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ORIGINAL RESEARCH

Susceptibility of *PON1/PON2* Genetic Variations to Ischemic Stroke Risk in a Chinese Han Population

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Background: Paraoxonases (PONs) are a family of orphan enzymes with multiple functions, including anti-inflammatory, antioxidative, antiatherogenic activities. Studies have suggested that genetic variations in *PON1* and *PON2* are associated with ischemic stroke (IS) risk; however, the conclusion remains unclear in the Chinese population.

Methods: To investigate the susceptibility of genetic variations in *PON1* and *PON2* to risk of IS and its subtypes, this case–control study was carried out on a Chinese population comprising 300 IS patients and 300 healthy controls. Genotypes of six genetic variations in *PON1* and *PON2* were identified with an improved multiplex ligase detection–reaction technique.

Results: *PON1* rs662 was associated with increased risk of IS (CT vs. TT — $OR_{adjusted}$ 1.79, 95% CI 1.08–2.97; p=0.025). Stratified analysis for patients by sex revealed that the significant association of *PON1* rs662 with IS risk was maintained in the male cohort (CT vs. TT — $OR_{adjusted}$ 2.59, 95% CI 1.29–5.21 [p=0.009]; CT/CC vs. TT — $OR_{adjusted}$ 2.03, 95% CI 1.05–3.93 [p=0.036]), but not in the female cohort. Analysis according to IS subtype revealed that *PON1* rs662 genetic variation was an increased risk in the subcohort of patients with large-artery atherosclerosis (CT/CC vs. TT — $OR_{adjusted}$ 2.31, 95% CI 1.09–4.91; p=0.029), but not in patients with other types of IS.

Conclusion: This study suggested that *PON1* rs662 presented a potential risk of IS, especially for males, and this association was more obvious for large-artery atherosclerosis.

Keywords: ischemic stroke, genetic variation, *PON1*, *PON2*

Introduction

Stroke has become one of the main causes of death and disability worldwide: >15 million people suffer ischemic stroke (IS), each year, causing 6 million deaths and 5 million disabilities. It is the second-leading cause of disability and death in the people >60 years old and the fifth-leading cause of death in people aged 15–59 years.[1] IS incidence varies depending on age, sex, race, and genetic factors. Epidemiological studies have revealed several factors, including age, sex, obesity, cigarette smoking, hypertension, diabetes mellitus, atherosclerosis, and dyslipidemias, contribute to the occurrence of stroke.[2–5]

IS, hemorrhagic stroke, and transient ischemic attacks are three main types of cerebrovascular events. Of these, IS occurs as a result of an obstruction within a blood vessel supplying blood to the brain and is the most common form of cerebrovascular disease, accounting for approximately 87% of all stroke cases. Actually, the etiology of IS affects risk of recurrence, disease prognosis, and choices for management. Therefore, the categorization of subtypes of IS based mainly on etiology was developed for the TOAST study,[6] which developed five subtypes of IS: 1) large-artery

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atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, 4) stroke of other determined etiology, and 5) stroke of undetermined etiology.

Studies on stroke etiology have indicated that a complex interaction of genetic and environmental factors contributes to the occurrence of stroke.[7,8] For genetic factors, a number of genes involved in cholesterol metabolism, inflammation, blood coagulation, homocysteine metabolism, and the renin–angiotensin system have been suggested to contribute to the development of stroke.[9] Specifically, genes involved in lipid metabolism have been implicated in the etiology of stroke insofar as the concentration of low-density lipoprotein (LDL) and the oxidation of LDL represent initial events in atherogenesis by producing proatherosclerotic and proinflammatory oxidized lipids, whereas high-density lipoprotein (HDL) in functional form is an atheroprotective factor through its multifunctionality, including reverse cholesterol transport and antioxidant, anti-inflammatory, and antithrombotic effects.

Paraoxonases (PONs), a family of orphan enzymes with multiple activities, are tightly associated with the HDL surface that decreases the peroxidation of LDL and have anti-inflammatory, antioxidative, and antiatherogenic activities. *PON1*, *PON2*, and *PON3* are three known members of this gene family, on the long arm of chromosome 7 between q21.3 and q22.1 in humans. These three types of PONs are antiatherogenic enzymes in terms of their antioxidant activities and inhibiting the oxidation of LDL, along with preventing oxidative modification of the cell membrane.[10] Of these, PON1 is a calcium-dependent glycoprotein associated with HDL particles, and exerts a cardioprotective function through its hydrolyzing effect on LDL-oxidized phospholipids. Studies have revealed that genetic variations in *PON1* can affect its concentration or activity and predict the risk of IS.[11] Genetic variations in the promoter region of *PON1* and the coding regions of *PON1* and *PON2* have been focused on for their susceptibility to IS; however, the results were not consistent,[12] which may be attributed to differences in genetic background among ethnicities. To investigate the susceptibility of genetic variations in *PON1* and *PON2* to the risk of IS and its subtype, this case–control study was carried out on a Chinese cohort.

Methods

Study Subjects

A total of 300 patients were enrolled as cases, and all patients were diagnosed with IS on the basis of clinical symptoms, physical examination, and brain computed tomography or magnetic resonance imaging, independently assessed by a technologist and a physician. All the patients suffered a sudden onset of focal or global neurologic deficit with signs and symptoms persisting for more than 24 h. Patients with a history or occurrence of transient ischemic attacks, hemorrhagic stroke, cerebral trauma, cardiogenic thrombosis, cerebrovascular malformations, coagulation disorders, autoimmune diseases, tumors, peripheral vascular disease, or chronic infection diseases were excluded. According to the criteria and characteristics of the enrolled patients, we divided the patients into four subtypes: 1) large-artery atherosclerosis, 2) cardioembolism, 3) small-vessel occlusion, and 4) stroke of other etiology.

Healthy control subjects were recruited from the Health Medical Center of Nanjing First Hospital during the same period. All these were confirmed as healthy according to the results of routine health examination and matched to cases in terms of age and sex. For the healthy controls, those with history of tumors, autoimmune diseases, genetic diseases, liver ailments, and hematologic diseases were excluded. Demographic characteristics and clinical information — including sex, age, drinking, smoking, diastolic blood pressure (DBP), systolic blood pressure (SBP), diabetes, fasting serum levels of total plasma cholesterol, triglycerides (TGs), HDL, LDL, glucose, and homocysteine — were abstracted from medical records at our hospital. All enrolled participants were heritably unrelated ethnic Han Chinese from the same geographic region: Nanjing City, Jiangsu, China. The protocol of this study was in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Nanjing First Hospital, and written informed consent was obtained from all participants.

SNP Selection and Genotyping

All potential genetic variations in *PON1* or *PON2* associated with risk of IS were retrieved from the National Center for Biotechnology Information dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP), and then potential genetic variations were selected: 1) positioned in exons, promoter regions, 5′UTRs, 3′UTRs, or splice sites; 2) minor-allele frequency ≥5%; and 3) had been reported to be associated with IS risk, previously. Finally, six genetic variations in *PON1* and *PON2* were selected (see Table 1 for details). Blood samples were collected from all participants with EDTA-coated tubes and stored in a refrigerator at −80°C. Total DNA was extracted from whole-blood samples and concentrated

Table 1 Enrolled Genetic Variations

SNP ID	Region	Allele	Chromosome	Position	Gene
rs705381	Promoter	−162A/G	7	94953949	*PON1*
rs854571	Promoter	−832C/T	7	94954619	*PON1*
rs854572	Promoter	−909G/C	7	94954696	*PON1*
rs3735590	3′UTR	+26080 T/C	7	94927495	*PON1*
rs662	Exon 6, Q192R	+16342 C/T	7	94937446	*PON1*
rs7493	Exon 9, C311S	+34610C/G	7	95034775	*PON2*

using a mini whole blood genomic DNA purification kit (GoldMag Xi'an, China) according to the manufacturer's instructions, and then DNA purity was measured with spectrometry (DU530; Beckman Instruments, Fullerton, CA, US).

All six genetic variations selected were genotyped using the improved multiplex ligase detection–reaction technique developed by Genesky Biotech (Shanghai, China). In brief, multiplex polymerase chain reaction was performed to amplify genetic-variation loci. Secondly, amplification products were purified by nuclease and shrimp alkaline enzyme. Finally, a connection the reaction was performed to have each site containing two 5′ terminal allele–specific probes and a 3′ terminal-specific probe of fluorescent tags, and then ligation products were analyzed with an ABI 3730XL. Of all subjects, 10% were randomly selected and subjected to repeated genotyping, and reproducibility of 100% attained.

Statistical Analysis

Differences in demographic characteristics between patients and controls were compared by univariate analysis with the use of Student's *t*-test. Hardy–Weinberg equilibrium in the healthy control group was tested using a goodness-of-fit χ^2 test. Logistic regression was applied to calculate ORs and 95% CIs. The dominant, codominant, and additive models were tested for all genetic variations. $p<0.05$ was considered statistically significant.

Results

A total of 300 patients with IS and 300 age- and sex-matched healthy controls were enrolled in this population-based case–control association study, and their demographic data and clinical characteristics are summarized in Table 2. There were no significant differences with respect to age ($p=0.136$), sex ($p=0.273$), smoking ($p=0.351$), or drinking ($p=0.854$) between the two groups. For clinical characteristics, levels of DBP ($p<0.001$), SBP ($p<0.001$), TGs ($p=0.024$), Glu ($p<0.001$), and homocysteine ($p=0.021$) were significantly higher in patients than in controls. In contrast, levels of HDL in patients were significantly lower than in controls ($p<0.001$), as shown in Table 2. A total of 117 patients were identified as having large-artery

Table 2 Demographic Data and Clinical Characteristics of Patients with Ischemic Stroke and Controls

	Patients, n (%)	Controls, n (%)	*p*-value
Total cases	300	300	
Age (mean ± SD, years)	68.23±11.33	66.95±9.60	0.136
Sex			0.273
Male	181 (60.33)	194 (64.67)	
Female	119 (39.67)	106 (35.33)	
Smoking			
Yes	47 (15.67)	39 (87.00)	0.351
No	253 (84.33)	261 (13.00)	
Drinking			
Yes	15 (5.00)	16 (5.33)	0.854
No	285 (95.00)	284 (94.67)	
SBP (mean ± SD)	142.73±18.86	124.52±13.08	<0.001
DBP (mean ± SD)	83.60±10.68	75.72±8.15	<0.001
TC (mean ± SD)	4.73±1.52	4.65±0.94	0.438
TGs (mean ± SD)	1.47±0.86	1.34±0.57	0.024
LDL (mean ± SD)	2.73±0.85	2.68±0.62	0.405
HDL (mean ± SD)	1.13±0.37	1.39±0.29	<0.001
Glu (mean ± SD)	6.41±2.65	5.41±0.72	<0.001
Hyc (mean ± SD)	16.77±7.23	15.70±3.38	0.021
Clinical stage			
Small-vessel occlusion	56 (18.67)		
Large-artery atherosclerosis	117 (39.00)		
Cardioembolism	28 (9.33)		
Other	97 (32.33)		

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total plasma cholesterol; TGs, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Glu, glucose; Hyc, homocysteine.

Table 3 Genotype Distribution of Polymorphisms in all Participants

	Patients, n (%)	Controls, n (%)	OR (95% CI)	*p*-value
rs705381				
CC	244 (81.33)	231 (77.00)	Reference	
CT	53 (17.67)	63 (21.00)	0.76 (0.50–1.15)	0.194
TT	3 (1.00)	6 (2.00)	0.43 (0.11–1.76)	0.241
CT/TT	56 (18.67)	65 (23.00)	0.74 (0.49–1.10)	0.133
rs854571				
CC	153 (51.00)	152 (50.67)	Reference	
CT	124 (41.33)	121 (40.33)	1.03 (0.73–1.45)	0.861
TT	23 (7.67)	27 (9.00)	0.82 (0.45–1.50)	0.517
CT/TT	147 (49.00)	148 (49.33)	0.99 (0.72–1.37)	0.957
rs854572				
CC	89 (29.67)	93 (31.00)	Reference	
CG	152 (50.67)	145 (48.33)	1.11 (0.77–1.61)	0.581
GG	59 (19.67)	62 (20.67)	0.98 (0.61–1.57)	0.939
CG/GG	211 (70.33)	207 (69.00)	1.07 (0.75–1.51)	0.720
rs3735590				
GG	223 (74.33)	231 (77.00)	Reference	
GA	70 (23.33)	64 (21.33)	1.17 (0.79–1.73)	0.428
AA	7 (2.33)	5 (1.67)	1.28 (0.40–4.15)	0.676
GA/AA	77 (25.67)	69 (23.00)	1.19 (0.82–1.74)	0.365
rs0662				
TT	34 (11.33)	48 (16.00)	Reference	
CT	154 (51.33)	129 (43.00)	1.79 (1.08–2.97)	0.025
CC	112 (37.33)	123 (41.00)	1.47 (0.87–2.50)	0.153
CT/CC	266 (88.67)	252 (84.00)	1.58 (0.98–2.55)	0.063
rs7493				
GG	200 (66.67)	193 (64.33)	Reference	
CG	87 (29.00)	92 (30.67)	0.93 (0.65–1.33)	0.678
CC	13 (4.33)	15 (5.00)	0.85 (0.39–1.85)	0.684
CG/CC	100 (23.33)	107 (35.67)	0.91 (0.65–1.28)	0.602

atherosclerosis, 28 with cardioembolism, 56 with small-vessel occlusion, and 97 with other etiologies (Table 2).

Observed frequencies of all tested genotypes in controls were not derived from the Hardy–Weinberg equilibrium (data not shown). Logistic regression analysis revealed that *PON1* rs662 genetic variation was associated with increased risk of IS (CT vs. TT — $OR_{adjusted}$ 1.79, 95% CI 1.08–2.97; p=0.025), as shown in Table 3. Subgroup analysis of patients stratified by sex revealed a significant association of *PON1* rs662 with IS risk was in the male cohort (CT vs. TT — $OR_{adjusted}$ 2.59, 95% CI 1.29–5.21 [p=0.009; CT/CC vs TT — $OR_{adjusted}$ 2.03, 95% CI 1.05–3.93, [p=0.036]) but not in the of female cohort, as shown in Table 4. Further, we evaluated the susceptibility of genetic variations to risk of subtypes of IS. *PON1* rs662 showed increased risk inpatients with large-artery atherosclerosis (CT/CC vs. TT — $OR_{adjusted}$ 2.31, 95% CI 1.09–4.91; p=0.029) but not in patients with any other type of IS, suggesting the risk of *PON1* rs662 for IS is modified by its type, as shown in Table 5.

Discussion

This population-based case–control association study with 300 paired cases and controls revealed that the *PON1* rs662 genetic variation was potentially associated with increased risk of IS, especially in the male population, and that the susceptibility of *PON1* rs662 to IS risk could be modified by its etiology. We observed that genotypes in controls were not derived from the Hardy–Weinberg equilibrium, indicating controls in this study

Table 4 Genotype Distribution of Polymorphisms in all Participants Stratified by Sex

	Male			Female		
	Patients/controls (%)	**OR (95% CI)[a]**	***p*-value[a]**	**Patients/controls (%)**	**OR (95% CI)[a]**	***p*-value[a]**
rs705381						
CC	151/159 (83.43/81.96)	Reference		93/72 (78.15/67.92)	Reference	
CT	28/33 (15.47/17.1)	0.88 (0.51–1.54)	0.656	25/30 (20.01/28.3)	0.56 (0.30–1.06)	0.073
TT	2/2 (1.10/1.03)	1.07 (0.15–7.82)	0.950	1/4 (0.84/3.77)	0.15 (0.02–1.36)	0.091
CT/TT	30/35 (16.57/18.04)	0.89 (0.52–1.53)	0.681	26/34 (21.85/32.08)	0.50 (0.27–0.94)	0.030
rs854571						
CC	94/107 (51.93/55.15)	Reference		59/45 (49.58/42.45)	Reference	
CT	73/72 (40.33/37.11)	1.20 (0.78–1.85)	0.413	51/49 (42.86/46.23)	0.83 (0.47–1.48)	0.526
TT	14/15 (7.73/7.73)	1.08 (0.49–2.36)	0.852	9/12 (7.56/11.32)	0.59 (0.22–1.58)	0.291
CT/TT	87/87 (48.07/44.85)	1.16 (0.77–1.76)	0.469	60/61 (50.42/57.55)	0.79 (0.46–1.36)	0.391
rs854572						
CC	53/58 (29.28/29.90)	Reference		36/35 (30.25/33.02)	Reference	
CG	89/89 (49.17/45.88)	1.10 (0.68–1.78)	0.687	63/56 (52.94/52.83)	1.12 (0.61–2.07)	0.710
GG	39/47 (21.55/24.23)	0.81 (0.45–1.45)	0.475	20/15 (16.81/14.15)	1.27 (0.55–2.94)	0.569
CG/GG	128/136 (70.72/70.71)	1.00 (0.64–1.56)	0.987	83/71 (69.75/66.98)	1.17 (0.65–2.09)	0.607
rs3735590						
GG	127/148 (70.17/76.29)	Reference		96/83 (80.67/78.3)	Reference	
GA	52/43 (28.73/22.16)	1.39 (0.87–2.23)	0.175	18/21 (15.13/19.81)	0.76 (0.37–1.55)	0.442
AA	2/3 (1.10/1.55)	0.76 (0.12–4.75)	0.769	5/2 (4.20/1.89)	4.32 (0.48–38.95)	0.193
GA/AA	54/46 (29.83/23.71)	1.35 (0.85–2.14)	0.203	23/23 (19.33/21.70)	0.92 (0.47–1.81)	0.807
rs662						
TT	15/31 (8.29/15.98)	Reference		19/17 (15.97/16.04)	Reference	
CT	95/76 (52.49/39.18)	2.59 (1.29–5.21)	0.008	59/53 (49.58/50.00)	1.06 (0.48–2.35)	0.891
CC	71/87 (39.23/44.85)	1.66 (0.82–3.38)	0.159	41/36 (34.45/33.96)	1.29 (0.55–3.04)	0.565
CT/CC	166/163 (91.71/84.02)	2.03 (1.05–3.93)	0.036	100/89 (84.03/83.96)	1.17 (0.55–2.49)	0.684
rs7493						
GG	120/126 (66.30/64.95)	Reference		80/67 (67.23/63.21)	Reference	
CG	53/57 (29.28/29.38)	1.05 (0.66–1.65)	0.851	34/35 (28.57/33.02)	0.73 (0.40–1.32)	0.294
CC	8/11 (4.42/5.67)	0.80 (0.31–2.08)	0.648	5/4 (4.20/3.77)	1.07 (0.24–4.74)	0.929
CG/CC	61/68 (33.70/35.05)	1.00 (0.65–1.55)	0.991	39/39 (32.77/36.79)	0.76 (0.43–1.34)	0.378

Note: [a]Adjusted for age, sex, smoking, and drinking.

were a large, randomly matching population with no selection, genetic drift, migration, or mutation, suggesting the controls we selected were reliable.

PON1 rs662 is a genetic variation in the coding region of *PON1*, causing a missense substitution at position 192 (^{192}Gln [Q]/Arg [R]). Studies have reported that this genetic variation is the major determining factor leading to PON1 activity in that the 192R variant can hydrolyze paraoxonase faster than the 192Q variant.[13–14] Therefore, *PON1* rs662 has been regarded as a risk factor of cardiovascular disease-[15] and IS, although results have often been conflicting.[11] This study based on a Chinese population also reported the *PON1* rs662 R(G) allele was a potential risk factor for IS, consistent with pooled results of published data verifying the association between *PON1* rs662 and stroke risk,[16,17] especially in Asian populations.[12] In addition, we observed that the risk of *PON1* rs662 in IS was more obvious in the male subcohort than the female one, indicating the interaction of sex and *PON1* rs662 contributed to different risk of IS[18,119] and that males with the *PON1* rs662 C allele are at higher risk of IS. Consistently, sex differences, including dyslipidemia, are regarded as predictors of IS,[20] which may contribute to the sex difference in susceptibility of *PON1* rs662 to IS. Despite the limited sample size, we observed the risk of *PON1* rs662 for IS was more obvious in patients with large-artery atherosclerosis, consistent with the result of previous report.[21] Actually, large-artery atherosclerosis-shares a similar etiology with atherosclerosis,[6] and *PON1*

Table 5 Associations Between Genetic Variations and Risk of Types Oflischemic Stroke

	Small-vessel Occlusion		Large-Artery Atherosclerosis		Cardioembolism		Other Etiology	
	OR (95% CI) [a]	*p*-value	OR (95% CI)[a]	*p*-value	OR (95% CI)[a]	*p*-value	OR (95% CI)[a]	*p*-value
rs705381								
CC	Reference		Reference		Reference		Reference	
CT/TT	0.70 (0.33–1.48)	0.353	0.64 (0.36–1.13)	0.125	0.61 (0.23–1.66)	0.333	0.88 (0.50–1.54)	0.647
rs854571								
CC	Reference		Reference		Reference		Reference	
CT/TT	1.04 (0.58–1.86)	0.892	1.05 (0.68–1.62)	0.826	0.91 (0.41–2.04)	0.824	0.88 (0.56–1.40)	0.600
rs854572								
CC	Reference		Reference		Reference		Reference	
CG/GG	1.35 (0.70–2.61)	0.365	1.17 (0.72–1.89)	0.527	0.58 (0.26–1.30)	0.184	1.04 (0.63–1.72)	0.867
rs3735590								
GG	Reference		Reference		Reference		Reference	
GA/AA	1.36 (0.72–2.59)	0.346	1.24 (0.75–2.04)	0.398	1.00 (0.38–2.64)	1.000	0.98 (0.57–1.70)	0.938
rs662								
TT	Reference		Reference		Reference		Reference	
CT/CC	1.16 (0.51–2.62)	0.728	2.31 (1.09–4.91)	0.029	0.71 (0.27–1.84)	0.480	1.76 (0.85–3.66)	0.131
rs7493								
GG	Reference		Reference		Reference		Reference	
CG/CC	0.68 (0.36–1.30)	0.243	1.29 (0.82–2.01)	0.270	0.92 (0.40–2.11)	0.835	0.72 (0.43–1.18)	0.193

Note: [a]Adjusted for age, sex, smoking, and drinking.

rs662 genetic variation presents a risk of atherosclerosis.[22] Therefore, the contradiction of published data regarding the susceptibility of *PON1* rs662 to IS risk may be due to lack of classification of stroke subtypes, which should be verified by further larger studies. To date, few studies have actually discussed the association between *PON1* rs662 polymorphism and risk of IS subtypes. The novelty of this study was that we firstly reported that *PON1* rs662 polymorphism was associated with risk of large-artery atherosclerosis in a Chinese population, although the sample was relatively small.

Three genetic variations in the promoter and one in 3′ UTR of *PON1* were also investigated in this study, and no significant association was observed. Although these genetic variations have been reported to regulate *PON1* expression[11] and contribute to susceptibility to IS,[23,24] in this study we failed to find any association of them to risk of IS, which should be confirmed by further large-sample studies. For *PON1* rs854571, the results of this study are consistent with previous reports.[23–25] The *PON2* rs7493 genetic variation causes a substitution (C311S) on exon 9 and has been reported not to be associated with IS risk in Chinese population.[23–26] Pooled results of published data have also revealed such an association[27] consistent with the results of this study.

In short, this study suggests that *PON1* rs662 is a potential risk of IS, especially for males, and this association is heightened in large-artery atherosclerosis.

Data-Sharing Statement

The data that support the findings of this study are available from the corresponding author Yanping Mei upon reasonable request.

Ethics Statement and Consent

The protocol of this study was in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the Nanjing First Hospital, and written informed consent was obtained from all the participants.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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