Determining association of rho kinase 1 gene polymorphisms with risk of Alzheimer's disease: a multicenter pilot study

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Background: In addition to the increasing evidence for a molecular mechanism of rho kinase 1 (*ROCK1*) in Alzheimer's disease (AD), there are several published studies regarding the relationship between *ROCK1* gene polymorphisms and neurological diseases. However, it is unknown whether there is an association between the polymorphisms of *ROCK1* and AD. We sought to identify the potential association between *ROCK1* gene polymorphisms and AD in the Chinese Han population.

Methods: A total of 295 patients with AD and 206 healthy controls from multiple centers were enrolled in this study. Three single-nucleotide polymorphisms (SNPs) (rs35996865, rs11873284, and rs2127958) in *ROCK1* gene were analyzed using Sanger sequencing.

Results: We did not find any significant differences between AD and control groups with regards to the frequency of these three *ROCK1* polymorphisms. Further, the three SNP genotype frequencies and allele frequencies did not show significant differences between patients of AD and controls in *APOE4*-stratified subjects (P>0.01). Additionally, the three SNPs did not show significant differences even when adopting a four-inheritance model by logistic regression.

Conclusions: This is the first multicenter pilot study to evaluate the contribution of *ROCK1* genetic variance to AD risk. Our data demonstrated that the *ROCK1* gene may not influence the risk of AD by interacting with *APOE* among Chinese Han people.

Keywords: Alzheimer's disease (AD); rho-kinase 1; apolipoprotein E (APOE); polymorphism

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Introduction

Alzheimer's disease (AD), the most common dementia, is a genetically complex disease which can be caused by genetic and environmental factors, individually and in interaction with each other. Mutations in three genes (*APP*, *PSEN1*, and *PSEN2*) have been identified to be associated with autosomal dominant forms of familial AD (1). As mutations that cause familial AD, other heritable genetic risk factors also contribute to individual's susceptibilities to AD. For

Table 1 Demographic detail of the sample set

Characteristics of participants	Cases with AD	Controls
Total sample	295	206
Male	116	92
Female	179	114
Age (years)	70.5±10.3	70.9±9.0
MMSE score	14.7±7.2	28.1±2.2

The ages and MMSE scores are means and standard deviations. AD, Alzheimer's disease; MMSE, mini-mental state examination.

example, *Apolipoprotein E* (*APOE*) ε4 allelic variants have been identified (2), and found to be linked to a major increase in the risk for susceptibility to AD in different populations (3). Furthermore, genome-wide association studies (GWAS) have identified more than 20 genetic loci associated with the risk of AD, including *BIN1*, *PICALM*, *CLU*, *CR1*, *MS4A6A* and others (4).

Rho kinases (ROCKs) are serine/threonine kinases first identified as downstream effector of Rho GTPase. *ROCK* genes consist of two paralogs: *ROCK1* and *ROCK2*, sharing 65% homology in amino acid sequences and 92% similarity in kinase domains (5). ROCKs constitute an important intracellular signaling system that participates in the regulation of a multitude of cellular functions, such as proliferation, differentiation, metabolism and apoptosis (6,7). Notably, several studies have suggested that ROCK kinases can induce the processing of APP into the toxic β-amyloid (Aβ) 1-42 peptide and furthermore, inhibitors of ROCKs, such as statins and NSAIDs, can inhibit this toxic APP processing (8,9). *ROCK1* is located on chromosome 18 (18q11.1) and is expressed mainly in hypothalamus and hippocampus. Recent research has found that *ROCK1* is elevated in mild cognitive impairment (MCI) and AD brain, and knockout of the *ROCK1* gene reduced the production of Aβ (10). Similarly, our recent study suggested that ROCK1 increases Aβ clearance by modulating autophagosome formation and is also involved in tau hyperphosphorylation and cytoskeleton disruption by miRNA-146a (11-13). These discoveries elucidated that ROCK1 is involved in modulating metabolism of both Aβ and tau, the constituents of hallmark AD pathologies.

Interestingly, there are several published studies in regard to the relationship between *ROCK1* gene polymorphisms and diseases that threaten the health of people (14-18) With increasing evidence for a molecular mechanism of ROCK1 in AD pathogenesis, *ROCK1* genetic variance as a potential contributor to AD risk is still not completely understood. Here, we put forward a hypothesis that *ROCK1* gene polymorphisms affect potential risk for AD, and we test this with a case-control study (n=501) to determine the prevalence of the common single-nucleotide polymorphisms (SNPs) of *ROCK1* (rs35996865, rs11873284, and rs2127958) among healthy and AD patients in the Chinese Han population, and investigate the association between these polymorphisms and AD risk.

Methods

Study population

A total of 295 AD patients of Chinese Han ethnicity (179 women and 116 men; 70.5±10.3 years old were recruited; the Mini-Mental State Examination (MMSE) score mean for these patients was 14.7±7.2) were enrolled from the outpatient Clinic at the Department of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, and at the Department of Geriatric Psychiatry, Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, and Department of Neurology & Institute of Neurology, Huashan Hospital, Fudan University. All subjects received a detailed neurological examination and underwent a psychiatric interview. The patients had a clinical diagnosis of probable dementia of Alzheimer type according to NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association). Two hundred and six gender, age, and ethnic background-matched non-demented elderly controls (NEC) (114 women and 92 men; 70.9±9.0 years old at the recruitment; average MMSE score, 28.1±2.2) were also recruited. The makeup of male and female subjects did not vary significantly (P>0.05) (*Table 1*). The NEC subjects were free of neurological or psychiatric disorders by medical history, physical examinations, laboratory examinations, and with a MMSE score over 26 (*Table 1*). All AD patients and NEC were unrelated Chinese Han. Informed consent for participation in the study was obtained either directly, or from a guardian of each patient. This study was approved by the Research Ethics Committee, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China.

SNP selection

The preliminary screening criteria for *ROCK1* gene polymorphisms were (I) minor allele frequency >5% in the Chinese Han population; (II) on the basis of previous literature data. This resulted in selection of three SNPs in *ROCK1* (rs35996865, rs2127958, rs11873284) for inclusion in this study.

Blood samples, DNA isolation and genotyping

Genomic DNA was isolated from peripheral blood through standardized phenol/chloroform extraction. Three single nucleotide polymorphisms (rs35996865, rs2127958, rs11873284) and *APOE* were genotyped through PCR using flanking primers and Sanger sequencing. Primers of *ROCK1* were designed according to the RefSeq gene sequence. Optimized primer sequences are listed in supplementary *Table S1*. Primers of *APOE* were used as described in Zivelin *et al.* (19). PCR conditions are available on request.

Statistical analysis

Results are expressed as mean ± SD or percentage as indicated. To compare the differences of mean values between two groups, unpaired Student's *t*-test was used. Goodness-of-fit to the Hardy-Weinberg equilibrium (HWE) and differences in genotype and allele frequencies between the cases and controls were calculated by Chi-squared analysis with Fisher's exact tests. The criterion for significance level was set at a P value of 0.01 for all tests. All probability values were based on two-tailed tests. SPSS was used for statistical analysis. Haplotype analysis was performed by using online software, SHEsis (http://analysis.bio-x.cn/myAnalysis.php), SNPStats (https://www.snpstats.net/snpstats/start.htm?q=snpstats/start.htm) was used to evaluate the association between SNPs and the risk of AD under 4 inheritance models, including codominant, overdominant, dominant, and recessive models.

Results

In the present study, a total of 295 patients with AD and 206 unrelated gender- and age-matched (P>0.05) controls were investigated. *Table 1* shows the clinical features of the study population.

Table 2 shows the distribution of genotypes and alleles for *ROCK1* and *APOE*ε4 between the case and control. No significant deviations from HWE were found for *ROCK1* in two groups. Our study demonstrated no significant differences between groups among three *ROCK1* polymorphisms in allele frequencies and genotype distributions (*Table 2*). Further, the three SNP genotype frequencies and allele frequencies also did not show significant differences between patients of AD and controls in *APOE*ε4-stratified subjects (*Table 3*) (P>0.01).

To further investigate the *ROCK1* genetic association of AD, four-inheritance models were assumed and analysis was performed by logistic regression among ε4-carriers. The three SNPs did not show significant differences adopting

Table 2 Genotype and allele frequencies for *ROCK1* and APOE

Gene	Genotype/alleles	Cases with AD		Controls		P
		n	Total[a]	n	Total[a]	
ROCK1	TT/TG/GG	217/62/4	283	168/36/1	205	0.282
rs35996865	T/G	496/70		372/38		0.128
ROCK1	TT/TC/CC	90/138/59	287	62/107/35	204	0.546
rs2127958	T/C	318/256		231/177		0.705
ROCK1	AA/AG/GG	219/58/4	281	172/33/1	206	0.242
rs11873284	A/G	496/66		377/35		0.100
APOE	(–,–)/(–,+)/(+,+)	164/104/27	295	169/35/2	206	<0.0001
ε4	–/+	432/158		373/39		<0.0001

P, P value (Chi square test or Fisher exact test); n, number; [a], numbers do not always add up to total numbers because of missing values in the sanger sequencing. AD, Alzheimer's disease.

Table 3 Association of *ROCK1* SNPs with AD in *APOE4* stratified samples

APOE	Gene	Genotype/alleles	Cases with AD		Controls		P
			n	Total[a]	n	Total[a]	
ε4-carrier	*ROCK1*	TT/TG/GG	96/30/2	128	27/10/0	37	0.689
	rs35996865	T/G	222/34		64/10		0.959
	ROCK1	TT/TC/CC	35/63/32	130	8/26/3	37	0.036
	rs2127958	T/C	133/127		42/32		0.395
	ROCK1	AA/AG/GG	96/26/3	125	29/8/0	37	0.636
	rs11873284	A/G	218/32		66/8		0.648
ε4-noncarrier	*ROCK1*	TT/TG/GG	121/32/2	155	141/26/1	168	0.375
	rs35996865	T/G	274/36		139/15		0.163
	ROCK1	TT/TC/CC	55/75/27	157	54/81/32	167	0.837
	rs2127958	T/C	185/129		189/145		0.548
	ROCK1	AA/AG/GG	123/32/1	156	143/25/1	169	0.397
	rs11873284	A/G	278/34		311/27		0.204

P, P value (Chi square test or Fisher exact test); n, number; [a], numbers do not always add up to total numbers because of missing values in the sanger sequencing. ε4-carrier, carriers of at least one 4 allele of the *Apolipoprotein E* gene; ε4-noncarrier, subjects not carrying an ε4 allele of the *Apolipoprotein E* gene. AD, Alzheimer's disease; SNPs, single-nucleotide polymorphisms

any of the four-inheritance model (*Table 4*).

Discussion

To the best of our knowledge, this is the first study to investigate the contribution of *ROCK1* gene variants to risk for AD. According to molecular neuropathogenic investigations distinct from GWAS, we put forward a novel hypothesis that ROCK1 variation could account for some risk of developing AD. Unfortunately, our outcomes do not show evidence of *ROCK1* associations to AD risk among AD patients, even when stratified by *APOE* genotype. As the strongest genetic determinant of AD, *APOE*ε4 allele associates with increased incidence of AD, whereas the ε2 allele decreases risk. Studies in humans and transgenic mice showed that APOE regulates Aβ metabolism, aggregation and clearance in the brain in an isoform-dependent manner (20-23). For example, APOEε4 astrocytes eliminate Aβ plaques less effectively than APOEε3 astrocytes (24), and clearance of Aβ by endocytosis is impaired by APOEε4 (25). Interestingly, Iizuka and coworkers found ROCK1 immunoreactivity was distributed in astrocytes and Bergmann glial cells that actually produce APOE in the CNS (26). Again, our previous study shows that ROCK1 colocalizes with highly fibrilized Aβ and that activated ROCK1 inhibits the autophagic clearance of Aβ (12). Both APOE and ROCK1 colocalize with plaque-associated amyloid and glial cells, suggesting a role for ROCK1 in pathogenesis of AD by interacting with APOE4. Follow-up functional studies are still needed to confirm these findings.

Interestingly, two studies have identified ROCK1 as a risk factor of brain diseases. Robert and colleagues investigated the role of *ROCK1* gene variations played in ischemic stroke and found that there were obvious associations between *ROCK1* gene variants (rs2127958, rs11873284) and ischemic stroke in Caucasian women (14). Evidence from clinical studies has shown that ROCK activity was elevated in patients with acute ischemic stroke (27) and inhibition of ROCK activity by statins probably helps to prevent ischemic stroke (28-30). Non-synonymous SNPs (rs111874856, rs112130712, rs112108028, rs73963110) are involved in the risk of Behcet's disease in the Turkish population (15). The frequencies of these four variants are extremely rare to absent in Chinese populations according to the NCBI database. Both studies looked at Caucasians, which have a different genetic background from Asians. Additionally,

Table 4 Four genetic modes of inheritance for the three studied polymorphisms in ε4-carrier (adjusted by gender and age)

ROCK1	Model	OR	95% CI	P
rs35996865	Codominant	1.09 (G/T)	0.36–2.03	0.57
		NA (G/G)	NA	
	Dominant	0.96	0.42–2.25	0.93
	Recessive	NA	NA	0.32
	Overdominant	0.89	0.38–2.08	0.78
rs2127958	Codominant	0.56 (C/T)	0.23–1.41	0.039
		2.37 (C/C)	0.56–9.97	
	Dominant	0.75	0.30–1.83	0.52
	Recessive	3.56	1.00–12.66	0.026
	Overdominant	0.41	0.18–0.91	0.025
rs11873284	Codominant	1.09 (A/G)	0.43–2.76	0.39
		NA (G/G)	NA	
	Dominant	1.29	0.52–3.21	0.58
	Recessive	NA	NA	0.18
	Overdominant	1.11	0.44–2.78	0.83

P, P value; OR, odds ratio; CI, confidence interval; NA, not applicable; codominant, the contributions of both alleles are visible in the phenotype; Dominant, the effect of one allele is visible when being homozygotes or heterozygotes; Recessive, the effect of one allele is visible only when being homozygotes; Overdominant, the phenotype of the heterozygote lies outside the phenotypical range of both homozygous parents.

the *ROCK1* gene polymorphism rs35996865 mapping to the 5'-UTR was significantly associated with colorectal cancer (16), obesity-related metabolic syndrome (17), renal cell carcinoma (18) and respiratory distress syndrome (31), but not AD. Nonetheless, we have detected elevated ROCK1 activity in the peripheral blood of AD patients (unpublished data) and found that ROCK1 plays an important role in pathogenic mechanisms of AD. However, our association study did not show that any of the three SNPs examined increases the risk of AD.

Results from this study may have some implications for future work, in order to clarify the role for increasing AD risk of the *APOE*ε4 allele in association with *ROCK1* gene variants. The transcription level of the *APOE* gene should be considered with *ROCK1* SNP status to determine their potential cooperative role in the progression of AD. Further, the combined effects of APOE4 and ROCK1 on senile plaque clearance and phagocytosis by astrocytes still remains to be examined. One of the limitations of this study is small sample size. Larger sample size and different ethnic groups would be helpful for explaining the involvement of ROCK1 in AD pathogenesis. Moreover, further analysis of the correlations of other polymorphisms in the *ROCK1* gene and clinical manifestations is warranted in future.

In summary, we present here the first multicenter pilot study to evaluate the contribution of *ROCK1* variants to AD risk. Our data demonstrated that the *ROCK1* gene may not influence the risk of AD by interacting with *APOE* among Chinese Han people.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by the Research Ethics Committee, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China (No. 2016-127). Informed consent for participation in the study was obtained either directly, or from a guardian of each patient.

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Supplementary

Table S1 Optimized primer sequences for rs35996865, rs11873284 and rs2127958

SNP	Primer sequences
rs35996865-fw	tct gtt tcc tct ggg ttg ca
rs35996865-rv	tca cct ttc ctc aca cca ca
rs11873284-fw	cca cct tag cct ccc aaa gt
rs11873284-rv	gcc ttc ttt tgg act gtt tgg
rs2127958-fw	tgg cac tag gaa agg aca gtt
rs2127958-rv	agc atc tgg cct aag gga ct

SNP, single-nucleotide polymorphism; fw, forward strand; rv, reverse strand.