665	rs9808232	1.031	0.888	1.198	0.6869	0.6720
		11286528	A	0.4839	rs1515219	0.980	0.844	1.138	0.7924	0.3251
		11303284	A	0.4470	rs6716817	0.940	0.808	1.094	0.4241	0.6806
		11369434	G	0.1289	rs10203916	0.921	0.729	1.163	0.4891	0.3066
		11377735	A	0.1843	rs6755337	0.914	0.749	1.116	0.3777	0.8616
		11392895	A	0.2825	rs12622447	1.062	0.903	1.250	0.4659	0.5279
		11406981	G	0.3750	rs7581184	1.072	0.917	1.254	0.3828	<0.0001
		11412302	A	0.2800	rs6432187	1.054	0.894	1.244	0.5316	0.0392
		11420801	C	0.2108	rs7424263	0.947	0.786	1.141	0.5675	0.5948
		11422663	A	0.2758	rs4533449	0.905	0.760	1.076	0.2586	0.4120
ARHGEF10	8p23	1735572	A	0.1795	rs4875960	0.948	0.778	1.154	0.5936	0.2474
		1740903	A	0.2279	rs4327894	0.909	0.758	1.090	0.3020	0.3517
		1743991	G	0.4076	rs6558545	0.905	0.776	1.055	0.2026	0.4554
		1752777	A	0.1041	rs6980781	1.065	0.841	1.349	0.6014	0.8330
		1755410	G	0.2793	rs6558550	0.899	0.758	1.066	0.2202	0.0998
		1769901	G	0.3649	rs6558551	1.044	0.894	1.219	0.5843	0.0811
		1772670	A	0.4483	rs4875952	1.046	0.900	1.214	0.5589	0.5333
		1776763	G	0.3855	rs11136430	1.139	0.979	1.325	0.0916	0.7295
		1778839	G	0.4267	rs11136431	0.903	0.777	1.051	0.1884	0.0062
		1791325	G	0.3029	rs9693412	0.978	0.831	1.150	0.7836	0.1668
		1795162	G	0.3013	rs7007884	1.196	1.019	1.402	0.0283	0.4533

Gene	Chromosome	Position	MA	MAF	dbSNP SNP_ID	HR	Lower 95% CI	Upper 95% CI	$P_{uncorrected}$	HWE
		1799279	A	0.4970	rs13277792	0.943	0.812	1.096	0.4453	0.4012
		1805367	A	0.2314	rs17829629	0.910	0.759	1.092	0.3118	0.0974
		1808062	A	0.2297	rs3735866	0.903	0.752	1.083	0.2708	0.0541
		1820250	C	0.3336	rs7014895	0.876	0.744	1.032	0.1135	0.0122
		1821207	C	0.1492	rs9657362	1.045	0.850	1.285	0.6735	0.7961
		1828103	C	0.3744	rs4242520	1.092	0.936	1.274	0.2646	<0.0001
		1828619	C	0.2149	rs7826500	1.073	0.897	1.284	0.4388	0.4126
		1833416	A	0.0541	rs4474061	0.855	0.594	1.230	0.3996	0.2473
		1833702	G	0.1546	rs2272712	1.111	0.910	1.356	0.3011	0.9398
		1844699	A	0.4609	rs2294035	0.973	0.838	1.131	0.7213	0.3979
		1844997	A	0.0393	rs2294039	1.369	0.981	1.912	0.0650	0.3842
		1847801	A	0.2258	rs6990129	1.020	0.854	1.218	0.8258	0.9104
		1849377	G	0.1508	rs11995882	0.958	0.774	1.187	0.6962	0.2599
		1853293	G	0.4765	rs7004405	1.138	0.979	1.322	0.0919	0.7227
		1856248	G	0.4285	rs11136442	1.013	0.872	1.177	0.8650	0.1471
		1858362	G	0.4217	rs4242549	0.953	0.818	1.110	0.5347	0.8191
		1864037	A	0.1367	rs2272611	1.186	0.970	1.450	0.0971	0.1012
		1864886	C	0.0714	rs17683288	1.348	1.042	1.744	0.0232	0.6925
		1867936	G	0.4588	rs4242548	1.024	0.879	1.194	0.7602	0.2098
		1875853	G	0.1069	rs3824141	0.822	0.630	1.071	0.1457	0.7570
		1877939	A	0.0773	rs7386016	0.858	0.637	1.156	0.3150	0.4896
		1880248	G	0.3712	rs999545	0.958	0.818	1.121	0.5905	0.0546
		1885063	G	0.1503	rs2280823	1.178	0.968	1.433	0.1015	0.5551
		1887097	G	0.3738	rs17830107	0.857	0.731	1.004	0.0563	0.3686
		1891563	G	0.3943	rs4876268	0.796	0.680	0.931	0.0044	0.3508
		1891670	A	0.3297	rs6999840	1.008	0.860	1.180	0.9262	0.0465
		1894036	C	0.0656	rs14375	0.972	0.717	1.319	0.8569	0.7479
		1895261	G	0.1009	rs6981540	1.015	0.796	1.294	0.9068	0.0249
		1898064	G	0.06500	rs12547074	0.993	0.734	1.343	0.9639	0.9140
		1903753	C	0.2684	rs4876265	1.054	0.892	1.245	0.5355	0.4042
		1905758	A	0.2800	rs6558568	1.056	0.896	1.243	0.5177	0.0097

Gene	Chromosome	Position	MA	MAF	dbSNP SNP_ID	HR	Lower 95% CI	Upper 95% CI	$P_{uncorrected}$	HWE
		1907867	G	0.2389	rs4242546	1.030	0.867	1.225	0.7343	0.4710
ROCK1	18q11.1	16863577	A	0.4686	rs288980	1.036	0.892	1.202	0.6450	0.0013
		16864064	A	0.0425	rs7239317	1.450	1.057	1.987	0.0210	0.3753
		16907607	G	0.4289	rs2127958	0.848	0.728	0.987	0.0334	0.1989
		16909448	A	0.3837	rs1481280	0.775	0.661	0.908	0.0016	0.1882
		16935290	A	0.0427	rs1006881	1.416	1.030	1.946	0.0322	0.6309
		16945763	A	0.0349	rs11874761	1.570	1.128	2.186	0.0075	0.3311
		16954952	G	0.0346	rs10083915	1.451	1.024	2.055	0.0363	0.7653
		16968988	G	0.0391	rs11873284	1.535	1.102	2.138	0.0112	0.8575

Table 3

Haplotype-based Cox regression analysis of the incidence of IsST

Results are adjusted for age, BMI, current smoking, treatment assignment, history of diabetes, hypertension and hyperlipidaemia, and current hormone use. Only haplotypes with a frequency greater than 1% are shown. Haplotype block was defined by Haploview v4.1. 1 denotes the major allele and 2 the minor allele. HF, haplotype frequency.

Gene	Haplotype block	HF	HR (95% CI)	$P_{\text{uncorrected}}$
ROCK1	rs2127958–rs1481280			
	11 (AC)	0.57043	Referent	
	21 (GC)	0.04587	1.710 (0.918–3.183)	*0.0908*
	22 (GA)	0.38316	0.630 (0.456–0.869)	*0.0049*