Multidrug resistance protein 2 genetic polymorphism and colorectal cancer recurrence in patients receiving adjuvant FOLFOX-4 chemotherapy

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Abstract. Multidrug resistance protein 2 (MRP2), encoded by the ATP-binding cassette C2 (ABCC2) gene, is an efflux pump located on the apical membrane of many polarized cells, which transports conjugate compounds by an ATP-dependent mechanism. The correlation of G1249A ABCC2 polymorphism with the development of colorectal cancer (CRC) and poor prognosis was evaluated in patients who were treated with fluorouracil/leucovorin (FL) plus oxaliplatin (FOLFOX-4). A total of 50 paraffin-embedded tissue samples collected from CRC patients were analyzed to identify the polymorphism. Patients were in stage II/III and received postoperative FOLFOX-4 chemotherapy. As a control group, an equal number of unrelated healthy subjects were enrolled in the study. The polymorphism was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, and results were compared with clinicopathological markers, early relapse and survival rates. During the 12 months of follow-up, local and distant recurrences were observed in 15 (30%) patients. No significant difference in the distribution of wild-type and polymorphic genotypes was observed between the patient and control groups and between the patients who experienced recurrence within 1 year and those who did not (all P>0.05). In conclusion, the G1249A polymorphism is not associated with CRC risk and early recurrence. However, significant correlation was observed between G1249A polymorphism and the overall survival and disease-free survival of the patients.

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Introduction

Adjuvant FOLFOX-4 chemotherapy following surgery is recommended as an effective therapy for patients with stage II and III colon cancer (1). Nevertheless, a high percentage of patients encounter recurrence as a primary cause of mortality, which is mainly associated with chemotherapeutic response (2-4). Over the past 10 years, combination chemotherapies have improved response rates and prolonged overall survival in colorectal cancer (CRC) patients (5). To date, oxaliplatin (a platinum drug) combined with fluorouracil (5FU)/leucovorin (FL) in the FOLFOX-4 regimen is frequently prescribed to treat CRC.

The prediction of sensitivity or resistance to chemotherapy by analysis of genetic variations is of major interest in choosing the first-line chemotherapy most likely to be efficient. A single nucleotide polymorphism (SNP) is a point mutation that is observed in 1% of a general population. Genetic polymorphisms in the sequences of drug transporter genes have been found to affect the therapeutic response, toxicity and survival of cancer patients. Furthermore, SNPs may affect CRC susceptibility when exposed to exogenous and endogenous carcinogenesis.

The formation of glutathione conjugates is a well-known mechanism by which platinating agents inhibit tumor growth. It is also known to be one of the mechanisms involved in the resistance to oxaliplatin in CRC (6). Glutathione conjugates may be substrates of ATP-binding cassette (ABC) multidrug transporters. For example, glutathione detoxifies oxaliplatin by conjugation (7,8); the conjugate is eliminated by MRPs/ABCCs, either alone, as a co-substrate, or in its conjugated form (9). Intracellular glutathione content may be reduced by the high expression of MRPs/ABCCs (10).

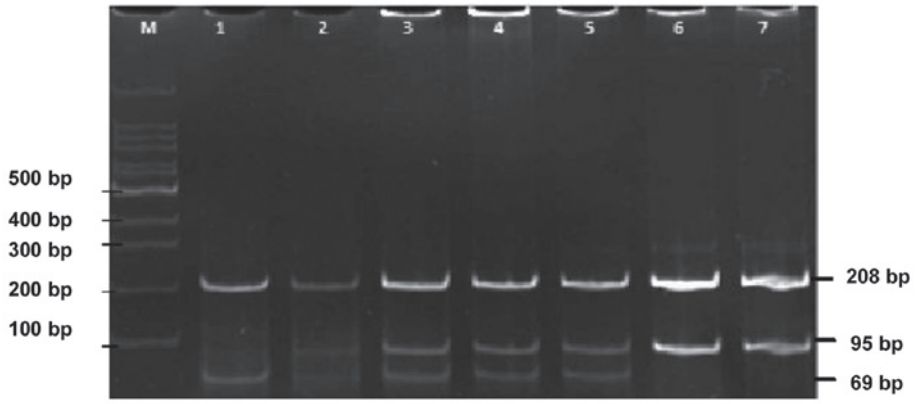

Figure 1. Electrophoresis pattern for MRP2 G1249A analyzed by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based assay on 10% polyacrylamide gel. M, marker: 100 bp; lane 1, homozygous AA: 208, 69 and 29 bp; lanes 2-5, homozygous GG: 208 and 95 bp; lanes 6 and 7, heterozygous GA: 208, 95, 69 and 29 bp.

The human multidrug resistance protein 2 (MRP2/ABCC2/cMOAT) gene is a member of the ABC transporter family that is located on chromosomal locus 10q24 and consists of 32 exons (11). MRP2 is presented at the apical membrane of polarized cells, including hepatocytes, renal proximal tubule epithelia and intestinal epithelia (12). It is the optimal pump to eliminate relatively hydrophilic compounds, including glucuronide, glutathione and sulfate conjugates (12). Among several MRP2 SNPs, the G1249A SNP is known to be associated with alterations in mRNA levels (13,14). This MRP2 polymorphism results in an amino acid alteration from Val to Ile at position 417, located in membrane spanning domain 2 of the protein. According to previous reports (15,16), mutations in this domain of MRP2 may alter the specificity of its substrates but not its transporter activity. In the present study, we investigated the association of G1249A MRP2 polymorphism with the poor response to FOLFOX-4 chemotherapy, a platinum-base regimen, and the short-term survival of CRC patients.

Materials and methods

Patients and treatment. The subjects included 50 primary CRC patients (30 male, 20 female; median age 57 years), who received radical resection in Chamran Hospital and Hazrat Rasuol Akram Hospital, Tehran, Iran. Patients were eligible if they had undergone adjuvant FOLFOX-4 chemotherapy following the radical resection of histologically confirmed stage II (T2 and T3, N0, M0) or stage III (any T, N1 and 2, M0) CRC. Patients who had received preoperative chemotherapy and radiotherapy were excluded. A total of 50 unrelated healthy individuals who did not have a family history of cancer and were gender/age-matched with the patients were also enrolled in this study. The protocol of the study was approved by the Faculty of Medicine and Health Sciences Ethics Committee, University Putra Malaysia.

The patients received 12 cycles of a FOLFOX-4 regimen which consisted a 2-week cycle of oxaliplatin (85 mg/m^2) combined with leucovorin (200 mg/m^2) on day 1, bolus 5FU (400 mg/m^2) and continuous infusion of 5FU (600 mg/m^2). Follow-up consisted of a carcinoembryonic antigen (CEA) test at 3-month intervals for 2 years and at 6-month intervals thereafter. Colonoscopy and CT scans were usually performed at 6-month intervals in the first 2 years and annually thereafter; however, these tests were mandatory following an elevated CEA level. Development of new recurrent or metastatic lesions following surgery was considered as a relapse. Local relapse was histopathologically/cytologically confirmed by the examination of specimens. Written informed patient consent was obtained from the patients.

DNA extraction and genotyping. Sections (5-10 μm) of paraffin-embedded tissues (n=50) were cut with a microtome and placed in a 1.5-ml microcentrifuge tube. To avoid intersample contamination, the blade was cleaned with xylene after cutting each paraffin block. DNA was isolated from the paraffin-embedded tissue samples using a QiAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was also extracted from 50 blood samples of the unrelated healthy individuals using a kit (Qiagen).

Single nucleotide polymorphism was analyzed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The following PCR primers were used for genotyping: forward, 5'-GGGCAAAGAAGTGTGTG GAT-3'; and reverse, 5'-ACATCAGGTTCACTGTTTCTC CCA-3' (17). The PCR amplification conditions consisted of an initial denaturation step at 94˚C for 3 min, followed by 35 cycles of a denaturation step at 94˚C for 20 sec, an annealing step at 56.4˚C for 15 sec and an extension step at 72˚C for 20 sec with a final extension step at 72˚C for 5 min. The PCR products were digested with 5 units *Nco*I at 37˚C for 15 h. The digested products were thereafter electrophoresed on a 10% acrylamide gel followed by ethidium bromide staining for genotype determination. The fragments obtained were 208 and 95 bp for the wild-type genotype GG; 208, 69 and 26 bp for the mutant genotype AA; and 208, 95, 69 and 26 bp for the GA genotype (Fig. 1). The accuracy of PCR-RFLP was confirmed by direct sequencing using a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

Data analysis. Data analysis was performed using the SPSS software V11.0 (SPSS, Inc., Chicago, IL, USA). The χ^2 and

Table I. Patients and tumor characteristics.

Characteristics	Total cases	(%)
Gender		
Male	30	60
Female	20	40
Age (years)		
>50	38	76
<50	12	24
Tumor size (cm)		
<5	28	56
>5	22	44
Location		
Colon	35	70
Rectum	15	30
Depth of tumor invasion		
T4	3	6
T3	47	94
Lymph node metastasis		
Negative	22	44
Positive	28	56
Stage		
II	22	44
III	28	56
Histology[a]		
WD + MD	48	96
PD	2	4

[a]WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

Fisher's exact tests were used to analyze the data. Any possible association between the development of the disease and the studied polymorphism was evaluated by calculating odds ratios (ORs) and 95% confidence intervals (CIs) from contingency tables and using a two-sided Fisher's exact test. Survival analysis was performed using the Kaplan-Meier method and differences between the survival curves were evaluated with a log-rank test. Disease-free survival (DFS) was defined in months from the day of surgery to the first event of documented relapse or death. For those patients who did not relapse, data were recorded during the last follow-up. Overall survival (OS) was defined as the time from the date of coloctomy to death; data on survivors were censored at the last follow-up.

Results

A total of 50 patients diagnosed with stage II/III CRC (median age 60 years and range 17-77 years) were enrolled in the study. Of these, 60 % were males and 52% were in UICC stage III. Other clinicopathological characteristics of the patients are listed in Table I. All patients were treated with 12 cycles of the FOLFOX-4 regimen for 6 months. The follow-up duration was 24-48 months. Fifteen cases (30%) experienced an early relapse, either a local recurrence or distant metastasis, within 1 year of follow-up.

Table II. Genotypes and allele frequencies of the G1249A polymorphism in 50 colorectal cancer (CRC) patients and healthy controls.

	Genotype N (%)			Allele (%)	
	GG	GA	AA	G	A
Patient	16 (32)	28 (56)	6 (12)	60	40
Control	22 (44)	23 (46)	5 (10)	67	33
P-value	0.151	0.121	0.500	0.189	-
Odds ratio	2.597	0.667	0.814	-	0.739

Table III. Characteristics of patients according to G1249A genotypes.

Characteristics	GG	GA or AA	P-value
Gender			0.247
Male (n=30)	8	22	
Female (n=20)	8	12	
Age (years)			0.120
>50 (n=38)	10	28	
<50 (n=12)	6	6	
Tumor size (cm)			0.120
<5 (n=28)	6	6	
>5 (n=22)	10	28	
Location			0.572
Colon (n=35)	11	24	
Rectum (n=15)	5	10	
Depth of tumor invasion			0.237
T3 (n=47)	14	33	
T4 (n=3)	2	1	
Lymph node metastasis			0.587
Negative (n=22)	8	14	
Positive (n=28)	8	20	
Stage			0.587
II (n=22)	8	14	
III (n=28)	8	20	
Histology[a]			-
WD + MD (n=48)	14	34	
PD (n=2)	2	0	

[a]WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

G1249A polymorphism and CRC risk. Table II presents OR estimates of CRC risk for each genotype of the MRP2 gene SNP. Of the 50 patients, the MDR2 wild-type genotype (1249GG) was observed in 32% of patients, whereas 56% were

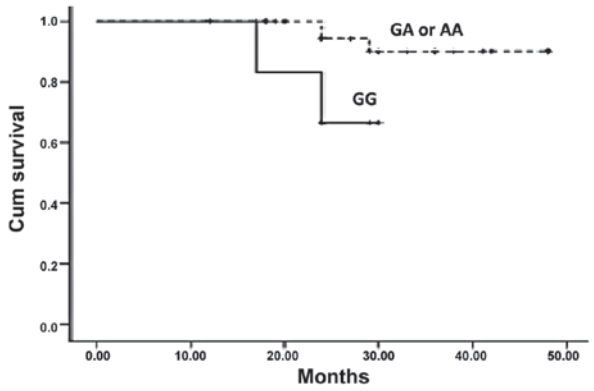

Figure 2. Effect of genotypes on the overall survival of patients with colorectal cancer (CRC). A significant association between G1249A polymorphism of MRP1 and overall survival was observed (P=0.043).

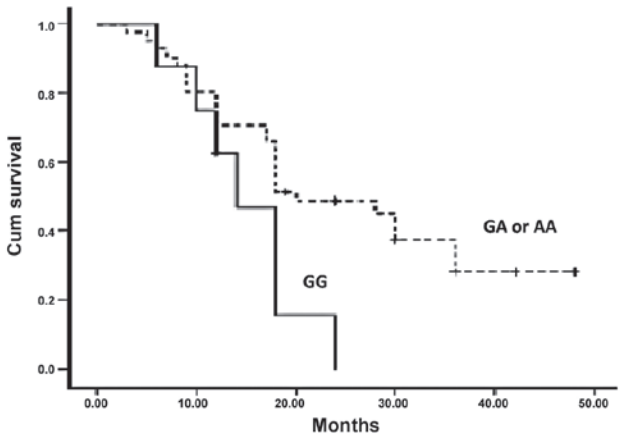

Figure 3. Effect of genotypes on the disease-free survival of patients with colorectal cancer (CRC). A significant association between G1249A polymorphism of MRP1 and disease-free survival was observed (P=0.043).

heterozygous (1249GA) and 12% were homozygous (1249AA) for the mutation. The 1249GG, 1249GA and 1249AA genotypes were found in 44, 46 and 10% of the controls, respectively. For the GG, AA and GA genotypes, no statistically significant association was identified between the SNP and CRC risk. In addition, no significant trend was identified in the number of alleles with respect to CRC occurrence.

Correlation between G1249A polymorphism and clinicopathological markers. No significant association was observed between the G1249A genotypes (GG, GA and AA) and the patient's characteristics, including age, gender, tumor location, tumor size, deep tumor invasion, disease stage and the presence or absence of lymph node metastasis (P>0.05; Table III).

Correlation between prognostic and clinicopathological markers. To investigate whether various clinicopathological data of the patients correlate with chemotherapy response, disease recurrence was evaluated according to the clinicopathological factors. None of those factors were identified to be associated with clinical response in the CRC patients (P>0.05). In addition, all factors were examined using the Kaplan-Meier method. These parameters did not correlate with the OS or DFS of the patients (all P>0.05), with the exception of deep tumor invasion. The correlation between deep tumor invasion and survival rates, although significant, was not suitable for evaluation, since the number of patients with T4 type tumors was very small.

Correlation between G1249A polymorphism and prognosis. To identify any possible correlation between the genotypes of the G1249A polymorphism and the survival rates and early recurrence, the patients were divided into the normal group (GG) and the mutation group (GA and AA). The polymorphic genotype of MRP2 was not different between the patients who responded and those who did not respond to the adjuvant FOLFOX-4 chemotherapy (P=0.468, P>0.05). However, a significant association between G1249A genotypes and survival rates was observed when Kaplan-Meier survival curves were plotted (Figs. 2 and 3). Thus patients with a mutant allele A, either homozygous or heterozygous, of G1249A had higher DFS and OS than patients with wild-type allele G (P=0.045 and 0.043).

Discussion

Among platinum drugs, oxaliplatin is the favored drug for the therapy of CRC. Oxaliplatin combined with 5FU/LV (the FOLFOX-4 regimen) is currently used for the treatment of stage II/III CRC patients who have undergone complete resection of the primary tumor. Expression of ABC-transporter proteins, particularly MRP2, has been demonstrated to be associated with resistance to platinum-based anticancer drugs, including cisplatin (18,19). Hence, variations in the MRP2 gene may be important for evaluating and/or predicting the response to platinum drugs. The role of MRP2 genetic polymorphisms on the response of CRC to platinum-based chemotherapy has not previously been reported. Among the polymorphisms of the MRP2 gene, G1249A has been found to be a common SNP that affects mRNA levels (13,14). Owing to its possible effect on gene expression, we anticipated that this polymorphism of the MRP2 gene may affect the tumor response to adjuvant FOLFOX-4 chemotherapy in CRC. To examine our hypothesis, we investigated whether functional polymorphism of G1249A in the MRP2 gene affected CRC occurrence and/or correlated with early recurrence in patients treated with the FOLFOX-4 regimen. We selected a homogeneous population in stage II/III who had not received any preoperative treatment. The genotype frequencies of the MRP2 G1249A polymorphism did not change during malignancy and were identical in the tumor and the matched normal tissues in our selected population. Therefore, *de novo* mutation of the MRP2 gene, which may affect cancer development and drug response, does not appear to occur in CRC. In addition, we analyzed the genetic polymorphism in the DNA isolated from paraffin-embedded samples obtained from the patients' matched normal tissues and in the peripheral blood of the controls. A previous study has verified that the genotyping results of DNA isolated from tissue are equivalent to those of DNA isolated from blood (20). The frequency of the A allele for the G1249A polymorphism in the controls was 33%, which is inconsistent with that reported in the literature (11). No association was observed in the present study between the SNP of

the MRP2 gene in exon 10 and the development of CRC. By contrast, an association between this polymorphic genotype and the risk of primary colorectal adenocarcinoma has been previously reported in Japanese patients (21).

We have also analyzed this SNP to investigate its significance in relation to platinum-based chemotherapy. The genotype frequencies of G1249A were not different between the patients who relapsed within one year and those who did not. Our results indicate that the mutant allele in exon 10 was not related to the response to adjuvant FOLFOX-4 chemotherapy. Similar results were obtained by Sun *et al* (11) in Chinese advanced non-small cell lung cancer (NSCLC) patients who were treated with a platinum-based drug. By contrast, a significant correlation between G1249A and the response to chemotherapy was observed in advanced ovarian cancer by Xingsheng *et al* (22). In addition, in Kaplan-Meier curves, the adenocarcinoma patients with a GG genotype of G1249A demonstrated a significantly shorter overall survival and disease-free survival than patients with AA or GA genotypes. Thus, we demonstrated that G1249A MRP2 is a molecular predictive marker for the survival of patients with stage II/III CRC treated with adjuvant FOLFOX-4 therapy following curative resection.

In conclusion, the MRP2 G1249A polymorphism was not associated with the incidence of CRC in the Iranian population. In addition, this polymorphism did not affect the prognosis of the disease in our population. Although, the considered patient population was small in size, it was very homogeneous, and hence was suitable for prognostic evaluation.

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