

MDR1 Gene Polymorphisms Are Associated with Glucocorticoid-Induced Avascular Necrosis of the Femoral Head in a Chinese Population

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P-glycoprotein (P-gp) is present in various tissue cells, required for the pumping of lipophilic drugs (including glucocorticoids) out of cells. We hypothesized that polymorphisms in the P-gp encoding gene (multidrug-resistant transporter-1 [*MDR1*]) are related to individual differences in glucocorticoid sensitivity and the development of glucocorticoid-induced avascular necrosis of the femoral head (GANFH). In this case-control study, we genotyped three known single-nucleotide polymorphisms (SNPs: C1236T, G2677T/A, and C3435T) within the *MDR1* gene in 662 Chinese subjects. Statistically significant differences between GANFH patients and either healthy controls or glucocorticoid-resistant patients (non-GANFH) were found for the T allele or TT genotype of C3435T. The haplotype TTT, composed of these three SNPs, exhibited a significant association with the disease. No associations were identified between C1236T or G2677T/A and GANFH. Our results suggest that the C3435T polymorphism of the *MDR1* gene is associated with susceptibility to GANFH in a Chinese population.

Introduction

AVASCULAR NECROSIS of the femoral head (ANFH) occurs when the bone tissue undergoes necrosis and the bone collapses, occurring in a particular anatomical site of the femoral head. ANFH may induce hip joint dysfunction and partial or complete loss of the ability to walk. Glucocorticoid therapy is considered as one of the most common risk factors of ANFH. However, it cannot be ignored that, while some people develop osteonecrosis others do not, under the same conditions. This phenomenon suggests that susceptibility to glucocorticoid-induced avascular necrosis of the femoral head (GANFH) and individual genetic factors are closely related. Specific single-nucleotide polymorphisms (SNPs) have previously been considered accountable for the susceptibility to ANFH (Jones and Hungerford, 2007). These SNPs are located in the genes encoding for plasminogen activator inhibitor-1 (PAI-1), methylenetetrahydrofolate reductase (MTHFR), apolipoprotein B (APOB), collagen type II alpha 1 (COL2A1), among others (Glueck *et al.*, 1999; Zalavms *et al.*, 2002; Liu *et al.*, 2005; Hirata *et al.*, 2007). However, it still remains unclear exactly which gene polymorphisms are responsible for GANFH.

Multidrug-resistant transporter-1 (*MDR1*) is a house-keeping gene, located on human chromosome 7 band q21.1. This gene spans 4.5 kb encoding a 1280 amino acid (aa) polypeptide. A 170-kDa of protein named P-glycoprotein (P-gp) can be formed after glycosylation of the amino acid polypeptide. P-gp is present in various tissue cells, required for pumping lipophilic drugs (including glucocorticoids) out of the cells. This causes the intracellular drug concentration to decrease and cytotoxic damage is subsequently reduced. In addition, P-gp can indirectly regulate other genes such as cytochrome p450 CYP450, which also has an important role in drug absorption, distribution, and metabolism (Pal and Mitra, 2006).

Almost 30 SNPs have been identified in the gene region of *MDR1*. The most widely studied SNPs are C1236T (exon 12, rs1128503), G2677T/A (exon 21, rs2032582), and C3435T (exon 26, rs1045642). The polymorphisms C1236T and C3435T do not change the amino acid, while G2677T/A induces the replacement of amino acid residues (alanine 893–serine 893 or alanine 893–threonine 893). These three SNPs have previously been shown to be associated with both disease development and drug response in different populations (Balcerczak *et al.*, 2010; Ozen *et al.*, 2011; Chen *et al.*, 2012).

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Chinese osteonecrosis cases account for more than half of the new cases in the world (Pei, 2010). Whether or not genetic factors increase GANFH disease susceptibility in Chinese is unclear, with controversy surrounding some reported results (Gong *et al.*, 2013). The present study aimed to investigate the frequency of the C1236T, G2677T/A, and C3435T polymorphisms of the *MDR1* gene, and furthermore, their association with GANFH disease susceptibility in a Chinese population.

Materials and Methods

Subjects

In total, 105 unrelated patients who had been diagnosed with GANFH were enrolled into the GANFH group. GANFH patients were undergoing GANFH treatment in the Bone-setting Hospital of Luoyang and Taizhou Hospital of Zhejiang Province between October 2010 and March 2013. These patients had received standard glucocorticoid therapy after being diagnosed with systemic lupus erythematosus or acute lymphoblastic leukemia or having had organ transplantation (average doses of glucocorticoid were 1400 ± 200 , 1500 ± 400 , and 1300 ± 500 mg of prednisone, respectively). The development of GANFH was diagnosed according to glucocorticoid therapy history; assessment by X-rays, magnetic resonance imaging (MRI), and bone scans (Sugano *et al.*, 2002; Zhang and Li, 2007). Two hundred seventeen patients who were confirmed to have no osteonecrosis in the hip, knee, shoulder, or ankle joints more than 1 year after using >2000 mg of prednisone were considered as the steroid-resistant group. Three hundred forty healthy blood donors were considered as the healthy control group. All individuals from the steroid-resistant and control groups were of the same ethnicity (Han) and geographical origin, and were living in the same region as the patients with GANFH (Central and Southeast China). All enrolled individuals in these three groups were aged between 18 and 48 years, and individuals who had a history of heavy drinking (alcohol intake >1000 mL/week) were excluded. This study was approved by the Ethics Committees of the Faculty of Medicine (Bone-setting Hospital of Luoyang and Taizhou Hospital of Zhejiang Province, China), and informed consent was obtained from all subjects before blood sampling. The informed consent obtained from patients and controls also collected basic personal data, for example, name, age, sex, race, place of origin, telephone number, and address.

Genotyping

Polymorphisms were detected by polymerase chain reaction (PCR) followed by direct sequencing. Genomic DNA

was extracted from blood samples using the salting-out method, as previously described (Rousseau *et al.*, 1994). PCR primers for individual SNPs are shown in Table 1. PCR amplifications were carried out in 25 μ L volumes containing ~ 30 ng genomic DNA, 0.3 mM upstream and downstream primers, 200 mM dNTPs, and 0.5 units Taq polymerase (Sangon Biotech, Shanghai, China). PCR conditions were as follows: 5 min at 94°C followed by 30 cycles of 45 s at 94°C, 45 s at 57–59°C, and 30 s at 72°C (Table 1). Amplified products were purified with the EZ-10 Spin Column PCR Product Purification Kit (Sangon Biotech, Shanghai, China). Sequencing reactions were performed using the BIG Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI, Foster City, CA). The sequenced products were purified by ethanol precipitation. The DNA pellet obtained was then dissolved in 4 μ L of loading dye (deionized formamide; Applied Biosystems, Foster city, CA) and analyzed on an Applied Biosystems Genetic Analyzer 3100 (PE Applied Biosystems).

Statistical analysis

Chi-square (χ^2) tests were used to compare allele and genotype distribution of the GANFH patients against their respective controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated. *p*-Values <0.05 were considered statistically significant. Hardy–Weinberg equilibrium (HWE) was assessed using a χ^2 -test in each control group and r^2 values were calculated to evaluate the magnitude of linkage disequilibrium (LD). SNPstats software (<http://bioinfo.iconcologia.net/SNPstats>) and SPSS 15.0 (SPSS, Chicago, IL) software were used for all statistical analyses.

Results

Clinical characteristics of patients and control subjects are illustrated in Table 2. Analysis included 340 healthy controls, 217 patients who were receiving glucocorticoid therapy, but did not develop GANFH (glucocorticoid resistant) and 105 GANFH patients (glucocorticoid sensitive). The mean age was 35.0 ± 10.4 years (range 18–48 years) in the healthy control group, 38.9 ± 8.4 years (range 20–48 years) in the glucocorticoid-resistant control group, and 36.3 ± 9.1 years (range 18–48 years) in the GANFH group. Females constituted 49.1% of the healthy control group, 51.6% of the glucocorticoid-resistant control group, and 41.9% of the GANFH group. Mean age and gender did not differ among these three groups ($p > 0.05$).

TABLE 1. SEQUENCES OF POLYMERASE CHAIN REACTION PRIMERS

SNPs	Primers (5'-3')	Annealing Temp. (°C)	Extension time (s)	Size (bp)
C1236 T	CACTTCAGTTACCCATCTCG GGTCATAGAGCCTCTGCATC	58	30	316
G2677 T/A	AGCAAATCTTGGGACAGGA GTCCAAGAAGCTGGCTTTGC	57	30	352
C3435 T	TGTTTTTCAGCTGCTTGATGG AGGCATGTATGTTGGCCTC	59	30	196

GenBank accession numbers were NC_000007.13 used as the reference sequence. SNP, single-nucleotide polymorphism.

TABLE 2. CHARACTERISTICS OF GANFH PATIENTS AND CONTROLS

	GANFH group (n = 105)	GC-resistant group (n = 217)	Healthy control group (n = 340)	p
Age, years range (mean ± SD)	18–48 (36.3 ± 9.1)	20–48 (38.9 ± 8.4)	18–48 (35.0 ± 10.4)	0.298 ^a
Gender: female, no. (%)	44 (41.9)	112 (51.6)	167 (49.1)	0.259 ^b
Body mass index (mean ± SD)	22.5 ± 3.1	23.8 ± 3.0	23.7 ± 3.6	0.247 ^a
Type of underlying disease				
Organ transplantation, no. (%)	27 (25.7)	72 (33.2)	ND	0.174 ^c
Systemic lupus erythematosus, no. (%)	33 (31.4)	50 (23.0)	ND	0.107 ^c
Acute lymphoblastic leukemia, no. (%)	18 (17.1)	52 (24.0)	ND	0.164 ^c
Others, no. (%)	27 (25.8)	43 (19.8)	ND	0.229 ^c

^ap-Value among three groups, for ANOVA test.

^bp-Value among three groups, for χ^2 -test.

^cp-Value between GANFH group and glucocorticoid-resistant group, for χ^2 -test.

GANFH, glucocorticoid-induced avascular necrosis of the femoral head; GC, glucocorticoid; ND, not determined; χ^2 , chi-square.

Genotypic and allelic frequencies of SNPs in GANFH patients, glucocorticoid-resistant patients, and healthy controls are shown in Table 3. The genotyping failure rate was 2.4–6.5% across the SNPs. The genotypes of each respective SNP in healthy control individuals were all in HWE ($p > 0.05$). The T allele of C3435T was significantly associated with GANFH (OR = 0.70; 95% CI, 0.50–0.98, GANFH group vs. healthy control group, OR = 0.56; 95% CI, 0.39–0.80, GANFH group vs. glucocorticoid-resistant group, respectively) (Table 3). Moreover, significant associations were observed between the TT genotype of C3435T and GANFH (OR = 0.36; 95% CI, 0.15–0.85, the GANFH group vs. healthy control group, OR = 0.23; 95% CI, 0.09–0.54, the GANFH group vs. glucocorticoid-resistant group, respectively) (Table 3). However, no significant differences between patients and control groups were observed in either allele or genotype distribution of C1236T and G2677T/A ($p > 0.05$) (Table 3).

SNP loci 1236, 2677, and 3435 were found to be in LD. The strength of pairwise LD between the three SNPs was as follows: 1236 and 2677, $r^2 = 0.42$; 1236 and 3435, $r^2 = 0.67$; 2677 and 3435, $r^2 = 0.73$. ($r^2 > 0.33$ was considered as thresholds of strong LD). A significantly higher occurrence of the haplotype TTT was found in the GANFH group than in the glucocorticoid-resistant group or healthy control group (OR = 0.72; 95% CI, 0.43–0.94, GANFH group vs. glucocorticoid-resistant group, OR = 0.67; 95% CI, 0.30–0.89, GANFH group vs. healthy control group, respectively) (Table 4).

The allele distribution in the three groups showed a linear trend (Fig. 1). The frequency of the T allele of C3435T was significantly lower in the GANFH group than both the healthy control group and glucocorticoid-resistant group ($p < 0.05$) (Fig. 1). Although not statistically significant, the same trends were found for the T allele of C1236T and G2677T/A (Fig. 1).

Discussion

Here we performed a case–control study to investigate the role of *MDR1* SNPs C1236T, G2677T/A, and C3435T in GANFH susceptibility. Our results suggest that SNP C3435T may be a risk factor for the development of GANFH in the Chinese population. Whereas no significant associations of SNPs C1236T and G2677T/A with GANFH susceptibility were found, loci C1236T, G2677T/A, and C3435T were in LD. The TTT haplotype, composed of these loci, may de-

crease the susceptibility to GANFH when compared with other haplotypes.

In the present study, the homozygosity of both the wild-type and variant alleles of *MDR1* (C1236 T, G2677T/A, and C3435T) in healthy control subjects was similar to that reported by other Asian studies, which were performed with healthy Chinese (Zhang *et al.*, 2008; Qiu *et al.*, 2012), Japanese (Komoto *et al.*, 2006), and Korean subjects (Choi *et al.*, 2007). However, the allele frequencies were significantly higher than those observed in European ethnicities, such as German (Cascorbi *et al.*, 2001), Russian (Gaikovitch *et al.*, 2003), and Serbian (Milojkovic *et al.*, 2011). *MDR1* C3435T has previously been reported to be associated with susceptibility to GANFH in a Chinese population with systemic lupus erythematosus (Yang *et al.*, 2007) and a Japanese population with kidney transplantation (Asano *et al.*, 2003). The current study verifies this finding in a larger Chinese population with underlying conditions that had not previously been studied in this context. Therefore, *MDR1* C3435T polymorphism and its respective association to susceptibility to GANFH may be consistent among different populations. However, further studies are required to confirm possible associations in other populations, especially non-Asians, since the allele distributions seem to vary significantly between Oriental and non-Oriental groups.

In our study, *MDR1* C1236T was not found to be associated with GANFH susceptibility. SNP G2677T/A was once found to have a weak association with GANFH in a Chinese population with systemic lupus erythematosus (Yang and Xu, 2007) and a Japanese population with kidney transplantation (Asano *et al.*, 2003). However, despite our larger sample size, we found no association ($p = 0.15$). These results suggest that C1236T and G2677T/A may have less functional influence on the *MDR1* gene than C3435T on GANFH susceptibility.

It has been widely accepted that glucocorticoids may induce blood to enter a hypercoagulable hypofibrinolysis state. This blood state mediates femoral vein thrombosis, resulting in bone ischemia, necrosis of the bone tissue structure, and function damage. However, individuals differ in their abilities to absorb and metabolize glucocorticoids, which may affect GANFH development. The membrane transporter protein P-gp, encoded by the *MDR1* gene, plays an important role in drug absorption and distribution. Therefore, we selected *MDR1* C1236T, G2677T/A, and C3435T, the most widely studied functional SNPs, to investigate a possible

TABLE 3. ALLELE AND GENOTYPE FREQUENCIES OF POLYMORPHIC VARIANTS OF THE *MDR1* GENE IN GANFH PATIENTS AND CONTROLS

SNP ID	Position	Allele and genotype	Frequency						GANFH vs. healthy control group		GANFH vs. GC-resistant group	
			GANFH group, n (Freq)		Healthy control group, n (Freq)		GC-resistant group, n (Freq)		p-Value	OR (95% CI)	p-Value	OR (95% CI)
			n (Freq)	n (Freq)	n (Freq)	n (Freq)	n (Freq)	n (Freq)				
rs1128503	C1236 T	CC	15 (0.16)	48 (0.15)	21 (0.10)	1	1	1	1	1	1	
		CT	44 (0.46)	144 (0.44)	92 (0.44)	0.95	0.98 (0.50–1.91)	0.30	0.67 (0.32–1.42)			
		TT	37 (0.38)	135 (0.41)	97 (0.46)	0.71	0.88 (0.44–1.74)	0.10	0.53 (0.25–1.15)			
rs2032582	G2677 T/A	T	118 (0.61)	414 (0.63)	286 (0.68)	0.64	0.92 (0.66–1.29)	0.11	0.75 (0.52–1.07)			
		GG	24 (0.24)	63 (0.19)	36 (0.17)	1	1	1	1			
		GT/A	49 (0.49)	170 (0.51)	109 (0.52)	0.34	0.76 (0.43–1.34)	0.21	0.67 (0.36–1.25)			
rs1045642	C3435 T	T/AT/A	27 (0.27)	99 (0.30)	64 (0.31)	0.30	0.72 (0.38–1.35)	0.19	0.63 (0.32–1.26)			
		T/A	103 (0.52)	368 (0.55)	237 (0.57)	0.33	0.85 (0.62–1.17)	0.22	0.81 (0.58–1.14)			
		CC	43 (0.43)	121 (0.37)	65 (0.32)	1	1	1	1			
		C/T	49 (0.50)	154 (0.46)	91 (0.45)	0.65	0.90 (0.56–1.44)	0.44	0.81 (0.49–1.37)			
		T/T	7 (0.07)	55 (0.17)	47 (0.23)	0.016 ^a	0.36 (0.15–0.85)	<0.001 ^a	0.23 (0.09–0.54)			
T	63 (0.32)	264 (0.40)	185 (0.46)	0.038 ^a	0.70 (0.50–0.98)	0.001	0.56 (0.39–0.80)					

^aFor χ^2 -tests, $p < 0.05$.

OR, odds ratios; CI, confidence intervals; n, number of alleles or genotypes; Freq, frequency; *MDR1*, multidrug-resistant transporter-1.

TABLE 4. HAPLOTYPE OF POLYMORPHIC VARIANTS OF THE *MDR1* GENE IN GANFH PATIENTS AND CONTROLS

C1236 T	G2677 T/A	C3435 T	Frequency						GANFH vs. healthy control group		GANFH vs. GC-resistant group	
			GANFH group, Freq		Healthy control group, Freq		GC-resistant group, Freq		p-Value	OR (95% CI)	p-Value	OR (95% CI)
			group, Freq	group, Freq	group, Freq	group, Freq	group, Freq	group, Freq				
T	T	T	0.26	0.35	0.41	0.03 ^a	0.72 (0.43–0.94)	0.007 ^a	0.67 (0.30–0.89)			
T	G	C	0.23	0.21	0.19	0.86	1.03 (0.54–1.57)	0.55	1.14 (0.61–1.45)			
C	G	C	0.24	0.17	0.15	0.24	1.18 (0.68–1.59)	0.10	1.23 (0.74–1.67)			
C	A	C	0.15	0.12	0.10	1	1	1	1			

^aFor χ^2 -tests, $p < 0.05$.

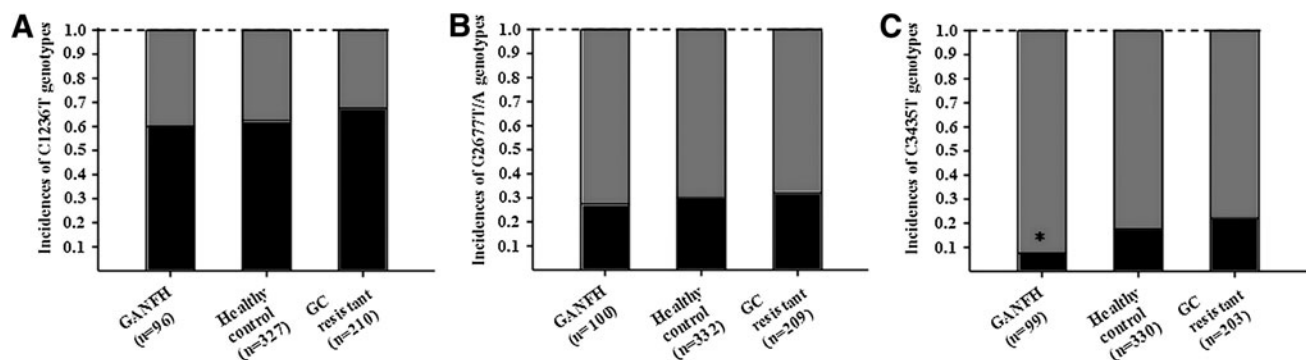


FIG. 1. Incidences of C1236T, G2677T/A, and C3435T genotypes in the glucocorticoid-induced avascular necrosis of the femoral head (GANFH) patients and control groups. (A) C1236T, black indicates TT genotype and gray indicates CC and CT genotypes; (B) G2677T/A, black indicates T/AT/A genotype and gray indicates GG and GT/A genotypes; (C) C3435T, black indicates TT genotype and gray indicates CC and CT genotypes; * for chi-square (χ^2) tests, $p=0.016$, GANFH versus healthy control group; $p<0.001$, GANFH group versus GC-resistant group.

association with femoral head necrosis. Our results showed a significant association of C3435T with GANFH in a Chinese population. We speculate that the 3435TT genotype reduces the incidence of GANFH by enhancing the pump activity of P-gp to clear the excess glucocorticoid out of cells. Our speculation is partially supported by Asano *et al.* (2003) who reported that patients with 3435TT genotype have a significantly higher dose/concentration ratio of tacrolimus than the 3435CC genotype, indicating enhanced P-gp pump activity of the 3435TT genotype in a Japanese population.

To obtain a more exact conclusion of the study, our experiments were somewhat different from others. First, subject to difficulty in acquiring the experimental samples, the previous studies only contained a small number of GANFH samples (no more than 30 cases), which may have some influence to statistical conclusions. We therefore obtained > 100 GANFH cases by using the plentiful case resources in China, which we believe has allowed us to conduct more conclusive analyses. Second, the previous studies often contain only a single underlying disease (such as systemic lupus erythematosus) with femoral head necrosis posthormone treatment. This situation presents a risk in terms of genetic association: is the genetic association related to GANFH or to the pathogenesis and special treatment of the underlying disease itself? Our study encompassed multiple underlying diseases in the hope of eliminating the influence of a single underlying disease on the result of any genetic association with GANFH.

In addition, age across both our cases and control subjects ranged from 18 to 48 years, ruling out factors such as senile osteoporosis (naturally increased post-middle age) and postmenopausal osteoporosis in females, which can both affect femoral head necrosis. Patients with a history of heavy drinking were also excluded to eliminate any effects due to alcohol. These efforts are useful in eliminating any background noise in our GANFH cases. Previous research has only contained the glucocorticoid-sensitive (GANFH) and glucocorticoid-resistant (no GANFH) groups, whereas we added the random healthy control group whose glucocorticoid sensitivity may lie between the levels of the other two groups. Our results showed that the T allele of the C3435T showed a linear trend increase in the glucocorticoid-sensitive group (GANFH), healthy control group, and glucocorticoid-resistant group (no GANFH). Therefore, we have confirmed

from a unique angle that the *MDR1* gene polymorphisms effect glucocorticoid sensitivity, with downstream effects on GANFH diseases.

Due to difficulties and challenges in defining the history of glucocorticoid treatments for underlying disease, especially when patients discharged from hospitals, exact doses of glucocorticoid used for some patients were not identified in this study. However, we obtained an average dose of glucocorticoid used in the patients according to hospital records and careful consultation with the patients. Another limitation was that complex phenotypes, polygenic diseases, or both could not be completely explained by SNPs in one single gene, as the SNPs in other genes were possibly involved in the availability of glucocorticoid metabolism.

In short, *MDR1* C3435T polymorphism may be a risk factor for susceptibility to GANFH in a Chinese population and could potentially be used to predict the development of GANFH.

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Author Disclosure Statement

No competing financial interests exist.

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