MDR1, *MRP1* and *MRP2* Genotypes and *In Vitro* Chemosensitivity in Japanese Patients with Colorectal Adenocarcinomas

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   In our previous paper, the chemosensitivity of human colorectal adenocarcinoma was evaluated against 12 anticancer drugs including 5-fluorouracil (5-FU), 7-ethyl-10-hydroxycamptothecin (SN-38), mitomycin C (MMC) and cisplatin (CDDP), and it was found that the anticancer drugs were effective against those with a relatively high growth rate.   MMC was effective for those with a relatively high mRNA expression of the multidrug resistance-associated protein 2 (MRP2), whereas no correlation was found for the multidrug resistant transporter MDR1 and MRP1.   In this study, 3 genotypes of *MDR1*, 4 genotypes of *MRP1*, and 6 genotypes of *MRP2* were additionally evaluated, and it was suggested that *MDR1* C3435T and *MRP2* G1249A were related with the susceptibility to colorectal adenocarcinoma.   The chemosensitivity against 5-FU, SN-38, MMC and CDDP was independent of *MDR1* C3435T, *MRP1* G2168A, and *MRP2* C-24T (C3972T), possibly due to no association with the growth rate of and mRNA expression levels of MDR1, MRP1 and MRP2 in the adenocarcinoma, however, *MDR1* C3435T tended to be accompanied with a higher expression of MDR1 mRNA.

INTRODUCTION

   Chemosensitivity tests are useful for optimizing the chemotherapeutic treatment of cancer by selecting anticancer drugs and/or therapeutic methods suitable for each patient, and various *in vitro* tests have been developed for the last 20 years, including the subrenal capsule (SRC) assay (1), human tumor clonogenic assay (HTCA) (23), thymidine incorporation assay (TIA) (24), succinic dehydrogenase inhibition (SDI) assay (12), methylthiazoletetrazolium (MTT) assay (14), and histoculture drug response assay (HDRA) (4, 26).   However, these assays have not yet been adopted on a widespread clinical basis

due to the requirements of relatively large specimens, low success rate of primary culture, contamination of fibroblasts, and relatively high concentration of anticancer drugs than clinically achievable concentrations.   To overcome these problems, the collagen gel droplet embedded culture drug sensitivity test (CD-DST) was developed in 1997 (10, 11). By embedding and culturing tumor cells in 30 μL collagen gel droplets, assessment is possible using a relatively small number of cells (3 x 10^3 cell/30 μL) with a high success rate of *in vivo*-like primary three-dimensional culture.   The exposure concentration and time of anticancer drugs has been defined by a comparative evaluation with clinical results (10, 11, 27).   The application of imaging analysis enables the selective quantification of tumor colonies without inaccuracy caused by fibroblast contamination.   Consequently, an excellent predictability for clinical response and usefulness have been demonstrated using breast cancer, lung cancer, gastric cancer and carcinomas of the pancreas and biliary tract (5, 6, 27).

In our previous paper, the CD-DST method was used to assess the chemosensitivity of human colorectal adenocarcinomas (15).   The chemosensitivity was evaluated against anticancer drugs often prescribed for patients with colorectal adenocarcinomas, that is, 5-fluorouracil (5-FU), irinotecan hydrochloride (CPT-11), mitomycin C (MMC) and cisplatin (CDDP).   For CPT-11, 7-ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of CPT-11, was used instead of CPT-11.   Additionally, the chemosensitivity was evaluated against 8 anticancer drugs to search more effective ones, and the mRNA expression was evaluated for the multidrug resistant transporter MDR1, and the multidrug resistance-associated proteins 1 and 2 (MRP1 and MRP2) (3, 18-20, 22, 25).   It was concluded that 1) the chemosensitivity was successfully evaluated for 64% of patients, 2) the anticancer drugs were effective against the samples showing a relatively high growth rate, 3) gemcitabine hydrochloride was more promising than those often prescribed for the treatment of colorectal adenocarcinoma, and 4) there was no correlation of the mRNA expression levels of MDR1 and MRP1 with the chemosensitivity of any anticancer drugs tested, but MMC was more effective for the colorectal adenocarcinoma with relatively high expression of MRP2 mRNA.

Recent advances in the pharmacogenetics and pharmacogenomics have strongly suggested potential implications of genetic polymorphisms of ABC transporters in inter-individual variations in cancer chemotherapy (3, 18-20, 22, 25).   In the present study, the distributions of *MDR1* genotypes of T-129C, G2677(A,T) and C3435T, *MRP1* genotypes of G128C, C218T, G2168A and G3173A, and *MRP2* genotypes of C-24T, G1249A, C2302T, C2366T, C3972T and G4348A were evaluated in Japanese patients with primary colorectal adenocarcinoma, and their effects on the chemosensitivity against 5-FU, SN-38, MMC and CDDP, and also on the growth rate of tumor and mRNA expression levels of MDR1, MRP1 and MRP2 in the adenocarcinoma were examined for future individualization of cancer chemotherapy based on the genotyping of these transporters.

MATERIALS AND METHODS

Human colorectal adenocarcinoma and chemosensitivity test. Twenty-five patients with primary colorectal adenocarcinoma diagnosed at Kobe University Hospital were enrolled in the previous (15) and this studies.   They had never undergone radiation or cancer chemotherapy.   Colorectal adenocarcinomas were obtained as surgical samples immediately after resection, quickly stripped of connective tissue, and stored at 4°C in culture medium for evaluation of the chemosensitivity for 12 anticancer drugs, which was conducted within 24 hours.   Chemosensitivity was successfully evaluated by the CD-DST

method as reported before for 16 of 25 patients, and the data was presented in the previous report (15).   Briefly, the tumor cells prepared were cultured in 30 μL collagen droplet (3 x 10^3 cells/droplet), and after overnight incubation, the droplet was treated with an anticancer drug at a designated concentration.   The concentrations of 1.0 μg/mL (7.69 μM) for 5-FU, 0.03 μg/mL (0.0897 μM) for MMC and 0.2 μg/mL (0.667 μM) for CDDP were used for CD-DST (5, 6, 10, 11, 27) and that of 0.03 μg/mL (0.0750 μM) for SN-38 was determined as the quotient of the area under the concentration-time curve by 24 hours, the exposure time for anticancer drugs in CD-DST (15).   Each droplet was washed after incubation for 24 hours, followed by culturing in the anticancer drug-free medium for 7 days.   The growth rate of the cells was evaluated as the ratio of total volume of tumor colonies after the culture for 7 days to that before the culture in the untreated group, and the data where the growth rate in drug free well was more than 0.8 were successfully adopted (4, 15).   The chemosensitivity was evaluated based on the ratio of total volume of tumor colonies in the drug-treated group (T) to that in the untreated group (C).   The assay was conducted in triplicate, and the average values of T/C% after the culture for 7 days were adopted as an index of chemosensitivity, and a T/C% less than or equal to 50% was considered effective and that greater than 60% ineffective.   Aliquots of the samples were snap-frozen and stored at -80°C for the assay of mRNA expression of three transporters by real time quantitative RT-PCR analysis (16, 17, 21).   In this study, the genotypes of these transporters were evaluated using the remaining samples as described below.   Informed consent was obtained from all subjects prior to their participation in the study.   The protocol was approved by the Institutional Review Broad of Kobe University Hospital, Kobe University, Japan.

MDR1, MRP1 and MRP2 genoptyping. Colorectal adenocarcinoma sample cut up into small pieces was placed in a 1.5-ml microcentrifuge tube, and then genomic DNA was extracted using a DNeasy Tissue Kit® (QIAGEN) according to the manufacturer's protocol. The polymorphism of T-129C in the promoter region, a missense polymorphism of G2677(A,T), and a silent polymorphism of C3435T in the *MDR1* gene, 4 missense polymorphisms of G128C, C218T, G2168A and G3173A in the *MRP1* gene, and a polymorphism of C-24T in the 5'-flanking region, 4 missense polymorphisms of G1249A, C2302T, C2366T and G4348A, and a silent polymorphism of C3972T in the *MRP2* gene were evaluated by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and confirmed by direct sequencing.   The details of *MDR1* genotyping were reported previously (8, 13, 17, 22). and the polymorphisms of *MRP1* and *MRP2* genes were identified according to the report of Ito *et al.*(9).

Statistical analysis. Values are given as the mean ± standard deviation (SD). Statistical significance of differences between mean values was calculated using the non-paired t-test, and multiple comparisons were performed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Table 1 lists the genotype frequency of *MDR1*, *MRP1* and *MRP2* in 13 Japanese patients with colorectal adenocarcinoma.   We have reported *MDR1* C3435T genotype and allele frequency in the healthy subjects showing that CC/CT/TT was 41/62/14 in genotype and C/T was 144/90 in allele frequency (8).   In addition, Ito *et al.*, have reported on *MRP2* G1249C position, showing that GG/GA/AA was 37/10/1 in genotype and G/A was 84/12 in allele frequency.   Compared with the healthy subjects, T-allele at C3435T of *MDR1* was more frequently found in patients with colorectal adenocarcinoma, whereas A-allele at G1249A of

MRP2 was found less frequently, although this is from a small number of patients with insufficient statistical power. Frequencies at other positions are comparable with the healthy subjects. A polymorphism of C3435T is reported to be a risk factor for a certain class of diseases such as inflammatory bowel diseases, Parkinson's disease and renal epithelial tumor, and this is considered to be explained by the effects on MDR1 expression and function (3, 18-20, 25). Further investigations with a larger number of subjects should be addressed.

TABLE 1. *MDR1*, *MRP1* and *MRP2* genotypes in 13 Japanese patients with colorectal adenocarcinomas

	Position	Genotype			Allele	
		w/w	w/m	m/m	w	m
MDR1	T-129C	11	2	0	24	2
	G2677(A,T)	3	6	4	12	14
	C3435T	3	6	4	12	14
MRP1	G128C	13	0	0	26	0
	C218T	13	0	0	26	0
	G2168A	10	3	0	23	3
	G3173A	13	0	0	26	0
MRP2	C-24T	7	4	2	18	8
	G1249A	12	1	0	25	1
	C2302T	13	0	0	26	0
	C2366T	13	0	0	26	0
	C3972T	7	4	2	18	8
	G4348A	13	0	0	26	0

The symbols "w" and "m" mean the polymorphisms with higher and lower frequency of allele, respectively.

The effects of *MDR1* C3435T, *MRP1* G2168A and *MRP2* C-24T (C3972T) genotypes on the chemosensitivity against 5-FU, SN-38, MMC and CDDP is shown in Table 2. Various anticancer drugs are considered to be the substrate for MDR1, including CPT-11, MMC, anthracyclines, taxans, *vinca*-alkaloids and etoposide (18, 19). MRP1 plays an important role in cancer chemotherapy with docetaxel, methotrexate, *vinca*-alkaloids and etoposide (25). MRP2 also indicates broad substrate specificity, and the chemotherapeutic effects of CPT-11, CDDP and vinblastine would be defined by MRP2 (2, 7, 25). However, these genotypes had no effects on the chemosensitivity against 5-FU, SN-38, MMC and CDDP (Table 2). 5-FU was not effective for 10 of 13 patients, and also SN-38, MMC and CDDP were not effective for 10, 9 and 11 patients (data not shown), respectively, suggesting insufficient concentrations used in CD-DST of 5-FU (1.0 µg/mL), SN-38 (0.03 µg/mL), MMC (0.03 µg/mL) and CDDP (0.2 µg/mL). However, these concentrations had been

defined by the comparative evaluation with clinical results (10, 11, 27), and those for 5-FU, MMC and CDDP were often used for CD-DST (5, 6, 10, 11, 27).

TABLE 2. Effects of *MDR1, MRP1* and *MRP2* genotypes on T/C% values of 4 anticancer drugs assessed by CD-DST in 13 Japanese patients with colorectal adenocarcinomas

	Position	Genotype	N	5-FU	SN-38	MMC	CDDP
MDR1	3435	CC	3	70.9±17.9	66.3±26.0	86.3±43.2	97.8±38.6
		CT	6	58.8±22.9	68.8±32.8	70.6±39.7	88.7±43.2
		TT	4	82.7±17.8	70.3±19.8	83.8±25.0	97.2±16.8
MRP1	2168	GG	10	68.6±23.6	69.8±27.3	80.2±35.9	95.6±34.9
		GA	3	70.0±17.0	64.7±24.2	71.7±34.6	86.1±33.6
MRP2	-24 & 3972	CC	7	72.1±16.1	70.1±31.1	84.0±31.4	98.9±37.1
		CT	4	68.4±31.6	65.9±26.1	71.2±42.7	88.1±26.7
		TT	2	38.9, 79.1	64.6, 74.0	38.5, 106.0	49.3, 120.3

The values are the mean ± SD.

In the previous report, it was found that the anticancer drugs were effective against the samples showing a relatively high growth rate, and MMC was more effective for the colorectal adenocarcinoma with relatively high expression of MRP2 mRNA, thus the association of these genotypes with the growth rate and expression was also evaluated (Tables 3 and 4). As shown in Tables 3 and 4, there was no statistically significant effect of genotypes, and therefore the lack of dependency of genotypes on the chemosensitivity seemed to be due to there being no effect on the growth rate and expression. As for MDR1, the mRNA expression tended to higher in homozygotes for T-allele, similarly to the effects on the mRNA expression in duodenal enterocytes (17). Recent pharmacogenetic studies on the drug transporters strongly suggest the importance of the haplotype analysis of the genetic polymorphisms. Therefore, it will be necessary to obtain the conclusions on the effects of genotypes on the chemosensitivity.

In conclusion, *MDR1* genotypes of T-129C, G2677(A,T) and C3435T, *MRP1* genotypes of G128C, C218T, G2168A and G3173A, and *MRP2* genotypes of C-24T, G1249A, C2302T, C2366T, C3972T and G4348A were evaluated in the Japanese patients with primary colorectal adenocarcinoma, and it was suggested that *MDR1* C3435T and *MRP2* G1249A were related with the susceptibility to colorectal adenocarcinoma. The chemosensitivity against 5-FU, SN-38, MMC and CDDP was independent of *MDR1* C3435T, *MRP1* G2168A, and *MRP2* C-24T (C3972T), possibly due to no association with the growth rate of and mRNA expression levels of MDR1, MRP1 and MRP2 in the adenocarcinoma, however, *MDR1* C3435T tended to be accompanied with a higher expression of MDR1 mRNA.

TABLE 3. Effects of *MDR1*, *MRP1* and *MRP2* genotypes on growth rate of colorectal adenocarcinomas

	Position	Genotype	N	Growth Rate
MDR1	3435	CC	3	1.91±1.02
		CT	6	3.28±2.74
		TT	4	1.36±0.77
MRP1	2168	GG	10	2.49±2.34
		GA	3	2.00±0.88
MRP2	-24 & 3972	CC	7	1.52±0.83
		CT	4	3.17±3.31
		TT	2	4.93, 2.64

The values are the mean ± SD.   It is noted that the sample did not remained for 1 patient.

TABLE 4. Effects of *MDR1*, *MRP1* and *MRP2* genotypes on expression of their mRNA in colorectal adenocarcinomas

	Position	Genotype	N	mRNA
MDR1	3435	CC	3	1.49±1.60
		CT	6	1.50±1.49
		TT	3	8.90±7.53
MRP1	2168	GG	9	2.48±2.31
		GA	3	2.25±1.42
MRP2	-24 & 3972	CC	7	0.06±0.06
		CT	3	0.15±0.15
		TT	2	0.03, 0.01

The values are the mean ± SD.   It is noted that the expression was not evaluated for 1 patient.

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