RESEARCH ARTICLE

Influence of Genotype and Haplotype of MDR1 (C3435T, G2677A/T, C1236T) on the Incidence of Breast Cancer - a Case-Control Study in Jordan

Ali M Abuhaliema*, Al-Motassem F Yousef, Nirmeen N El-Madany, Nailya R Bulatova, Nemah M Awwad, Muhammad A Yousef, Khalil Z Al Majdalawi

Abstract

Background: Breast cancer is the leading cause of cancer death among women and the second in humans worldwide. Many published studies have suggested an association between MDR1 polymorphisms and breast cancer risk. Our aim was to study the association between genetic polymorphism of MDR1 at three sites (C3435T, G2677A/T, and C1236T) and their haplotype and the risk of breast cancer in Jordanian females. Materials and Methods: A case-control study involving 150 breast cancer cases and 150 controls was conducted. Controls were age-matched to cases. The polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) technique and sequencing were performed to analyse genotypes. Results: The distribution of MDR1 C3435T genotypes differed between cases and controls [cases, CC 45.3%, CT 41.3%, and TT 13.3%; controls, CC 13.4%, CT 43.3%, and TT 30.2%, p < 0.001]. Similarly, the distribution of G2677A/T significantly differed [cases, GG 43.1 %, GT+GA 50.9% and AA+TT 6%; controls, GG 29.6 %, GT+GA 50.9%, and AA+TT 19.4%, p = 0.004]. On the other hand, genotype and allelotype distribution of C1236T was not statistically different between cases and controls (p=0.56 and 0.26, respectively). The CGC haplotype increased the risk to breast cancer by 2.5-fold compared to others, while TGC and TTC haplotypes carried 2.5- and 5-fold lower risk of breast cancer, respectively. Conclusions: Genetic polymorphisms of MDR1 C3435T and G2677A/T, but not C1236T, are associated with increased risk of breast cancer. In addition, CGC, TGC and TTC haplotypes have different impacts on the risk of breast cancer. Future, larger studies are needed to validate these findings.

Keywords: Breast cancer - MDR1 - polymorphism - haplotype - Jordan

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Introduction

Breast cancer is the most common type of cancer among women (Kim et al., 2014; Payandeh et al., 2015). Nearly 1.7 million new cases were diagnosed in 2012, and over 508 000 women died in 2011 due to breast cancer worldwide (Global Health Estimates, 2013). In Jordan, breast cancer is the most common among cancers in females, accounting for 37 % of all female cancers and for 19% of human cancer, and is the leading cause of cancer deaths among Jordanian women(Al Rifai and Nakamura, 2015; Awwad et al., 2015). Breast cancer cells often spread undetected by contiguity, lymph channels, and through the blood early in the course of the disease. The most common metastatic sites are lymph nodes, skin, bone, liver, lungs, and brain (Kalinsky et al., 2015).

Numerous studies have inducted that the complex interplay of genetics, environmental exposures, hormones, and behaviours may predispose to breast cancer (Lecarpentier et al., 2015; Payandeh et al., 2015; Sufian et al., 2015). Many studies suggested that polymorphism of multi-drug resistance 1 (MDR1) gene may modulate the incidence of cancer risk (Zhu et al., 2013; de Oliveira et al., 2014). MDR1 gene is a member of the ABC (ATP-binding cassette) family that encodes a membrane-bound phosphoglycoprotein (P-gp). The MDR1 gene is located on chromosome 7 and contains 28 exons, and the coding region that leads to change in protein sequence accounts for less than 5% of the total (Ozdemir et al., 2013) . It acts as an efflux pump and provides cell protection against various substances such as organic cations, amino acids, polysaccharides, proteins, and some antibiotics (Kreile et al., 2014; Pongstaporn et al., 2015). Accordingly, kidney, adrenal gland, liver, blood-brain barrier, placenta, and testis contain high amount of P-gp (Ozdemir et al., 2013).

It has been suggested that single nucleotide polymorphisms (SNPs) of MDR1 could influence the level of expression of enhancer and promoter sequences

*Department of Biopharmaceutics and Clinical Pharmacy, Faculty of Pharmacy, the University of Jordan, Amman, Jordan *For correspondence: mr_abuhaliema@yahoo.com*

Table 1. Primer Sequences Used in Genotyping of MDR1 SNPs

SNP	Positiona	rs name	Primers sequence	Tm	GC%	PCR product size
C3435T	87509329	rs1045642	5'ACATGCTCCCAGGCTGTTTAT'3	59.71	47.62	432 bp
			5'TGACAGTTCCTCAAGGCATACA'3	59.36	45.45	
G2677T/A	87531302	rs2032582	5'TTTAGTTTGACTCACCTTCCCG'3	58.26	45.45	229 bp
			5'TGTTTTGCAGGCTATAGGTGCC'3	61.2	50	
C1236T	87550285	rs1128503	5'CTCGAAAAGAAGTTAAGGTACAGTG'3	57.89	40	321 bp
			5'ATCTCACCATCCCCTCTGTG'3	58.5	55	

a:NC_000007.14; Tm: melting temperature; bp: base pair

that may influence the efficacy of processing of the pre-mRNAs, and as a consequence, may influence mRNA stability(Ozdemir et al., 2013; Du et al., 2014). Modulation of the P-gp transporting function may contribute to mutagenesis through accumulation of exogenous toxins and increase the risk of cancer development. Among more than 50 SNPs identified in ABC gene, the most clinically relevant SNPs are C3435T (rs1045642, NC_000007.14; 87509329) in exon 26, G2677A/ T in exon 21 (rs2032582, NC_000007.14; 87531302) and C1236T (rs1128503, NC_000007.13; 87550285). Synonymous SNPs as C3435T and C1236T decrease the levels of mRNA expression and, hence, overall P-gp activity (Pongstaporn et al., 2015), whereas, G 2677A/T leads to change of alanine amino acid with serine or threonine. Many studies discussed the relation between the three SNPs and the incidence of breast cancer (Ji et al., 2012; Tulsyan et al., 2014; Gutierrez-Rubio et al., 2015; Kim et al., 2015). However, the findings of these studies are still inconclusive (Wang et al., 2013a; Gutierrez-Rubio et al., 2015).

The aim of this study is to discuss the association between genetic polymorphism of MDR1 at three sites (C3435T, G2677A/T, and C1236T) and their haplotype and the risk of breast cancer among Jordanian women.

Materials and Methods

Study population

A hospital-based study of 150 breast cancer (BC) women and 150 age-matched healthy individuals was conducted at Al-Basheer Hospital/Amman which is a referral hospital for cancer patients. The study lasted from the 15th of March till the 21st of December 2014. The research was approved by the Ethics Committee [Institutional Review Board, (IRB)] at Al-Basheer Hospital in accordance with the ethical standards on human experimentation (Institutional and National) and with the Helsinki Declaration (IRB no. 17443, 28/11/2013). Each subject gave a sample of his/her blood after detailed explanation of the purpose of the study followed by obtaining an informed consent.

MDR1 Genotyping

Venous blood (4 mL) was collected from patients and healthy subjects in K3EDTA-coated tubes. The tubes were kept in icebox and DNA extraction was performed on the same day using "DNA Genomic Wizard" purification kit (Promega Corporation, Wisconsin, USA) according to manufacturer's instructions. Amplification of the 3 position was done using primers as shown in Table (1) (Princess Haya Biotechnology Centre at the King Abdullah University Hospital, Jordan University for Science and Technology, Irbid, Jordan). The PCR conditions were: 4 minutes of initial denaturation at 95ºC, followed by 39 cycles of 95ºC for 15 seconds, 60ºC for 12 seconds, and 72ºC for 15 seconds, with a final extension at 72ºC for 10 minutes (Bio-Rad, S1000 Thermal cycler™ , USA).

The PCR products of C3435T were digested with Dpn II restriction enzyme, the products of C1236T were digested with Hae II, while the genotyping of G2677A/T was done by DNA sequencing for all samples (BigDye Terminator Cycle Sequencing on 3730xl DNA sequences, Genewiz® Co., USA). The digestion fragment sizes for the MDR1C3435T genotypes were: 93, 167, and 172 bp bands for CC; 167, and 265 bp bands for TT; and 93, 167,172, and 265 bp bands for CT, whereas for C1236T; 35, 87, and 199 bp bands for CC; 87, and 234 bp bands for TT; and 35, 87,199, and 234 bp bands for CT. Resulting fragments were separated on 2% agarose gel electrophoresis and visualized using RedSafe™ (New England Biolab, USA) staining.

Findings of the PCR-RFLP were validated by the following: 1) A negative control was run simultaneously with every PCR run. A negative control contained all PCR components except the DNA template; 2) 20 % of all samples were repeated to confirm findings of the PCR-RFLP; 3) Randomly selected 10 % PCR-RFLP results were confirmed by DNA sequencing. The concordance between repeated samples, sequencing and PCR-RFLP results was 100%.

Haplotype analysis

We analyzed the haplotype frequencies of the three loci (C3435T, G2677A/T and C1236T) and compared them between BC cases and controls. Haplotype frequencies were calculated using Haploview software for analysis and visualization of LD and haplotype maps (Barrett et al., 2005), and Multiallelic Interallelic Disequilibrium Analysis Software (MIDAS®, University

Table 2. Characteristics of BC Patients

Patient characteristic	Mean ±SD or N (%)
Age, years	49.9±10.8
Median age at diagnosis(range), years	49 (23-74)
BMI, kg/m2	28.8 ± 6.3
Number of children	4.2 ± 3
Age at first birth, years	19.4 ± 9.9
Nulliparous	22 (16.1)
Age at menarche, years	13.5 ± 1.6
Age at menopause, years	49.0 ± 4.6

SD: standard deviation; BMI: body mass index

Table 3. MDR1 Genotype and Allelotype Distribution among Cases and Controls

Variables	Genotypes				Alleles				
	Cases N (%)	Controls N (%)	P	OR (95% CI)	Allele	Cases, N (%)	Controls, N (%)	P	OR (95% CI)
MDR1 C3435T	150	150	< 0.0001		C	198 (66)	145 (48.3)	< 0.0001	2(1.49- 2.88)
CC	68 (45.3)	40 (26.7)		2.28(1.41-3.70)					
CT	62 (41.3)	65 (43.3)		0.92(0.58-1.45)	T	102 (34)	155 (51.7)		
TT	20 (13.3)	45 (30)		0.36(0.19-0.65)					
MDR1 G2677A/T	116	108	0.004		G	159(67.3)	119(55.1)	0.026	1.68(1.15- 2.46)
GG	50(43.1)	32(29.6)		1.79(1.03-3.12)					
GT+AT	59(50.9)	55(50.9)		0.99(0.59-1.68)	T	73(31)	93(43.1)		0.59(0.4-0.87)
AA+TT	7(6.0)	21(19.4)		0.26(0.11-0.65)	A	4(1.7)	4(1.8)		0.91(0.23-3.69)
MDR1 C1236T	148	126	0.56		C	169 (57.1)	132 (52.4)	0.26	1.2(0.86-1.7)
CC	56(37.8)	40(31.7)		1.3(0.79-2.16)					
CT	57(38.5)	52(41.3)		0.89(0.55-1.44)	T	127 (42.9)	120 (47.6)		
TT	35(23.6)	34(27)		0.83(0.48-1.44)					

N: sample size; P: P value based on chi square or fisher exact test; OR: odd ratio; CI: Confidence Interval

of Southampton, Highfield, Southampton, UK) (Gaunt et al., 2006) and linkage disequilibrium was represented by Lewontin's coefficient; D prime (D'), r2, and Chi square.

Statistical analysis

Data were coded and entered into SPSS software version 16 (Chicago, IL). Data of categorical nature were summarized as counts and percentages. Data of continuous nature were summarized as mean ± standard deviation. The relation between categorical vs. categorical variables were evaluated by Chi-square or Fisher exact test as appropriate. The strength of association was assessed by calculating odds ratio (OR) and 95% confidence interval (95% CI) (Cochran, 1954). The relationship between categorical and continuous variables were evaluated by independent sample t-test, ANOVA, Mann Whitney or Kruskal Wallis tests as appropriate. Normality of distribution was assessed by Kolmogorov-Smirnov or Shapiro-Wilk test as appropriate. Homogeneity of variance was evaluated by Levene's test. A p value<0.05 was considered statistically significant. Hardy-Weinberg Equilibrium was assessed for genotypes assuming degree of freedom =1 (Rodriguez et al., 2009).

Results

A total of 150 breast cancer patients and 150 control subjects were included in this study. BC patients and control groups were of the same age (49.9±10.8 years). Among BC patients, the median age at diagnosis was 49 years, that at menarche was 13.5±1.6 years and at menopause 49±4.6 years. Table 2 summarizes the demographic and clinical characteristicsof BC patients.

Genotype distribution

Study of MDR1 C3435T polymorphism revealed that the homozygote mutant type was found in 13.3% among cases, while among controls wild 30.2%, and higher prevalence of wild type in BC patients compared to controls (p < 0.001). Subjects with (C3435T) T allele were 2 time less likely to suffer from breast cancer (p< 0.0001). As for G2677A/T polymorphism, the prevalence of wild type (GG) was higher among cases compared to controls [50 (43.1%) vs 32 (29.6%), respectively], while the mutant type (AA+TT) was lower in BC [7(6%) vs 21 (19.4%) in controls], (p = 0.004). On the other hand, genotype and allelotype distribution of C1236T was not significantly different between cases and controls (p=0.56) (Table 3).

Table 4. Haplotype Distribution of MDR1 3435, 2677, and 1236 among Cases and Controls

Haplotype	total (%)	Cases, N=300	Controls, N=286	OR (95% CI)
C3435T-G2677A/T-C1236T		N (%)	N (%)	N (%)
CGC	35	135 (45)	70 (24.4)	2.5 (1.8-3.6)
TTT	19	54 (18)	57 (20)	0.9 (0.6-1.3)
CTT	13.9	37 (12.4)	45 (15.6)	0.8 (0.5-1.2)
TGC	13.9	25 (8.4)	55 (19.4)	0.4 (0.2-0.7)
TGT	6.4	18 (6)	20 (6.9)	0.8 (0.4-1.6)
CGT	5.8	21 (7)	13 (4.5)	1.6 (0.8-3.2)
TTC	3.2	4 (1.3)	15 (5.2)	0.2 (0.08-0.7)
CTC	2.8	5 (1.6)	12 (4.2)	0.4 (0.1-1.1)

N: No of chromosomes; OR: odd ratio; CI: Confidence Interval

MDR1 Haplotype

The most common haplotypes were CGC and TTT, while the least common haplotypes were TTC and CTC among both cases and controls. Three haplotypes were distributed differently between cases and controls. CGC haplotype is associated with 2-fold increase in the risk of breast cancer compared to others [OR=2.5, 95%CI (1.8-3.6), p <0.001] while the TGC and TTC haplotypes were protective. Carriers of TGC had 2.5times lower risk of breast cancer compared to others [OR=0.4, 95% CI (0.2-0.7), p <0.001], while carriers of TTC haplotype had 5 times lower risk of breast cancer [OR=0.2, 95%CI (0.08-0.7), p=0.007]. Table 4 summarises the association between haplotypes and incidence of breast cancer.

Additionally, every two SNPs were analyzed together. Considering C3435T and G2677A/T haplotypes, patients who carry CT and TG haplotype were 5 times less likely to develop breast cancer than those who carry CG haplotypes. Considering C3435T and C1236T haplotypes, patients who carry TC haplotype were 6.4 times less likely to develop breast cancer than those who carry CC haplotypes. Regarding G2677A/T and C1236T haplotypes, patients who carry TC haplotypes are 10.6 times less likely to develop breast cancer than those carrying GC haplotypes.

Table 5. Haplotype Distribution for Each two SNPs

Variable	Cases			Controls		
Haplotype	D'	r^2	X^2	D'	r^2	X^2
3435-2677	0.6	0.31	36.6	0.05	0.002	0.21
3435-1236	0.57	0.22	32.4	0.004	0	0.0017
2677-11236	0.92	0.52	60.6	0.54	0.29	26.11

D': theoretical range of the linkage disequilibrium coefficient; r2: the square of the correlation coefficient between two indicator variables; X2: Chi-Squared with one degree of freedom

The strongest linkage disequilibrium (LD) was observed between SNPs at 2677 and 1236 in cases and controls [cases: D'=0.92; controls: D'=0.54] (Table 5). The linkage disequilibrium was significant between all pairs of SNPs among cases but not among controls.

Discussion

MDR1 gene is a member of the ABC family that encodes P-gp, an ATP-dependent efflux pump that helps the cell to get rid of toxins and exogenous materials (Kreile et al., 2014). Many studies suggested that polymorphism of MDR1 gene may modulate risk of breast cancer due to the absence of protective effect provided by this pump (Ikeda et al., 2015). We studied the association between genetic polymorphism of MDR1 and the risk of breast cancer among Jordanian patients.

Our results indicate that C3435T CC genotype is significantly associated with increased risk of breast cancer. Zubor et al. (2007), reported that, among patients in Slovak Republic, those carrying CC genotype of C3435T had increased risk of breast cancer. In a study by Macias-Gomez et al. (2014) conducted among Mexicans the prevalence of the TT genotype was 8% for patients with fibrocystic changes (FCC), 13% for infiltrating ductal breast cancer (IDBC) and 24% for control samples.

In a meta-analysis published in 2012 that involved 7 studies, MDR1 C3435T polymorphism was significantly associated with increased risk of breast cancer (TT vs CC, OR = 1.66, 95% CI (1.24-2.21) (Wang et al., 2012). A meta-analysis published in 2013 based on 52 studies, included 9 studies that analysed the association of breast cancer and C3435T polymorphism showed similar results [TT vs CC: OR=1.18, 95%CI (0.869-1.621), p=0.001] (Wang et al., 2013b). There are some limitations in this meta-analysis including asymmetry of its funnel plot suggesting potential publication bias, a language bias, and lack of publication of trials with opposite results. The pre-existing publication bias would question the findings of this meta-analysis. Additionally, this meta-analysis suffered from significant heterogeneity.

On the other hand, a meta-analysis conducted in 2011 did not find any association between MDR1 C3435T polymorphism and risk of breast cancer (Lu et al., 2011). Fawzy et al. (2014) studied 190 Egyptian females with breast cancer and showed that the frequency of TT genotype of C3435T and T allele were significantly higher in breast cancer patients compared to the controls ($P < 0.05$) while no significant differences were found between the frequencies of MDR1 G2677A/T and C1236T genotypes and haplotypes.

The conflicting results of many studies may be caused by multi gene interactions. It may be assumed that TT genotype leads to defect in P-gp function resulting in increasing drug accumulation inside the cells, and resistance to the chemotherapy, though it may be a consequence from other mechanisms. On the other hand, in patients with the CC genotype, there might be a strong linkage disequilibrium with other polymorphisms in the MDR1 gene as well as alterations in the post translational pathway which influences the efficacy and stability of P-gp.

Genotypes distribution of C3435T vary widely among different populations. In Caucasians, the 3435 frequency was reported as CC (22%), CT (50%), and TT (28%) (Hamidovic et al., 2010). In a study conducted among Spanish breast cancer patients MDR1 C3435T allele distribution was (C) 0.52 and (T) 0.48 which were not different from controls, while genotypes were distributed as CC 14 (28%), CT 24 (48%) and TT 12 (24%) (Henriquez-Hernandez et al., 2009). On the other hand, in Korean population, CC, CT, and TT genotypes were found in 50.9%, 10.2%, and 38.9% BC patients, respectively. A study conducted among Jordanian and Sudanese population by Salem, et al (2014) reported that T allele was more prevalent among Jordanians than C allele (CC: 20.7%, CT: 51.7%, TT: 27.6%; C: 47%, T: 53%). Jordanians resembled Asians and Europeans but were different significantly from Africans (Salem et al., 2014).

Our results indicate that the wild type of 2677G polymorphism is associated with increased risk of breast cancer. These findings are concordant with those in a study by Rubis et al. (2012) that involved 209 patients and 202 controls from Poland [cases: GG = 43.5, GT+GA = 44.5 and AA+TT = 12%; controls: GG = 34%, GT+GA = 52.5% and AA+TT = 13.5%]. On the other hand, six studies (2 involving BC patients and 4 involving patients with all cancers) from seventeen studies that evaluated G2677A/T polymorphism that were included in a meta-analysis revealed TT genotypes of G2677A/T to be associated with cancer risk in general (Wang et al., 2013a). Wu et al. (2012), reported that T allele of G2677A/T carried 1.83 higher risk of developing breast carcinoma.

Regarding C1236T polymorphism, our results showed no significant association with the incidence of breast cancer. These findings are consistent with a recent meta-analysis, which reported lack of association between C1236T genetic polymorphism and the incidence of cancer in general (Wang et al., 2013a). In a study conducted by Alsaif et al. (2013) indicated that C1236T CC genotype is protective against breast cancer, while mutant genotypes of CT, and TT were more prevalent in cases compared to controls ($p < 0.001$). Gutierrez-Rubio et al. (2015), a study involved BC patients reported C1236T CT genotype is protective against breast cancer.

Little is known regarding the influence of MDR1 C3435T- G2677A/T- C1236T haplotypes in terms of the potential impact on risk of breast cancer. Our results revealed that CGC, TGC and TTC haplotypes strongly modulate the risk of breast cancer. CGC haplotype increases the risk to breast cancer by 2.5 folds compared to others while, TGC and TTC haplotypes were protective

against breast cancer. On the other hand, Wu et al. (2012) reported that TTT haplotype is significantly increases the risk of breast carcinoma. The frequencies of haplotypes reported in this study are in line with a recently published MDR1 haplotypes among apparently healthy Jordanians by Al-Diab et al. (2015) with the most prominent haplotype being CGC (37.6%), followed by TTT (18.6%), and the least frequent haplotype being CTC (1.8%).

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