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Original Article

A linkage and association analysis study in the multidrug resistance gene 1 (MDR1) in renal patients

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Abstract: Several investigations demonstrated that the polymorphisms of multidrug resistance gene *(MDR1*) gene contribute to interindividual variability in bioavailability and tissue distribution of its substrates. Genotyping of closely spaced single-nucleotide polymorphism (SNP) markers frequently yields highly correlated data, owing to extensive linkage disequilibrium (LD) between markers. The product of multidrug resistance gene (P-gp) is an important molecule, which regulating the bioavailability of many drugs, including calcineurin inhibitors. It also reported that some MDR1 gene polymorphisms (such as 3435C>T) was associated with significantly reduced intestinal P-gp expression in T/T homozygotes. The aim of this study is to develop genotyping assays for polymorphisms of the *MDR1* gene, which are believed to have functional properties and to assess the distribution of variant alleles in renal patients (UK Caucasoid). A total of ten polymorphisms in the MDR-1 gene were selected for analysis. Haplotype assays were performed by using EH programme in 172 individuals. The following possible haplotype was apparent (G-41, C-145, C-129, C+139, C+1236, G+2677, G+2956, C+3435, C+4030 and A+4036). This finding suggests the importance of haplotype assignment for the *MDR1* gene.

Keywords: MDR, gene, single-nucleotide polymorphism, linkage disequilibrium, haplotype, immunosuppressive

Introduction

P-glycoprotein/MDR1/ABCB1 is a drug transporter that is classified into the ATP-binding cassette (ABC) protein family. ABC transporters have conserved catalytic domains for ATP hydrolysis and actively pump out substrates against a concentration gradient in an ATP-dependent fashion [1, 2].

The *MDR1* gene is located at chromosome 7q21 [3] and consists of a core promoter region and 28 exons ranging in size from 49 to 209 bp encoding an mRNA of 4.5 kb [4].

The human MDR1 mRNA encodes a polypeptide with 1280 amino acids which has an apparent molecular weight of 170 kDa. The protein is defined as having two halves, each containing six hydrophobic trans-membrane domains, and an ATP binding domain. The two halves are separated by a flexible linker region, and the two ATP-binding domains are structurally similar. All 12 trans-membrane domains are found in the plasma membrane [5].

P-gp is expressed in the apical membrane of cells with excretory functions, such as those in the liver, kidney, small intestine, stomach, and the blood–brain barrier [6].

The physiological expression of P-gp in tissues is one of the determinants for drug detoxification in various cells, and thus provides a cellular defence mechanism against potentially harmful compounds [7, 8]. This protein also mediates the transfer blocking of hydrophobic xenobiotics across the placenta [9] and prevention of the entry of substrates into the central nervous system as a part of the blood–brain barrier [10, 11].

Genetic variants of *MDR1* can influence interindividual variability in the bioavailability and pharmacokinetics of various drugs [12]. For example, Hoffmeyer et al. reported that

3435C>T was associated with significantly reduced intestinal P-gp expression in T/T homozygotes in comparison with subjects homozygous for C allele (C/C), leading to higher steady-state plasma concentrations after the oral administration of digoxin. However, a large number of the subsequent human studies shown the inconsistent observations even with the same probe drugs and among the same disease/racial populations [12, 20-23].

Of the more than 50 exonic single nucleotide polymorphisms (SNPs) in MDR1 [13-15], SNPs 1236C>T (exon-12), 2677G>T/A (exon-21), and 3435C>T (exon-26) occur in different populations at high (>0.1) but various frequencies [16, 17]. These three SNPs are in strong but varied linkage disequilibrium (LD) in different populations, accounting for the two most common haplotypes (1236C–2677G–3435C, and 1236T–2677T–3435T) [13, 15, 18]. To date, detailed linkage disequilibrium analysis of the different polymorphisms of the MDR1 gene within different population/ethnics have been documented (such as Japanese, Asian and Caucasians) [30-32].

3435C>T, in combinations with 1236C>T and/or 2677G>T, has been found in some investigation to play a key role in modifying the function of MDR1. Although the mechanism of this effect is not fully understood at the molecular level, evidence suggests that 3435C>T affects the timing of P-gp cotranslational folding, which results in changes in substrate specificity [13, 19].

Numerous studies have investigated the influence of polymorphisms on side effects of and clinical responses to important drugs, especially anti-neoplastic agents, antidepressants and immunosuppressants. Furthermore, a large number of studies have been investigated the associations between *MDR1* polymorphisms and susceptibility to or the etiology of several diseases like: Parkinson's disease, epilepsy, depression, SLE, inflammatory bowel diseases, cancers and renal disease (cirrhosis and nephritic syndrome), gingival hyperplasia, rheumatoid arthritis, hypertension [24] and FMF (Familial Mediterranean fever) [25].

Genetic epidemiology studies of unrelated subjects are now widely used to study the role of genetic susceptibility and gene-environment interactions in the etiology of complex diseases such as end stage renal patients. These environmental factors could be smoking, blood pressure, age, race, diet and treating the distribution of the environmental exposures might be completely inclusive in our results therefore the environmental factors are our study limitation.

The calcineurin inhibitors, cyclosporine and tacrolimus are immunosuppressant drugs used for the prevention of organ rejection following transplantation. Both agents are metabolic substrates for cytochrome P450 (CYP) 3A enzymes, in particular, CYP3A4 and CYP3A5, and are transported out of cells via P-glycoprotein [30]. To determine the influence of *MDR1* SNPs on the pharmacokinetics of calcineurin inhibitors, we recruited 172 UK renal allograft recipients receiving maintenance treatment with either cyclosporine or tacrolimus. The following SNPs of *MDR1* were chosen for analysis in this study due to their high degree of heterozygosity or their potential for functional significance in Caucasoid populations. Those are included, the non-coding SNPs G-41A, G-145C, C-129T (5´-untranslated region), A4036G and C4030G (3´-untranslated region), C139T (intron 6) and the coding SNPs C1236T (Gly412Gly), G2677T (Ala893Ser), G2956 (Met986Val) and C3435T (ILe1145ILe).

Analysing SNPs or marker haplotypes for candidate genes and assessing their frequencies in cases are likely to be the most informative and rigorous way of performing association studies. To gain absolute confidence in defining such haplotypes in cases, it is necessary to construct haplotypes from segregation analysis of family material. Unfortunately, this is seldom available and other mathematical based methods have been devised to estimate these haplotypes and their frequencies. These are usually based on maximum likelihood calculations and the programme used in this study (EH) followed such procedures. For such estimates to be useful, a reasonably large data set is required.

Materials and methods

172 renal allograft recipients visiting the outpatient clinic of the Manchester Royal Infirmary were recruited into the study (45±5 years). During routine visits, blood samples were

Table 1. Estimates of haplotype frequencies for MDR1 (-41/-129/-145)

Allele		Association
-41/-129	GC	0.58[1]
-41/-145	GG	0.57[2]
-129/-145	CG	0.63[3]
-41/-145/-129	GCG	0.45[4]

[1]χ^2=0.62, D=0.01, D_{max}= 0.16, D'=0.08, L=-173.20, p=0.8918; [2]χ^2=40.53, D=-0.03, D_{max}=0.05, D'=0.60, L=-159.76, p=0.0001; [3]χ^2=0.36, D=-0.01, D_{max}=0.04, D'=0.03, L=-156.50, p=0.9484; [4]χ^2=33.8, L=-243.71, p=0.0001, DF=7.

Table 2. Estimates of haplotype frequencies for MDR1 (1236/2677/3435)

Allele		Association
1236/3435	CC	0.32[1]
2677/3435	GC	0.34[2]
1236/2677	CC	0.37[3]
1236/2677/3435	CGC	0.30[4]

[1]χ^2=7.33, D=0.07, D_{max}=0.16, D'=0.44, L=-162.99, DF=3, p=0.0621; [2]χ^2=50.08, D=0.16, D_{max}=0.23, D'=0.70, L=-144.94, DF=3, p=0.0001; [3]χ^2=12.97, D=0.1, D_{max} = 0.17, D'=0.55, L=-156.31, DF=3, p=0.0047; [4]χ^2=67.68, L=-215.88, p=0.0001, DF=7.

drawn for the determination of drug trough concentrations, as well as for genotyping. Purified genomic DNA was extracted from EDTA anticoagulated peripheral blood using GenoM™-6 robotic DNA extraction (Genovision, Norway). An ARMS-PCR technique was developed for MDR-1 genotyping. In brief, 100ng genomic DNA was used in a reaction volume of 25µl containing 10x NH_4Cl buffer, 1.5mM $MgCl_2$, 0.2mM each deoxyribonucleoside triphosphates, 0.3ρmole each forward and reverse primer (including hGh primers as internal control) and 1U *Taq* DNA polymerase. PCR primers designed Using primer3 web software. Thermal cycling conditions were: 95°C for 5 minutes; 10 cycles of 1 minute at 94°C, 1 minute at 62°C, 1 minute at 72°C; 20 cycles of 1 minute at 95°C, 1 minute at 57°C and 1 minute at 72°C; followed by a final extension at 72°C for 5 minutes. Amplification was monitored by electrophoresis of 10µl PCR product in 2% agarose gel stained with ethidium bromide.

The expected allele frequency for each genotype was compared with the population frequency obtained using Hardy-Weinberg Equilibrium analysis. Allele frequencies obtained were compared using Chi-Squared and 3x2 contingency tables, using Fisher's Exact Probability test. Potential haplotype frequencies were estimated using the Estimated Haplotype (EH) programme. Pair wise linkage disequilibrium was calculated based on Centre for Genetic Improvement of Livestock (CGIL) informatics web site at the University of Guelph, Ontario, Canada.

Results

We investigated the ten SNPs for any linkage disequilibrium (LD) to determine whether they were randomly associated in the same patient. Pair wise linkages between SNPs were not randomly distributed, and only two blocks of linkages were observed (defined as ≥10% occurrence). Those are included, the non-coding SNPs G-41A, G-145C, C-129T (5´-untranslated region) as first group and the coding SNPs C1236T (Gly412Gly) in exon 12, G2677T (Ala893Ser) in exon 21 and C3435T (ILe1145ILe) in exon 26, as the second block. It is interesting that the association of C139T (intron 6), G2956 (Met986Val) in exon 24, A4036G and C4030G (3´-untranslated region) were never observed with any of these LD blocks. Estimated Haplotype analysis of renal diseases populations revealed that the "G" allele at G-41A and "G" allele at G-145C were inherited in strong linkage (D'=60%, p=0.0001 and χ^2=40.53) but when the SNP C-129T was included as a third potential allele, the "GGC" haplotype frequency reduced to 45% in transplant recipients (**Table 1**). Estimated Haplotype analysis of renal diseases populations revealed that the "G" allele at G2667T and "C" allele at C3435T were inherited in strong linkage (D'=70%, p=0.0001 and χ^2=50.08) but when the SNP C1236T was included as a third potential allele, the "CGC" haplotype frequency reduced to 30% in transplant recipients (**Table 2**). Conversely, a mutant allele in the exon 12 SNP was usually associated with mutant alleles in the exon 21 and 26 SNPs. **Table 3** shows the genotype distribution of the ten *MDR1* SNPs in the 172 renal transplant recipients recruited into the study. Genotype frequencies for each SNP were in Hardy-Weinberg equilibrium. In the patient population, an increased frequency of the coding variant allele at G2677T was observed. More than 55% of the patients exhibited the heterozygosity for variant allele "T" at position G2677T, and more than 30% were "TT" homozygotes. The silence exonic variants, C1236T, C3435T and the non-coding position

Table 3. MDR1 polymorphisms in post transplant renal patients

		N=172	%
MDR-1 -41			
Genotype	G/G	143	83.1
	G/A	25	14.5
	A/A	4	2.4
Allele	G	311	90.4
	A	33	9.6
MDR-1 -145			
Genotype	G/G	154	89.5
	G/C	14	8.1
	C/C	4	2.4
Allele	G	322	93.6
	C	22	6.4
MDR-1 -129			
Genotype	C/C	143	83.1
	C/T	21	12.2
	T/T	8	4.7
Allele	C	307	89.2
	T	37	10.8
MDR-1 +139			
Genotype	C/C	68	39.5
	C/T	81	47.1
	T/T	23	13.4
Allele	C	217	63.1
	T	127	36.9
MDR-1 +1236			
Genotype	C/C	65	37.8
	C/T	80	46.5
	T/T	27	15.7
Allele	C	210	61.1
	T	134	38.9
MDR-1 +2677			
Genotype	G/G	36	20.9
	G/T	81	47.1
	T/T	55	32
Allele	G	153	44.5
	T	191	55.5
MDR-1 +2956			
Genotype	G/G	146	84.8
	G/A	17	10
	A/A	9	5.2
Allele	G	309	89.8
	A	35	10.2
MDR-1 +3435			
Genotype	C/C	29	16.9
	C/T	69	40.1
	T/T	74	43
Allele	C	127	36.9
	T	217	63.1
MDR-1 +4030			
Genotype	C/C	165	95.9
	C/G	5	2.9
	G/G	2	1.2
Allele	C	335	97.4
	G	9	2.6
MDR-1 +4036			
Genotype	A/A	97	56.4
	A/G	60	34.9
	G/G	15	8.7
Allele	A	254	73.8
	G	90	26.2

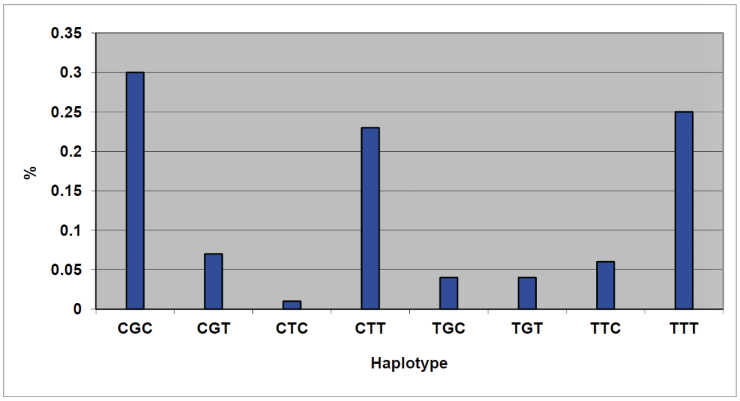

Figure 1. MDR1 haplotype for (1236/2677/3435) in renal cases.

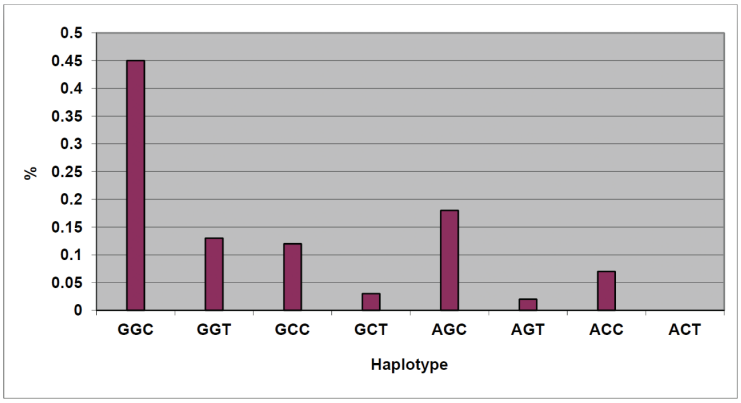

Figure 2. MDR1 haplotype for (-41/-145/-129) in renal cases.

of A4036G, were also observed with increased frequency, with homozygosity for these mutations in 38.9%, 63.1% and 26.2% of patients, respectively. Comparison of the linkages between the three LD blocks with the correlation coefficient showed that the block one of SNPs was not significantly associated with the genotypes at any of the positions of the other SNPs group. The intragenic recombination was performed on the Caucasian variant data sets. Intragenic recombination appeared to be minimal throughout the *MDR1* gene and similar in this sample population. The following possible haplotype was apparent (G-41, C-145, C-129, C+139, C+1236, G+2677, G+2956, C+3435, C+4030 and A+4036). Linkage disequilibrium was evaluated for the Caucasian sample population. And this LD between site pairs by a Fisher's exact test is shown in **Figure 1** and **2**. The highest linkage disequilibrium (D'=0.70) occurred between the two SNPs in the common haplotype *MDR1* at the second block. Linkage disequilibrium between the two SNPs of block2 occurred across distances ranging of 758 bp.

Discussion

Drug transporters are increasingly recognized as a key determinant of drug disposition and response [27]. The multidrug resistance gene-1 (*MDR1*, adenosine triphosphate-binding cassette transporter: *ABCB1*, P-glycoprotein) encodes membrane proteins that play a crucial role in protecting cells from xenobiotics, chemicals, and drugs [28]. It is now widely appreciated that expression of *MDR1* gene in organs such as the gastrointestinal tract, liver and kidney significantly alters the extent of drug absorption and excretion [29]. Moreover, expression of *MDR1* at the level of the blood–brain barrier limits the entry of many drugs into the central nervous system [6]. Given such an important role of MDR1 in the drug disposition process, it is not surprising to see increasing focus on the role of single nucleotide polymorphisms (SNPs) in this transporter as a potential determinant of intersubject variability in drug disposition. There is increasing evidence that gene-based haplotype approaches that take into account the combination of SNPs present in an allele make it easier to predict changes in response to drugs than SNP-based approaches [18]. Therefore the aim of this study is to develop genotyping assays for polymorphisms of the *MDR1* gene, which are believed to have functional properties and to assess the distribution of variant alleles in Caucasoid population.

All of the allele frequencies were in the Hardy–Weinberg equilibrium. The LD between 10 polymorphisms was analyzed pair wise for the value of Chi Square and the data indicated that a very high LD was found in the region covering the two SNPs G667T and C3435T. From these findings, the *MDR1* gene was divided into two blocks. Those are included, the non-coding

SNPs G-41A, G-145C, C-129T (5´-untranslated region) as first group and the coding SNPs C1236T (Gly412Gly) in exon 12, G2677T (Ala893Ser) in exon 21 and C3435T (ILe1145ILe) in exon 26, as the second block. Among 10 SNPs, strong associations were found only for the two pairs of G2677T/C3435T and G-41A/G-145C. Therefore, the *in vitro* effects of isolated mutations are difficult to evaluate because of the high frequency of the SNPs and the close linkage disequilibrium detected between the exon 21 and 26 SNPs. This prompts the pharmacogenetic studies to perform a multipoint haplotype determination of the *MDR1* gene to identify the associations between the genomic variations represented by each haplotype and immunosuppressive drugs dose requirements.

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