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ORIGINAL ARTICLE

Polymorphisms of the *MDR1* and *MIF* genes in children with nephrotic syndrome

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Abstract Oral steroid treatment is the first line of therapy for childhood nephrotic syndrome (NS). Nonetheless, some patients are resistant to this treatment. Many efforts have been made to explain the differences in the response to steroid treatment in patients with NS based on the genetic background. We have investigated single nucleotide polymorphisms of the *MDR1* [C1236T (rs1128503), G2677T/A (rs2032582), and C3435T (rs1045642)] and *MIF* (G-173C, rs755622) genes in 170 children with NS. Of these children, 69 (40.6%) were initial steroid non-responders, and 23 (13.5% of total) developed chronic kidney disease. Renal biopsy findings, which were available for 101 patients, showed that 35 patients had minimal change lesion and 66 had focal segmental glomerulosclerosis. The frequencies of the *MDR1* 1236 CC (18.8 vs 7.2%) or TC (53.5 vs 43.5%) genotype and C allele (45.5 vs 29.0%) were significantly higher in the initial steroid responders than in the non-responders. Analysis of *MDR1* three-marker haplotypes revealed that the frequency of the TGC haplotype was significantly lower in the initial steroid responders than in the non-responders (15.8 vs 29.0%). There was no association between the *MIF* G-173C polymorphism and clinical parameters, renal histological findings, and steroid responsiveness. These data suggest that the initial steroid response in children with NS may be influenced by genetic variations in the *MDR1* gene.

Keywords Childhood nephrotic syndrome · Macrophage migration inhibitory factor gene · Multiple drug resistance 1 gene · Single nucleotide polymorphism

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Introduction

Idiopathic nephrotic syndrome (NS) is one of the most common primary glomerular diseases in children. It can be clinically classified as steroid sensitive (SSNS) and steroid resistant (SRNS) according to the responsiveness to oral steroid treatment, which is the first line of therapy in childhood idiopathic NS. Responsiveness to the initial oral steroid treatment is one of the major prognostic factors of this disease. However, the detailed therapeutic mechanism of steroids on idiopathic NS is currently unknown, as is the pathogenesis.

Strenuous efforts have been made to explain the differences in the response to steroid treatment in patients with NS with various genetic backgrounds. These efforts have focused on the analysis of polymorphisms in a number of

genes, including those coding for angiotensin-converting enzyme (*ACE*) [1–6], cytokines or growth factors [7–18], apolipoprotein E (*APOE*) [19–21], paraoxonase 1 (*PON1*) [22], multiple drug resistance 1 (*MDR1*, also known as *ABCB1*, ATP-binding cassette, sub-family B member 1) [23], and glucocorticoid receptor (*NR3C1*) [24, 25]. However, the results of these studies have not been consistent.

In the study reported here, we have focused on three of the above-mentioned genes, which are known to have some direct functional role in glucocorticoid metabolism: (1) the *NR3C1* (glucocorticoid receptor) gene, (2) the *MIF* gene encoding macrophage migration inhibitory factor (MIF), a proinflammatory cytokine with a unique role as the physiological counter-regulator of the immunosuppressive effects of glucocorticoids [26], and (3) the *MDR1* gene encoding P-glycoprotein (P-gp), which has a function in the elimination of specific corticosteroids from the cytoplasm [27]. In a previous study, we analyzed three *NR3C1* gene polymorphisms [ER22/23EK (rs6189/rs6190), N363S (rs56149945), and *Bcl*I (rs number was not assigned)] in 190 Korean children with NS and 100 Korean control subjects [28]. Variant alleles of 22/23EK and N363S were not found in any of the patients or control subjects, and the *Bcl*I polymorphism was not found to be correlated with the development of NS, onset age, initial steroid responsiveness, renal pathologic findings, or the progression to end-stage renal disease (ESRD). In this study, therefore, the genotypes of the *MDR1* and *MIF* SNPs were identified in children with NS to determine the correlation between the genotypes/allotypes and clinico-pathological features.

Materials and methods

Patients

A total of 170 Korean children diagnosed with idiopathic NS by the Department of Pediatrics, Seoul National University Children's Hospital, Seoul, Korea between 1985 and 2006 were enrolled in this study. NS was defined as massive proteinuria of ≥40 mg/h/m^2 with hypoalbuminemia of ≤2.5 g/dL without known causes [29]. In all patients, oral prednisolone 60 mg/m^2/day or an equivalent dose of deflazacort was administered for 4 weeks at initial presentation, followed by 40 mg/m^2 every other day for 4 weeks. Remission of NS was defined as the absence of proteinuria (≤4 mg/h/m^2 or negative dipstick test) for 3 or more consecutive days. The responsiveness to later treatment with steroids and/or other immunosuppressive or cytotoxic drugs in relapsing patients or initial steroid non-responders could not be evaluated because the treatment modalities for those patients were not uniform. Chronic kidney disease (CKD) was arbitrarily defined as persistently high serum creatinine of ≥2.0 mg/dL.

Patients with a positive family history of NS and patients with a syndromic form of NS were excluded. Mutational analyses of the *NPHS2* and the *WT1* (exons 8 and 9) genes were performed on all patients with SRNS or focal segmental glomerulosclerosis (FSGS), and patients with mutations in either of these genes were excluded. Patients with congenital or infantile NS were also excluded.

Informed consent for the genetic analysis was obtained from all patients and/or their parents.

Genotyping

The genotypes of three known SNPs in the *MDR1* gene (C1236T, G2677T/A, and C3435T) and one SNP in the *MIF* gene (G-173C) were determined by PCR–restriction fragment length polymorphism (RFLP) (Table 1). Genomic DNA was extracted and purified from peripheral blood using a QIA Amp DNA Blood Mini kit (Qiagen, Hilden, Germany), and the PCR products were purified using a QIA Quick PCR Purification kit (Qiagen), following which they were digested with the corresponding restriction enzymes. The digested PCR products were visualized in an ethidium bromide-stained 2.5% agarose gel using a UV camera. The

Table 1 The primer sets and restriction enzymes used for the PCR–RFLP analyses

Gene	Variations (rs number)	Restriction enzymes	Primers used for PCR analysis
MDR1	C1236T (rs1128503)	*Hae*III	F: 5′-TATCCTGTGTCTGTGAATTGCC-3′
			R: 5′-CCTGACTCACCACACCAATG-3′
	G2677T/A (rs2032582)	*Bsr*I/*Bse*YI	F: 5′-TGCAGGCTATAGGTTCCAGG-3′
			R: 5′-CTTAGAGCATAGTAAGCAGTAGGGAG-3′
	C3435T (rs1045642)	*Dpn*II	F: 5′-TCTTGTTTTCAGCTGCTTGATG-3′
			R: 5′-GAAGGCATGTATGTTGGCCT-3′
MIF	G-173C (rs755622)	*Alu*I	F: 5′-CTAAGAAAGACCCGAGGCGA-3′
			R: 5′-GGCACGTTGGTGTTTACGAT-3′

RFLP, Restriction fragment length polymorphism; F, forward primers; R, reverse primers

genotypes of 100 healthy blood donors who comprised the normal control group were also determined.

Analysis of *MDR1* three-marker haplotypes

Three-marker *MDR1* haplotype analysis (C1236T, G2677T/A, and C3435T) was performed using software package PHASE ver. 2.1.1 (available online at: http://www.stat.washington.edu/stephens/software.html) [30, 31].

Statistical analyses

The Hardy–Weinberg equilibrium (HWE) assumption was assessed for both the patient and control groups by comparing the observed numbers of each genotype with those expected under the HWE for the estimated allele frequency. Logistic regression analysis was used to estimate the odds ratios (OR) and 95% confidence intervals (CI) for the association between the genotypes, alleles or haplotypes and the risk of NS or initial steroid responsiveness. The results are presented as the mean values ± 1 standard deviation (SD), and a P value of ≤ 0.05 was considered to indicate statistical significance.

Results

The clinico-pathological profiles of the patients

Of the 170 patients enrolled in this study, 119 (70.0%) were male. The mean age at the onset of NS was 5.17±3.31 years (range 1 year 2 months to approx. 16 years). Sixty-seven (39.4%) patients were resistant to initial steroid treatment, and 23 (13.5% of total) of these progressed to CKD. Renal histological examinations were available for 101 patients; 66 (65.3%) were FSGS patients and 35 (34.7%) were minimal change nephrotic syndrome (MCNS) patients (Table 2).

Table 2 Characteristics of patients with idiopathic nephrotic syndrome (n=170)

Parameter	Number of patients (%)
Age (mean ± SD)	5.17±3.31 years
Male gender	119 (70.0%)
Initial steroid resistance	67 (39.4%)
Progression to chronic renal failure	23 (13.5%)
Renal biopsy findings (n=101)	
Minimal change nephrotic syndrome	35 (34.7%)
Focal segmental glomerulosclerosis	66 (65.3%)

SD, Standard deviation

Influence of genotype on the NS onset in the patients

There was no significant difference in the distribution of genotypes and allotypes in all four SNPs in both genes between the patient group and the control group (Table 3). Also, the onset age of NS was not affected by any genotype or allotype variations in either gene (data not shown).

Influence of genotype on initial steroid responsiveness of the patients

The frequencies of the *MDR1* 1236 CC (18.8 vs 7.2%; OR 4.61, 95% CI 1.53–13.93, P=0.007) or CT (53.5 vs 43.5%; OR 2.19, 95% CI 1.12–4.27, P=0.022) genotype and C allele (45.5 vs. 29.0%; OR 2.05, 95% CI 1.29–3.25, P=0.002) were significantly higher in the initial steroid responders than in the non-responders. The distribution of the genotypes and allotypes of *MDR1* C2677T/A, *MDR1* C3435T, and *MIF* G-173 C was comparable between the groups (Table 4).

Influence of genotype on the renal pathology and the renal functional outcome of the patients

There were no significant differences in genotype and allotype distribution for both genes between patients with MCNS and those with FSGS. In addition, progression to ESRD was not influenced by any of the genotype or allotype variations (data not shown).

Analysis of *MDR1* three-marker haplotypes

Haplotype analysis of three polymorphisms (C1236T, G2677T/A, and C3435T) in the *MDR1* gene revealed five major haplotypes; there was no difference in the distribution of these haplotypes between the patients and the control subjects (Table 5). When each haplotype was controlled for the remaining haplotype set, the frequency of the TGC haplotype in one or both alleles was significantly lower in the initial steroid responders than in the non-responders (15.8 vs 29.0%; OR 0.46, 95% CI 0.27–0.78, P=0.004) (Table 6). Haplotype variations did not influence renal pathology or progression to ESRD (data not shown).

Discussion

In this study, we genotyped four known SNPs in the *MDR1* and *MIF* genes in a group of pediatric patients with idiopathic NS to determine the correlation between the genotypes/allotypes and their clinico-pathological features. Two positive results emerged from this study: (1) the frequencies of the *MDR1* 1236 CC or TC genotype and C allele were

Table 3 Comparison of the genotype distribution in patients and control subjects

Single nucleotide polymorphism	Genotype	Patient group (n=170)	Control group (n=100)	OR (95% CI)	P value
MDR1 C1236T	TT	62 (36.5%)	40 (40%)	1 (reference)	
	TC	84 (49.4%)	49 (49%)	1.11 (0.65–1.88)	0.710
	CC	24 (14.1%)	11 (11%)	1.41 (0.62–3.19)	0.413
	T allele	61.2%	64.5%	1 (reference)	
	C allele	38.8%	35.5%	1.15 (0.80–1.66)	0.441
MDR1 G2677T/A	GG	31 (18.2%)	12 (12%)	1 (reference)	
	TT	23 (13.5%)	17 (17%)	0.52 (0.21–1.31)	0.166
	AA	6 (3.5%)	4 (4%)	0.58 (0.14–2.43)	0.456
	GT	60 (35.3%)	35 (35%)	0.66 (0.30–1.46)	0.307
	GA	21 (12.4%)	15 (14%)	0.54 (0.21–1.39)	0.201
	TA	29 (17.1%)	17 (17%)	0.66 (0.27–1.62)	0.364
	G allele	42.1%	37.0%	1 (reference)	
	T allele	39.7%	43.0%	0.81 (0.55–1.20)	0.296
	A allele	18.2%	20.0%	0.80 (0.49–1.31)	0.374
MDR1 C3435T	CC	75 (44.1%)	35 (35%)	1 (reference)	
	CT	76 (44.7%)	47 (47%)	0.76 (0.44–1.30)	0.308
	TT	19 (11.2%)	18 (18%)	0.49 (0.23–1.05)	0.068
	C allele	66.5%	58.5%	1 (reference)	
	T allele	33.5%	41.5%	0.71 (0.50–1.02)	0.063
MIF G-173C	GG	109 (64.1%)	65 (65%)	1 (reference)	
	GC	59 (34.7%)	32 (32%)	1.10 (0.65–1.87)	0.725
	CC	2 (1.2%)	3 (3%)	0.40 (0.07–2.44)	0.319
	G allele	81.5%	81.0%	1 (reference)	
	C allele	18.5%	19.0%	0.97 (0.62–1.52)	0.892

OR, Odds ratio; CI, confidence interval

significantly higher in the initial steroid responders than in the non-responders, and (2) the frequency of the *MDR1* TGC haplotype was significantly lower in the initial steroid responders than in the non-responders.

The *MDR1* gene encodes a membrane P-gp that functions as an ATP-dependent exporter of xenobiotics from cells [32]. P-gp is expressed in normal tissue, such as the intestine, liver, and kidneys, which have excretory functions, as well as in capillary endothelial cells of the brain, placenta, and testis and in peripheral blood cells [32]. In the kidney, P-gp is expressed in the brush border membrane of proximal tubular epithelial cells [32, 33]. Various types of structurally unrelated drugs, including steroids, are known to be substrates for P-gp [34, 35]. Several studies have been conducted to evaluate the association of P-gp expression with the responsiveness to steroids in patients with idiopathic NS [36–38]. Wasilewska et al. [38] measured P-gp expression on CD3-positive lymphocytes and found that CD3/P-gp expression was significantly higher in patients with NS than in the controls and that the difference was higher in steroid-dependent and frequent relapsing groups than in the non-frequent relapsing group. Funaki et al. [36] reported that the *MDR1* mRNA expression level in nucleated cells of peripheral blood was variable in patients with idiopathic NS prior to remission, but apparently decreased after complete remission. In another study [37], MDR1 activity and mRNA expression in peripheral lymphocytes were higher in patients with steroid-, cyclophosphamide-, or cyclosporine-resistant NS than in patients who were sensitive to those drugs.

To date, approximately 50 SNPs have been reported in the *MDR1* gene [39, 40]. Among these SNPs, C1236T, G2677T/A, and C3435T are the most common variants in the coding region of *MDR1* [41]. While C1236T and C3435T are synonymous SNPs, G2677T/A causes an amino acid substitution (Ala899Ser/Thr). These three SNPs are in strong linkage disequilibrium, and we identified five major haplotypes (TTT, CGC, TGC, CAC, and TTC) in the patients and control groups. This haplotype distribution is similar to that reported in other studies conducted in Korean or Chinese subjects [42, 43]. Many studies have revealed that some SNPs of the *MDR1* gene result in changes in P-gp expression and function among different ethnicities and subjects, with most of the attention focused on the *MDR1*

Table 4 Comparison of genotypes among the initial steroid responders and initial steroid non-responders

Single nucleotide polymorphism	Genotype	Responders (n=101)	Non-responders (n=69)	OR (95% CI)	P value
C1236T in *MDR1*	TT	28 (27.7%)	34 (49.3%)	1 (reference)	
	TC	54 (53.5%)	30 (43.5%)	2.19 (1.12–4.27)	0.022
	CC	19 (18.8%)	5 (7.2%)	4.61 (1.53–13.93)	0.007
	T allele	54.5%	71.0%	1 (reference)	
	C allele	45.5%	29.0%	2.05 (1.29–3.25)	0.002
G2677T/A in *MDR1*	GG	17 (16.8%)	14 (20.3%)	1 (reference)	
	TT	11 (10.9%)	12 (17.4%)	0.75 (0.26–2.23)	0.610
	AA	4 (4.0%)	2 (2.9%)	1.65 (0.26–10.36)	0.595
	GT	34 (33.7%)	26 (37.7%)	1.08 (0.45–2.58)	0.868
	GA	13 (12.9%)	8 (11.6%)	1.34 (0.43–4.14)	0.613
	TA	22 (21.8%)	7 (10.1%)	2.59 (0.86–7.82)	0.092
	G allele	40.1%	44.9%	1 (reference)	
	T allele	38.6%	41.3%	1.05 (0.65–1.69)	0.848
	A allele	21.3%	13.8%	1.73 (0.92–3.26)	0.087
C3435T in *MDR1*	CC	42 (41.6%)	33 (47.8%)	1 (reference)	
	CT	48 (47.5%)	28 (40.6%)	1.35 (0.70–2.59)	0.371
	TT	11 (10.9%)	8 (11.6%)	1.08 (0.39–2.99)	0.882
	C allele	65.3%	68.1%	1 (reference)	
	T allele	34.7%	31.9%	1.13 (0.72–1.80)	0.595
G-173C in *MIF*	GG	67 (66.3%)	42 (60.9%)	1 (reference)	
	GC	33 (32.7%)	26 (37.7%)	0.80 (0.42–1.51)	0.486
	CC	1 (1.0%)	1 (1.4%)	0.63 (0.04-10.29)	0.744
	G allele	82.7%	79.7%	1 (reference)	
	C allele	17.3%	20.3%	0.82 (0.47–1.43)	0.490

C3435T SNP. Although this SNP is a synonymous variation, it is associated with altered protein expression [35]. The mechanism by which the *MDR1* C3435T SNP affects P-gp expression may be through possible linkage disequilibrium between the C3435T SNP and other *MDR1* variants that control expression, including the nonsynonymous G2677T/A and synonymous C1236T SNPs [40]. In addition, the *MDR1* C3435T SNP has been known to result in functional alteration of P-gp by affecting the timing of cotranslational folding and insertion of P-gp into the membrane, thereby altering the structure of substrate and inhibitor interaction sites [44].

There has been only one study [23] conducted to evaluate *MDR1* SNPs in children with idiopathic NS. In that study, Wasilewska et al. evaluated *MDR1* C1236T, G2677T/A, and C3435T SNPs in 108 children with idiopathic NS [23] . The results showed that the frequencies of the minor alleles in these three SNPs (1236 TT, 2677 TT, and 3435 TT) and the TTT haplotype were higher in late responders to oral prednisone (time to remission >7 days) than in early responders (time to remission <7 days). These researchers also observed the presence of three minor alleles and the 2677 TT genotype were more frequently in frequent relapsers than in non-frequent relapsers; there was

Table 5 Comparison of the haplotype distribution of the *MDR1* gene in patients and control subjects

Haplotype	Patients (n=170)	Control (n=100)	OR (95% CI)	P value
TGC	21.2%	18.0%	1.22 (0.78–1.91)	0.373
TTC	8.8%	5.5%	1.66 (0.81–3.40)	0.159
TTT	36.5%	30.3%	0.76 (0.52–1.09)	0.137
CGC	18.5%	18.0%	1.04 (0.66–1.63)	0.878
CAC	17.6%	15.5%	1.17 (0.73–1.88)	0.520

The order of the single nucleotide polymorphism (SNP) loci in the haplotype is *MDR1* C1236T, G2677T/A and C3435T, and each haplotype was controlled for the remaining haplotype set

Table 6 Comparison of the haplotype distribution of the *MDR1* gene in the initial steroid responders and the initial steroid non-responders

Haplotype	Responders (n=101)	Non-responders (n=69)	OR (95% CI)	P value
TGC	15.8%	29.0%	0.46 (0.27–0.78)	0.004
TTC	7.4%	10.9%	0.66 (0.31–1.39)	0.272
TTT	30.2%	30.4%	0.99 (0.62–1.58)	0.963
CGC	20.8%	15.2%	1.46 (0.82–2.60)	0.194
CAC	20.8%	13.0%	1.75 (0.96–3.19)	0.066

The order of the SNP loci in the haplotype is *MDR1* C1236T, G2677T/A and C3435T, and each haplotype was controlled for the remaining haplotype set

no genotype–phenotype correlation in other clinical features, including the age of NS onset, the use of cytotoxic drugs, and total dose of steroids. However, the influence of the genotypes/allotype on the steroid responsiveness could not be evaluated because all patients in their study had SSNS. In contrast, our study found that the presence of the *MDR1* 1236 CC genotype and the C allele predicted a better initial response to steroids in children with idiopathic NS. Unfortunately, we could not compare early and late responders because the exact date of remission was unclear in some patients.

One of the more recent issues attracting the attention of researchers is the association of *MDR1* genetic variation with the development and progression of certain diseases, especially those that involve impaired cellular barrier function at the level of the blood–brain barrier (Parkinson's disease) [45, 46], small intestine (ulcerative colitis) [47], or kidney (non-clear cell renal carcinoma) [48]. Most of these studies [46–48] have revealed that the frequency of the *MDR1* 3435 TT genotype and/or T allele was significantly higher in patients with these diseases than in control subjects. Such an increased disease risk is explained by a lower P-gp expression and/or impaired tissue barrier function in patients with the TT genotype or T allele, which results in an ineffective protection of the body from potential environmental and metabolic toxins [49, 50]. Nonetheless, in our study, the genotype and the allele distribution of three *MDR1* SNPs, including C3435T, were not significantly different between the patient and the control groups, suggesting that the expression level of P-gp may not be an important pathogenetic factor in idiopathic NS in children.

In a Chinese study [51] of 244 patients with ESRD, *MDR1* 3435 TT homozygotes were found to have a significantly higher serum creatinine level than CC homozygotes. This finding suggests that a low renal P-gp expression in 3435 TT homozygotes could lead to the exposure to higher concentrations of toxic agents, resulting in increased susceptibility to their effects. However, in our study, progression to ESRD was not influenced by any of the genetic variations studied.

We studied three *MDR1* SNPs in the coding region. However, nucleotide and/or haplotype variants not only in the coding region but also in the promoter region of the *MDR1* gene may be important for interindividual differences in P-gp expression [52]. In addition, the methylation status of certain CpG sites on the *MDR1* promoter and/or other epigenetic mechanisms play a critical role in switch-on or -off of *MDR1* gene expression [53].

MIF is expressed in glomerular parietal and visceral epithelial cells and in tubular epithelial cells in the kidney [51]. This factor plays important physiological roles in the regulation of macrophage function, lymphocyte immunity, and endocrine functions [18], as well as a pathogenetic role in some immunologically induced kidney diseases [26, 54–56]. Glucocorticoid counter-regulates MIF expression; low glucocorticoid level up-regulates MIF expression, while a high glucocorticoid level down-regulates it [26]. The association of *MIF* G-173C SNP with the progression of disease and response to steroid treatment in childhood NS has been studied in several recent studies [17, 18]. Vivarelli et al. [17] genotyped *MIF* G-173C in 257 Italian children with idiopathic NS and found that the frequency of the C allele (high producer) was higher in patients than in controls and also higher in patients with SRNS than with SSNS. This difference was particularly evident in patients with FSGS. In addition, carriers of the C allele were found to have a significantly higher probability of ESRD when compared with GG homozygous patients. Similarly, Berdeli et al. [18] investigated the *MIF* G-173C SNP in 214 children with idiopathic NS and found that the frequencies of the GC genotype and C allele were higher in the patients than in the control subjects. They also found an increased CC genotype frequency in patients with SRNS compared to those with SSNS, as well as an increased C allele frequency in patients with FSGS compared to other histopathological groups. Both the CC genotype and C allele were detected more frequently in patients with ESRD. Furthermore, age of onset was younger in patients with the CC genotype than in patients with the GC and GG genotypes. These findings suggest that the *MIF* -173C allele confers an increased risk of susceptibility to childhood idiopathic NS and plays a

crucial role in steroid responsiveness. However, in our study, the *MIF* G-173C SNP was not associated with disease susceptibility, renal pathology, steroid responsiveness, or renal functional outcome in Korean children with idiopathic NS.

In summary, we found that the frequencies of the *MDR1* 1236 CC or TC genotype and C allele were significantly higher in the initial steroid responders than in the non-responders and that the TGC haplotype in *MDR1* was significantly lower in the initial steroid responders than in the non-responders in Korean children with idiopathic NS. These findings suggest that the *MDR1* 1236 CC genotype and C allele may be a predictor for better initial steroid responsiveness. Further studies focusing on *MDR1* mRNA and protein expression in these patients are needed to confirm a correlation.

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