RESEARCH COMMUNICATION

MDR1 Gene Polymorphisms: Possible Association with its Expression and Clinicopathology Characteristics in Colorectal Cancer Patients

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Abstract

Aim: Over-expression of some genes, such as *MDR1*, can increase the level of chemotherapy drug afflux and limit the effectiveness of treatment. We aimed to investigate the effect of *MDR1* polymorphisms on its expression level and related characteristics in Iranian colorectal cancer patients. Methods: Tumor, normal mucosal tissue and blood samples from CRC patients and blood samples from healthy controls (n=60) were obtained for genotyping and measuring the expression level of MDR1. Results: The expression of the *MDR1* gene showed a significant increase in cancerous regions compared to adjacent normal tissue. Furthermore, the GG2677 genotype was correlated with highest while the AT 2677genotype was associated with the lowest levels of expression. In addition only the G2677T/A polymorphism showed association with histological grade of colorectal tumors. Conclusion: Our study once more emphasizes effects of *MDR1* SNPs which may indirectly impact on response to drugs.

Keywords: Chemotherapy - colorectal cancer - multi drug resistance - Iranian patients

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Introduction

Although chemotherapy is one of the most effective ways of cancer treatment resistance to chemotherapy drugs evolved in many cancers is a major obstacle in cancer therapy (Gregory et al., 2003; Martin, 2004; Luqmani, 2005; Isabel et al., 2007). Multi drug resistance (MDR) is a phenomenon in which cancerous cells become resistant to wide range of structurally and functionally different drugs (Darinka et al., 2008).

Colorectal cancer (CRC) is the fourth cause of the overall cancer mortality by 655,000 deaths yearly in the world. A chemo-resistant form of tumor and MDR-based mechanisms has been suspected to participate in the general unresponsiveness of colorectal tumors to drugs (Linn and Giaccone, 1995).

ATP Binding Cassette (ABC) transporters super-family is a class of trans-membrane proteins that increase the ability of tumor cells to transport drugs out of the cells by using ATP molecule (Longley and Johnston, 2005). These transporters are one of the best known mechanisms responsible for MDR phenotype (Childs and Ling, 1994). The *multi drug resistance* (*MDR1*) gene belongs to the ABC gene family and encodes a 170Kd glycoprotein (van der Deen et al., 2005). This protein has been reported to correlate with the degree of drug resistance in several cell lines (Yukihiko, 2001). In some studies the over-expression of this gene is considered to be correlated in MDR phenotype in some cancers including colorectal cancer (Peters et al., 1992; Darinka et al., 2008).

The *MDR1* gene with 28 exons is a polymorphic gene. So far, over 50 SNPS has been found in this gene. It is believe that some of these SNPs may affect the expression level of MDR1 gene (van der Deen et al., 2005).

In this study we aimed to evaluate the possible influence of MDR1 sequence variant C3435T, C1236T and G2677T/A on the expression level of MDR1.Furthermore, we wished to investigate the possible association between these polymorphisms and clinicopathology characteristics of CRC tumors.

Materials and Methods

Tissue and blood samples

From 2008 to 2010, 60 specimens of colorectal cancer tissues were obtained from patient who underwent surgery at Hazrat Rasool Hospital. Tumor, normal mucosal tissue and blood samples from patient and blood samples from healthy controls (n=60) were obtained after subjects provided informed consent. Tissue samples were frozen and stored at -70.

The project was approved by the local ethics committee of the National Institute for Genetic Engineering and Biotechnology (NIGEB).

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Real Time RT PCR

RNA extraction was carried out with the Tripure Isolation Reagent (Roche Applied Sciences). For cDNA synthesis, 1 μg of total RNA from each sample was used to synthesize first-strand cDNA according to the manufacturer's protocol (Fermentas).

Real-time RT-PCR

Evaluation of the expression level of MDR1 was performed by real-time quantitative PCR using the LightcyclerTM system (Roche Applied Sciences) and Fast-Start DNA Master SYBR-Green I kit (Roche Applied Sciences). The following primers were used for evaluating MDR1 expression: MDR1 forward 5'-TGACATTTATTCAAAGTTAAAAGCA- 3' MDR1-reversed 5'-TAGACACTTTATGCAAACATTTCAA-3'. GAPDH was selected as the housekeeping gene for assessment of expression. The primer sequences for GAPDH were as follows: forward 5'-GCAGGGGGGAGCCAAAAGGGT -3' and reverse 5'-TGGGTGGCAGTGATGGCATGG -3' (Au et al., 2007; Kimchi-Sarfaty et al., 2007).

All reactions were carried out in a total volume of 20 μL in capillary tubes. Each reaction mix contained 0.6 μM of each primer, 2.5 mM MgCl2 and 2 μL of Fast Start Master solution. A total of 18 μL of this reaction mix was placed in glass capillaries, and 2 μL of cDNA was added as template. The capillary tubes were capped and placed in the carousel under reduced light conditions.

Thermal cycling consisted of an initial denaturation step at 95°C for 10 min followed by an amplification program (primer annealing, amplification and quantification) repeated for 55 cycles with temperature ramp rate of 20°C/s. The amplification program was 95°C for 10s, 62 °C for 20s and 72°C for 15s, with a single fluorescence acquisition at the end of the elongation step. The third segment consisted of a melting curve program at 95°C for 0s, 70 °C for 10s and 9 °C for 0s with a linear temperature transition rate of 0.1°C/s with continuous fluorescence acquisition. Finally, a cooling program cooled the reaction mixture to 40 °C.

DNA extraction

Genomic DNA extraction from blood samples (60 patients and 60 control individuals) was carried out by Diatom DNA Prep 200 (Isogen Lab ltd Russ).

RFLP method: MDR1 C3435T polymorphism was detected using a PCR-RFLP assay. The following primers were used: 5'-GCTGGTCCTGAAGTTGATCTGTGAAC-3' as forward and 5'-ACATTAGGCAGTGACTCGATGAAGGCA -3' as reverse primer (Turgut et al., 2007). The PCR mixture included 1 μM primer, 200 μM of each dNTP (KBC), 2.5μl, Taq DNA polymerase 1X reaction buffer with1.5 mM Mgcl2 and 1 unit Taq polymerase (5 U/μL,KBC). The PCR protocol for C3435T was as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles, consisting of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 30 s. Final extension was performed at 72 °C for 4 min. Amplified

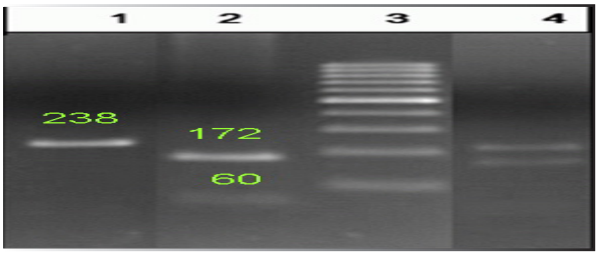

Figure 1. Gel Electrophoresis of *MDR1* C3435T Genotype Using RFLP Method. The PCR product (248 bp in size) was digested for 3 h at 37 with 2 U MboI restriction enzyme (Frementas, Germany). The expected fragment sizes were: a 238-bp fragment for TT genotype, a 172-bp and 60-bp fragments for the CC genotype, and a 238-bp, 170- bp and 60 bp for the CT genotype. Lane1: TT3435 lane2: CC3435 Lane 3: ladder 50bp Lane4: CT3435

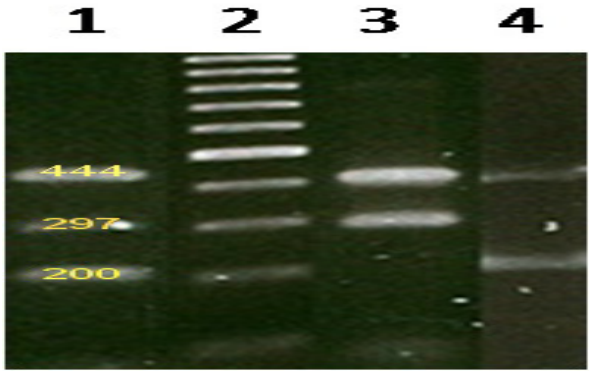

Figure 2. Gel Electrophoresis of *MDR1* C1236T Genotype Using ARMS Method. The outer primers produce a 444 bp amplicon and allele-specific primers produce a 200 bp amplicon for 1236C and a 297 bp amplicon for 1236T. Lane 1: CT1236 genotype; Lane 2: Ladder 100bp; Lane 3: CC1236 genotype; Lane 4: TT1236 genotype

segments were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

The PCR product (248 bp in size) was digested for 3 h at 37 °C with 2U MboI restriction enzyme (Frementas, Germany). The expected fragment sizes were: a 238-bp fragment for TT genotype, a 172-bp and 60-bp fragments for the CC genotype, and a 238-bp, 170 bp and 60 bp for the CT genotype. DNA fragments generated after restriction enzyme digestion were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and observed with an ultraviolet transilluminator (Figure 1).

ARMS method: G2677T/A and C1236T polymorphisms were detected using the ARMS assay. For each reaction we used tetra primer in a reaction tube. Reactions consisted of a total volume of 25 μL containing 250 μM dNTPs (Bioneer), 0.4 μM of each primer, 1.5 mM MgCl2, 2.5μL, Taq DNA polymerase 1X reaction buffer and 1 U Taq DNA polymerase (Bioneer) and 50 ng of genomic DNA (Figure 2). The primers data and amplicon sizes are shown in Table 1.

Data analysis

The raw data were analyzed using version 3.03 of the Lightcycler software. The software calculates the relative amount of the target gene and the reference gene

Table 1. Primer Sequences for Determining Genotype of C1236T and G2677T/A

Amplicon size	dsSNP: 1128503 C1236T
444	Forward outer primer (5' - 3'):
227 TTCGAAGAGTGGGCACAAACCAGATAA 253	
444	Reverse outer primer (5' - 3'):
670 GATGTGCAATGTGACTGCTGATCACC 645	
200	Forward inner primer (C allele):
472 CTCACTCGTCCTGGTAGATCTTGAAGTGC 500	
297	Reverse inner primer (T allele):
523 CCACTCTGCACCTTCAGGTTCCGA 500	
AMPLICON SIZE	G2677T/A dsSNP: 2032582
447	Forward outer primer1 (5' - 3'):
246 GAAAATAGAAGCATGAGTTGTGAAGA 271	
447	Reverse outer primer1 (5' - 3'):
692 CTGGCTTTGCTACTTTCTGTAAGTTT 667	
216	Forward inner primer1 (A allele):
478 CACTGAAAGATAAGAAAGAACTAGAAGCTA 507	
287	Reverse inner primer1 (T allele):
532 TATTTAGTTTGACTCACCTTCCCTGA 507	
453	Forward outer primer2 (5' - 3'):
243 TCAGAAAATAGAAGCATGAGTTGTGA 268	
453	Reverse outer primer2 (5' - 3'):
695 GAACTGGCTTTGCTACTTTCTGTAAG 670	
219	Forward inner primer2 (G allele):
478 CACTGAAAGATAAGAAAGAACTAGAAGATG 507	
290	Reverse inner primer2 (T allele):
532 TATTTAGTTTGACTCACCTTCCCGGA 507	

(housekeeping gene) based on the crossing point which was defined as the cycle number at which the fitted line in the log-linear portion of the plot intersected the threshold level. An external standard curve for MDR1and GAPDH was generated from a serial dilution of mRNA of each gene. For each sample, the amounts of MDR1 and the housekeeping gene were measured. Finally, the relative expression was calculated as the ratio of MDR1 to GAPDH in each sample. Statistical analysis was performed using the SPSS for software V16.0 (SPSS, Inc., Chicago, IL). Differences between groups were analyzed by Independent Sample T Test. The difference in genotype frequencies between controls and colorectal cancer patients was determined using the chi-square test. A P value less than 0.05 was considered statistically significant.

Results

The patient and tumor characteristics, gathered from the pathology reports, are listed in Table 2. Among the patient the allele frequency of 3435C was 46.2% and the allele frequency of 3435T was 53.8%, in control group frequencies were 39.6% for C3434 and 60.4% for T3435 allele. The allele frequency was not significantly different between patients and healthy controls (P= 0.6).

MDR1 wild type genotype 3435CC was observed in 24.5% of patients; whereas 3435CT and 3435TT were detected in 32.1% and 43.4% of the patients respectively. In healthy control group, MDR1 genotype frequency of 3435CC, 3435CT and 3435TT, were respectively 17%, 45.3%, and 37.7%. The genotype differences between two groups was not significant (P=0.6).

In patients the allele frequency of 1236C was 41.5% and for 1236T was 58.5% which was not significantly different with control group (46.6% 1236C and 53.4% 1236T). The genotype frequency were respectively 9.4%, 64.2% and 26.4% for 1236CC, 1236CT and 1236TT in patients and 15.3% 1236CC, 62.7% 1236C and 22% 1236TT, in healthy individuals. Statically analyses show no significant difference between two groups.

Table 2. Patient and Tumor Characteristics

Characteristic		No (%)
Total Patient		60 (100)
TNM staging	Polyp	10 (17)
	Stage 0	2 (3)
	Stage 1	6 (10)
	Stage 2	14 (23)
	Stage 3	18 (30)
	Stage4	10 (17)
Histological grade	Grade1	8 (13)
	Grade2	6 (10)
	Grade 3	46 (77)
Tumor size	<5cm	32 (53)
	5-8cm	22 (37)
	8-10cm	4 (7)
	>10cm	2 (3)
Lymph node metastasis	Positive	28 (47)
	Negative	32 (53)
Tumor position	Colon	25 (42)
	Rectum	35 (58)
Gender	Male	32 (53)
	Female	28 (47)

Table 3. Correlation of *MDR1* Gene Expression with Clinicopathology Characteristics of Patients

	Expression of MDR1
Age	NS (P>0.05)
Gender	NS(P>0.05)
Tumor position	NS(P>0.05)
Size of tumor	NS(P>0.05)
Lymph node involvement	NS(P>0.05)
Grade	NS(P>0.05)
Histological grade	S (P<0.05)

NS, not significant; S, significant

G2677T/A polymorphism genotype and allele frequency were compared between healthy and patient groups.In healthy group no carrier of GG, GT, GA in 2677 was observed so the frequency of T2677 and A2677 were 57.5% and 42.52%, genotype frequency was 18.3% for 2677TT, 3.4% for 2677 AA and 78.3% for 2677ATgenotype.In contrast in patients group there was no carrier of AA genotype and allele frequencies was 27.4% for wild type allele (G) and 66%, 6.6% for mutant alleles T and A. genotype frequencies for GG2677, GT2677, GA2677, AT2677 and TT2677 were respectively 5.7%, 41.5%, 2%, 11.3% and 39.5% respectively.

The expression level of *MDR1* gene in colorectal cancer specimens and adjacent normal tissues were determined by Real Time RT-PCR assay and the final data were standardized against GAPDH mRNA levels in samples.The expression level of MDR1 in tumoral tissue was higher than normal ones without considering genotype of individuals. The expression level of MDR1 in patients who were homozygous (CC) for both C3435C and C1236C polymorphisms was higher compared with

CT and TT genotypes. However, there was no considerable association between MDR1 expression level and C3435T or C1236T polymorphism in our results (P>0.05). On the other hand, G2677T/A polymorphism showed to have an effect on expression level of MDR1 but only patient group and not in control group.

Mean expression level of GG, GT, GA, TT and AT in patients group were 0.8967 ± 0.0057, 0.8645 ± 0.0646, 0.8250 ± 0.1767, 0.7349 ± 0.0785 and 0.7000 ± 0.212, the expression level of MDR1 in the GG2677 genotype was higher than TT2677 genotype, and in TT2677 genotype was also higher than AT2677 (P<0.05).There was significant correlation between *MDR1* G2677T/A, SNP and histological grade of patients tumor. Meaning that the GG2677 genotype was more frequent in well differentiated tumors compared to less differentiated tumors.

No correlation observed between age, gender, tumor position, size of tumor, lymph node involvement and this SNP as shown in Table 3.

Discussion

Intrinsic drug resistance in untreated colorectal cancers is thought to be due in part to MDR1 because normal colon tissues themselves express MDR1 (Goldstein et al., 1989). Many researchers reported that expression of *MDR1* increases in colon cancers compared to non-cancerous region (Fojo, 1987; Cohen et al., 1989; Peters et al., 1992; Meijer et al., 1999). In addition, there is much evidence supporting that *MDR1* polymorphisms may up regulate the expressing of this gene. Therefore, in this study we attempted to investigate whether or not the expression level of MDR1 gene is associated with different SNPs of MDR1.

We first compared the allele and genotype frequencies between colorectal cancer patient and healthy controls. For C3435 and C1236T polymorphisms there was no significant different between patients and healthy individuals, but the distribution of G2677T/A polymorphism was significantly different between patients and controls. For example, the GG, GT and GA genotypes were not detected in any control individual while it the frequency of this genotypes were 5.7% , 41.5% and 2% among the patients.

Darinka Todorova et al. (2008) evaluated Bulgarian CRC patients and healthy Bulgarian volunteers for C1236T and C3435T. No differences were found between the frequencies of the two mutant alleles in the tumor tissue from the cases and lymphocytes from the controls [frequencies of 2677T: 43.5% in patients and 44.1% in controls; frequencies of 3435T: 48.3% in patients and 50.9% in controls (both P > 0.05)].

Furthermore we evaluated the possible association between different genotypes and the expression level of MDR1 gene. We found that C3435T and C1236T had no effect on expression level of MDR1 in neither patients nor controls. There have been few reports on the effect of C3435T on MDR1 expression in intestine. A study conducted by Kurzawski et al (2005), on 184 CRC patients showed that the 3435TTgenotype was associated with lower MDR1 protein. However, Toshiyuki (2005) on Japanese healthy individuals found no correlation between C3435T polymorphism and expression level of MDR1 in intestine. In our study it was shown that the MDR1 expression level was significantly higher in patients with 2677GG genotype compared to GT,GA, TT, AT and AA genotypes.

In summary the result of the present study revealed that the GG2677 genotype was correlated with highest while AT 2677genotype was associated with the lowest levels of expression of MDR1 gene. In addition only G2677T/A polymorphism showed association with histological grade of colorectal tumors. Our study one more time emphasizes on the effect of SNPs on the gene regulation which may indirectly impact on response to drugs. Further study by enrolling more patients could help to evaluate the possible role of polymorphisms of MDR1 gene and also other ATP genes in conducting drug resistance in colorectal patients.

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