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Multiple Drug Resistant1 Gene C3435T Polymorphism and its Relation to Digoxin Blood Level in Cardiac Patients

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ABSTRACT

Background: The MDR1 gene was the first ABC transporter gene described. Genetic variations of MDR1 gene have over than 48 single nucleotide polymorphisms. Polymorphism in exon 26 C3435T results in 3 genotypes; homozygous genotype CC, homozygous genotype TT, and heterozygous genotype CT. It is a silent mutation, but is associated with altered P-glycoprotein expression, and subsequently its substrate drug pharmacokinetics. The objectives were detection of the MDR1 gene C3435T polymorphism at exon 26, the frequency of each genotype, and its relation to digoxin blood level in cardiac patients under digoxin therapy. Forty cardiac male patients were chosen after exclusion of factors affecting serum digoxin level or predisposing to digoxin toxicity. Their ages ranged from 12 to 60 years. The patients were either newly diagnosed cases who received digoxin for the first time or chronic cardiac cases on digoxin therapy. Following selection of cases measurements of serum digoxin level after reaching the steady state concentration and PCR-RFLP for detection of MDR1 C3435T polymorphism were performed. The frequency of MDR1 C3435T genotypes was TT genotype in 20 cases (50%), CT genotype in 18 cases (45%) and CC genotype in 2 cases (5%). This frequency was in concordance with Hardy-Weinberg equilibrium. It was found that there was significant difference in serum digoxin levels between patients with TT and CT genotypes, and also between patients with TT and CC genotypes. The TT genotype was the most

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frequent among cardiac patients of the current sample. There is variation in the digoxin level according to the MDR1 genotypes. This may imply the need to consider this genotype polymorphism in monitoring digoxin level among cardiac patients.

Key words: *Digoxin, MDR1 gene, C3435T, polymorphism.*

INTRODUCTION

Different patients respond in different ways to the same medication. Some individuals can be especially sensitive to the effects of a particular drug, whereas others can be quite resistant.[1] The existence of large population differences with small intra-patient variability is consistent with inheritance as a determinant of drug response. These differences in drug response are due to sequence variants in genes encoding drug-metabolizing enzymes, drug transporters, or drug targets.[2,3] One of the most studied drug transporters is the P-glycoprotein (P-gp), a member of ATP-binding cassette (ABC) family of membrane transporters. It is a product of human multiple drug resistance 1 gene (MDR1); it plays an important role in drug efflux.[4-6]

The MDR1 gene was the first ABC transporter gene described.[7,8] It is located at chromosome 7p21, it comprises 28 exons, and it spans 200 kb.[8,9] It encodes a 170-kDa integral membrane protein called P-glycoprotein (P-gp) that regulates the transport of a large span of substrates.[10,11]

Genetic variations of MDR1 gene have been studied reporting over than 48 single nucleotide polymorphisms (SNPs).[4,11,12] The polymorphism in exon 26 (C3435T) is extensively studied. It is a silent polymorphism, which does not change the encoded amino acid but is associated with altered P-glycoprotein expression, and subsequently

substrate drug pharmacokinetics. Three genotypes have been identified: homozygous genotype CC, homozygous genotype TT, and heterozygous genotype CT.[13] Individuals with the CC genotype have higher levels of P-gp expression compared with individuals with the TT genotype who have a reduced expression of the protein. Heterozygous have intermediate expression levels.[14]

Digoxin is one of the most commonly used drugs in medicine. Despite its widespread use and a history of over 200 years of clinical use and research, much controversy continues concerning its efficacy and safety.[15] The principle clinical uses of digoxin are in the treatment of congestive heart failure and in the treatment of supraventricular tachycardia.[16]

Therapeutic drug monitoring (TDM) for digoxin was introduced more than 30 years ago, and resulted in a marked reduction in the incidence of digoxin toxicity.[17] The narrow margin between toxic and non-toxic serum digoxin concentrations, coupled with substantial variability among patients, has lead to the routine use of serum digoxin concentration to guide therapy.[18]

Digoxin is an important substrate for P-gp. In the intestine P-gp limits oral absorption of digoxin and exports it back into the intestinal tract subsequent to its passive diffusion, also P-gp transports digoxin out of the renal tubular cells and liver canalicular cells into the lumen of the renal tubules and liver canaliculi to be excreted in the urine and the bile respectively.[19]

The influence of MDR1 SNP (C3435T) on disposition of P-gp substrates or treatment outcome has been exemplified in digoxin.[20] Most of the studies were conducted on healthy volunteers after a single

dose of digoxin,[21-26] however; the effect of (C3435T) polymorphism on patients maintained on digoxin therapy was not fully investigated.

Our aim in this study is the detection of the MDR1 gene C3435T polymorphism at exon 26, the frequency of each genotype, and its relation to digoxin blood level in cardiac patients under digoxin therapy.

SUBJECTS AND METHODS

This study was carried out on 40 cardiac male patients; all of them were inhabitants of Alexandria and admitted to the cardiology unit in "Alexandria Main University Hospital". Their ages ranged from 12-60 years. The studied cases were categorized into 2 groups; newly diagnosed cases receiving digoxin therapy for the first time for at least 1 week prior to sampling and chronic cardiac cases receiving digoxin therapy for more than 6 months. The daily digoxin dose was maintained at 0.25 mg/day after reaching the steady state for digoxin concentration. For the majority of cases (75%), digoxin was prescribed for rate control of atrial fibrillation, while in 25% of patients; it was given for, control of heart failure.

Their body mass index was less than 25 kg/m^2 to exclude obesity as a confounding factor. Patients suffering from renal impairment, liver impairment, chronic obstructive pulmonary diseases, thyroid disorders, diabetes mellitus, gastrointestinal tract disorders, and electrolytes disturbance were excluded. In addition; patients receiving concomitant drugs known to affect serum digoxin level as: Amiodaron, Verapamil, Niefedipine, Diltiazem, Quinidine, Propafenone, Captopril, Carvidilol, Triamterene, Salbutamol, Macrolides, Tetracycline, Indomethacin,

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Alprazolan, Itraconazol, Rifampan, Sacrolfate, Cholystyramine, or Cyclosporine were excluded.

All the patients were evaluated thoroughly with special stress on exclusion criteria that was further confirmed by radiological examinations and laboratory tests. Tests for selection of cases included fasting blood sugar,[27] renal function tests; blood urea nitrogen and serum creatinine,[28] liver function tests; alanine aminotransferase (ALT) and aspartate aminotransferase (AST),[29] serum sodium and potassium,[30] and serum TSH.[31]

All included cases were subjected to measurement of serum digoxin trough level (before the daily dose and not less than 8 hours after administration of the last dose of the drug). The digoxin assay was performed using electrochemi-luminescence immunoassay in Roche Elecsys 2010 immunoassay analyzers.[32]

To detect MDR1 C3435T polymorphism PCR amplification of the targeted area was carried out, followed by digestion of the amplified sequence (PCR- RFLP) by Sau3A1 restriction enzyme. First DNA was extracted from buccal epithelial cells using 5% chelex-100 resin suspension (Bio-Rad) (It binds ions and protects DNA from degradation).[33] PuReTaq Ready-To-Go PCR Beads (Amersham, Bioscience, UK. Product code: 27-9557-02) were used in amplification reaction using the following primers:

MDR1-E26f : GAT CTG TGA ACT CTT GTT TCT A.

MDR1-E26r : GAA GAG AGA CTT ACA TTA GGC.

For each reaction the following were added to PCR bead contained in 0.2 ml sterile tube;18 µl of autoclaved double distilled water, 1 µl of forward (26f) primer, 1 µl of reverse (26r) primer (each 20pM/µl), and 5

µl of extracted genomic DNA .The reaction mixture (25µl total volume) was then subjected to the following PCR protocol; Initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of; denaturation at 94ºC for 1 minute, annealing at 55ºC for 1 minute, and extension at 72ºC for 1 minute, then final extension at 72ºC for 10 minutes using (Techne thermal cycler TC-312). The expected size of the amplified product is 244 base pair (bp) that involves a Sau3A1 restriction site.

Ten µl of the amplified PCR products were digested by addition of 0.5 µl of Sau3A1 (equivalent to 2 units) specific for the sequence (A↓GACT). When mutation occurs, T substitutes C (A GATT) so the site of action of the enzyme is lost. The reaction mixture was incubated at 37ºC for 2 hours.[33]

The amplified PCR products were detected by submarine electrophoresis through 2% Nusieve agarose gel and visualized by UV transilluminator after staining with ethidium bromide.[33]

Interpretation

The digested PCR products (244 bp) are expected to produce either 2 fragments of 172 and 72 bp in homozygous CC genotype or 3 fragments of 172, 72, and 244 bp in heterozygous CT genotype. Homozygous TT remains undigested due to loss of restriction site with amplified product of 244bp. (Figure 1)

Statistical analysis

SPSS version 11.0 was used for statistical analysis. χ^2 test and one way analysis of variance (ANOVA) were used to test for significant differences. Pearson correlation coefficient (r) was used to test the correlation between digoxin level and age.

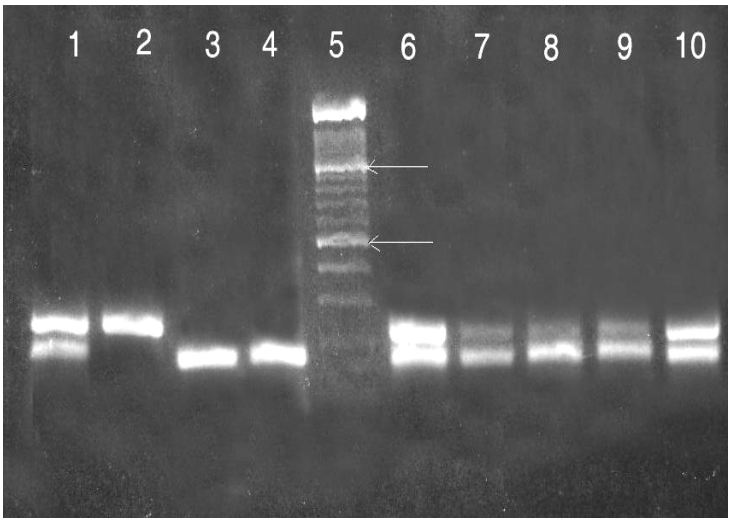

Figure 1: Ethidium Bromide Stained 2% Nusieve Agarose Gel Illustrating the Fragments Size Generated by Sau3A1 Enzyme of PCR Product of MDR1 Gene.

Lane 5 represents 100 bp ladder with intense band at 1000 bp and 500 bp (Arrows). Lane 2 represent homozygous TT genotype, it contains 1 undigested band at 244 bp. Lanes 1, 6, 7, 8, 9 and 10 show heterozygous CT genotype, containing 2 bands at 172, and 244 bp. Lane 3 and 4 represent homozygous CC genotype; they contain 1 band at 172 bp. The small cut-fragment 72 bp is not seen.

Testing for Hardy-Weinberg equilibrium

To test the observed genotype frequencies for Hardy-Weinberg equilibrium, the expected frequencies of the different genotypes were calculated and were compared with the observed frequencies using Chi square goodness of fit test.[34] The proportion of allele T was considered as p, while that of the C allele was considered q. The expected proportion of each was calculated according to the following:

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[2 X observed frequency of the homozygous genotype + observed frequency of the heterozygous genotype]/ 2 X total sample

Accordingly

Expected p = [2Xfrequency of TT+frequency of TC]/2Xtotal sample

Expected q = [2 frequency of CC+frequency of TC]/2Xtotal sample

The expected proportions were used to calculate the expected frequencies p^2, q^2, and 2pq which represent TT, CC and CT genotypes expected frequencies for Hardy-Weinberg equilibrium.

RESULTS

Distribution of C3435T MDR1 genotypes among studied cases

Homozygous TT genotype had the highest frequency observed in 20 cases (50%), followed by the heterozygous CT genotype in 18 cases (45%), and homozygous CC genotype which was detected in 2 cases (5%). The corresponding expected frequencies of these genotypes if the population is in Hardy-Weinberg equilibrium were 52.5, 40 and 7.5% respectively. There was no statistical significant difference between the observed and the excepted frequencies of the genotypes using χ^2 goodness of fit test (Table 1).

Table (1): Genotype Frequency (Observed & Expected) of MDR1 C3435T Polymorphism among the Studied Cardiac Patients

Genotypes	TT genotype		CT genotype		CC genotype		Total	
	No.	%	No.	%	No.	%	No.	%
Observed frequency	20	50.0	18	45.0	2	5.0	40	100
Expected frequency	21	52.5	16	40.0	3	7.5	40	100
$\chi^2 = 0.63$ p = 0.733 (not significant)								

Serum Digoxin levels among studied cases

Serum digoxin level ranged from (0.2 - 2.74) ng/ml with mean (±SD) of 1.31 (±0.58). 11 cases (27.5%) were found to have serum digoxin below the therapeutic range, 25 cases (62.5%) within the therapeutic range, and 4 cases (10%) above the therapeutic range but were not associated with clinical signs and symptoms of toxicity (Table 2).

Table (2): Serum Digoxin Level (ng/ml) according to the Therapeutic Range among Studied Cases

	Below the therapeutic range (< 0.8 ng/ml)	Within the therapeutic range (0.8-2.0 ng/ml)	Above the therapeutic range (> 2.0 ng/ml)
Number	11	25	4
Percent	27.5%	62.5%	10.0%
Minimum	0.2	0.8	2.3
Maximum	0.6	1.6	2.74
Mean	0.565	1.31	2.54
Std. Deviation	0.130	0.588	0.185

No significant difference was found between serum digoxin levels as regards duration of treatment and different age groups (Table 3). Among recent cases the mean of serum digoxin level was 1.21 (±0.634) ng /ml and among chronic cases it was 1.07 (±0.556) ng /ml (P=0.649). In patients less than 40 years, the mean digoxin level was 1.09 (±0.557), and in patients between 40 and 60 years, the mean serum digoxin was 1.160 (±0.655) with no statistically significant difference (P= 0.326). No significant correlation between serum digoxin level and age (r = 0.159, P= 0.321) was found.

Table (3): Serum Digoxin Level according to Duration of Treatment and Age Group among Studied Cases

Digoxin level (ng/ml)	Duration of treatment		Age group	
	Recent N=17	Chronic N=23	< 40 year N=18	> 40year N=22
Minimum	0.2	0.53	0.58	0.2
Maximum	2.61	2.74	2.52	2.74
Mean	1.21	1.07	1.09	1.16
Standard deviation	0.634	0.556	0.557	0.655
T test	**P = 0.649 (NS)**		**P = 0.326 (NS)**	

Comparing serum digoxin levels among different MDR1 C3435T genotypes (Table 4); the mean serum digoxin among cases with TT genotype was 1.39 (±0.675), while in cases with CT genotype it was 0.93 (±0.295) ng/ml, and in cases with CC genotype it was 0.37(±0.24), with a significant difference in serum digoxin level between cases with TT genotype and cases with CT and CC genotypes. But no significant difference in digoxin level between CT and CC genotypes.

Table (4): Comparison between Serum Digoxin Levels (ng/ml) among Different MDR1 C3435T Genotypes

Genotype / Serum Digoxin	TT N =20	CT N = 18	CC N = 2
Minimum	0.53	0.56	0.20
Maximum	2.74	1.50	0.54
Mean	1.391	0.926	0.370
Std. Deviation	0.675	0.295	0.240
F test = 5.916		**P = 0.006 ***	
LSD: TT with CT and CC, CT with TT, CC with TT			

The two patients with CC genotype (100%) showed digoxin levels below the therapeutic range, while only 3 cases (15%) of TT genotype and 6 cases (33%) of CT genotype were below this range. On the other hand, 4 cases of TT genotype were above the therapeutic range and it was the only genotype that showed levels above the range although it did not reach toxicity (Table 5).

Table (5): Distribution of Digoxin Level according to the Therapeutic Level among Different Genotypes of Studied Groups

Genotype / Therapeutic Range	TT		CT		CC	
	N=20	Percent	N=18	Percent	N=2	Percent
Below therapeutic range (< 0.8 ng/ml)	3	15%	6	33%	2	100%
Within therapeutic range (0.8-2.0 ng/ml)	13	65%	12	67%	0	0
Above therapeutic range (> 2.0 ng/ml)	4	20%	0	0	0	0

DISCUSSION

One objective of this study was to determine the frequency of C3435T MDR1 genotypes in the studied population. The detected frequencies were in conformity with the Hardy-Weinberg equilibrium (Table 1). According to Hardy-Weinberg equilibrium principle, the relative proportions of genotypes in population remain constant from one generation to another.[34] Conformity with Hardy-Weinberg equilibrium may indicate that the observed frequencies reflect that of the total population, not only frequencies among cardiac patients.

Our study showed that; TT genotype has the highest frequency followed by the heterozygous form CT. Although the C allele is

considered as the wild type it showed the lowest frequency in its homozygous CC form. There is marked difference in the genotype frequency of MDR1 C3435T observed between ethnic groups. In Caucasian population as well as in Asian population, there was interethnic variability in the frequency of different genotype. Studies from UK, Germany, Portugal, and Russia showed that the most frequent genotype was the CT genotype followed by TT genotype then CC genotype.[35-38] The same was also reported for China, India, and Malay.[38-40] While studies from Poland, Italy and Spain showed that the most frequent genotype was CT genotype followed by CC genotype then TT genotype.[41-43] The same was reported by studies from Filipines, Japan, Saudi Arabia, and another study from China.[21,38-43] On the other hand studies done among black African-Americans and in Ghana, Kenya, and Sudan showed completely different frequency, as the most frequent genotype was the CC genotype whereas the TT genotype was found in very small percent.[38,39]

Studies regarding the relationship between different genotypes of MDR1 C3435T and digoxin level were conducted on healthy volunteers with fixed age and fixed duration of digoxin administration.[21-26] Our study was conducted on cardiac patients, with different age range and variable durations of treatment with digoxin. Beside the small sample size, age range and variable durations of treatment represented limitations for our study. They are mainly due to the strict inclusion criteria followed in this study to avoid concomitant factors (medications or diseases) that may affect digoxin level. In this study both age and duration of treatment were not significantly associated or correlated with digoxin level (Table 3). On the contrary the significant effect on the level of digoxin was detected with the different genotypes in spite of the small sample size (Table 4). In addition the monitoring of the digoxin

level was done after reaching a steady state and the samples were taken in the trough level as in clinical TDM. In most of the reported articles the regimen of digoxin administration was a single dose and the pharmacokinetics of digoxin was followed from 0 time till 4 hours after administration in some studies or up to 24 hours in others.[14,21-26,44] On the other hand, Johne et al., (2002)[45] used the steady state regimen among Caucasians and also reported the same outcome like our study; significant differences were observed between CC, CT, and TT carriers in Digoxin maximum concentration and serial measurement of digoxin from the time of administration till 4 hours; the mean plasma concentration was higher in genotype TT than that with genotype CC, and digoxin trough values were 36% higher in TT subjects compared with those subjects with CC genotypes.

The relation between digoxin level and the genotypes detected by our study was in agreement with other previous studies[14,24,26] including that of Hoffmeyer et al. (2000)[14] which was the first investigation of the effect of MDR1 genotypes on pharmacotherapy. On the contrary, different relation between digoxin and MDR1 C3435T genotypes was reported.[21,44] However, on conducting the investigation again, it was consistent with our results.[26]

Gerloff et al., (2002)[25] reported that serum digoxin levels were not significantly different in any of the genotype groups tested. This lack of effect of the major MDR1 SNPs on digoxin absorption might be explained by saturation of the maximum transport capacity of intestinal P-gp at the dose used (1 mg), so further digoxin absorption is merely dependent on passive absorption.

In the current study; variations of the drug level within a single genotype (Table 5) could be attributed to differences in MDR1

haplotypes, as haplotypes consisting of a combination of SNPs G2677T and C3435T.[45] Other genetic polymorphisms may also affect the digoxin level, as those in other drug transporters, such as organic anion transporting polypeptides (OATPs), which influence systemic availability of certain drugs including digoxin.[46,47]

In conclusion, the TT genotype was the most frequent among cardiac patients of the current sample. There is variation in the digoxin level according to the MDR1 genotypes; with high bioavailability with TT genotype and the lowest levels with CC genotype that may not reach a therapeutic effect, while CT genotype have values in between with tendency to lower values. This may imply the need to consider this genotype polymorphism in monitoring digoxin level among cardiac patients to tailor the digoxin dose accordingly.

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