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Combination of O^6-methylguanine-DNA methyltransferase and thymidylate synthase for the prediction of fluoropyrimidine efficacy

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ABSTRACT

We investigated the correlation between the response to fluoropyrimidines as first-line therapy and the expressions of genes in patients with primary colorectal cancer (CRC). The study group comprised 92 patients with metastatic CRC. Total RNA was isolated from laser-captured tumour cells in surgically resected primary lesions, and gene expression was quantitatively evaluated by real-time RT-PCR assay. Low thymidylate synthase (TS), low γ-glutamyl hydrolase, high reduced folate carrier 1, high O^6-methylguanine-DNA methyltransferase (MGMT) and low cyclin E expressions were associated with a good response ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively) on univariate analysis. On multivariate logistic regression analysis, TS and MGMT remained independent predictors of the response. The clinical response rates were 63.2% in the low TS or high MGMT group and 14.3% in high TS and low MGMT group ($P < 0.0001$). The combination of high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment.

1. Introduction

The median survival time of patients with colorectal cancer (CRC) has improved in the past 10 years because of the development of new agents with advantages over 5-fluorouracil (5-FU), including irinotecan hydrochloride (CPT-11) and oxaliplatin. CPT-11 or oxaliplatin monotherapy, however, was not shown to be more effective than bolus 5-FU/leucovorin (LV) in terms of response and median survival time. CPT-11 or oxaliplatin plus bolus or infusional 5-FU/LV regimens were found to be clearly more effective than 5-FU/LV, resulting in a doubling of the tumour response rate and prolongation of median survival time by 2–3 months. Regimens combining CPT-11 or oxaliplatin with fluoropyrimidines are now key first- and second-line chemotherapies for CRC. Response rates with these regimens, however, remain around 40–50%, prompting investigations of molecular predictors of the response to specific chemotherapeutic regimens. In this study, we evaluated molecular markers that could be used to predict the clinical outcomes of treatment with fluoropyrimidine-based

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regimens, now widely used to treat CRC. Because data on CPT-11-based regimens not including fluoropyrimidines will be difficult to obtain in the future, we also investigated such regimens used for second-line treatment in this study.

Evaluations of regimens including fluoropyrimidines alone as well as those including CPT-11 without fluoropyrimidines are required to produce benchmarks for predicting the efficacy of combined treatment with fluoropyrimidines and CPT-11.

Many potential predictors of the response to fluoropyrimidines have been reported. Several enzymes involved in the targeting, metabolism and catabolism of fluoropyrimidines have been extensively studied, including thymidylate synthase (TS) and dihydropyrimidine dehydrogenase (DPD).[1–3] The enzymes concerning folinic acid metabolism and transport are also the important factors involved in the efficacy of biochemical modulation of 5-FU by LV; among these being folylpolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH) and reduced folate carrier 1 (RFC1).[4,5]

The role of molecular markers in predicting the response to CPT-11-based chemotherapy remains largely unclear, as compared with oxaliplatin-based chemotherapy for CRC, for which several promising markers have been identified.[6] Recently, comprehensive analysis based on the microarray gene expression also have been performed to clarify the predictive markers for CPT-11/5-FU/LV treatment.[7] DNA topoisomerase I (TOPO I) may be a useful predictor of the response to CPT-11-based treatments in colon cancer cell lines as well as in patients with metastatic CRC.[8] Factors involved in DNA-repair systems, such as excision repair cross-complementing 1 (ERCC1) and O^6-methylguanine-DNA methyltransferase (MGMT), have also been investigated recently with respect to their role in resistance to CPT-11.[9,10] The relations between response and factors involved in drug detoxification, such as glutathione S-transferase pi (GSTpi), have been studied for many chemotherapeutic agents, including CPT-11.[9,10] On the other hand, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and cell-cycle-regulation genes, such as cyclin E, have been reported to be related to the outcomes of patients with CRC.[11]

To gain further insight into potentially useful markers, we investigated the correlation between clinical response and the expressions of genes involved in the targeting, metabolism or catabolism of fluoropyrimidines, drug detoxification, cell cycles and DNA-repair systems in patients with metastatic or recurrent CRC who received first-line fluoropyrimidine-based regimens with or without LV or second-line CPT-11-based regimens.

2. Patients and methods

2.1. Patient selection and chemotherapy

This clinical–biological correlative study was performed retrospectively in a consecutive series of patients who underwent surgery for primary CRC at our hospital between 1996 and 2003 and received first-line fluoropyrimidine-based regimens for metastatic or recurrent CRC. Their responses to treatment and outcomes were confirmed. Patients who received second-line CPT-11-based chemotherapy were analysed as a subgroup. Approval for this study was obtained from the institutional review board of the National Cancer Center Hospital, Tokyo.

Fluoropyrimidines included 5-FU/*l*-LV (5-FU 600 mg/m^2 bolus and *l*-LV 250 mg/m^2 div, weekly × 6, q 8 weeks), continuous infusion of 5-FU (5-FU 250 mg/m^2/day), uracil-tegafur (UFT)/LV (UFT 300 mg/day and LV 75 mg/day per os, 4 weeks on and 1 week off), UFT alone (UFT 300 mg/day per os, 4 weeks on and 1 week off) and TS-1 (TS-1 80, 100 or 120 mg/day per os, 4 weeks on and 2 weeks off). CPT-11-based chemotherapy included CPT-11 alone (CPT-11 150 mg/m^2 div, biweekly) and CPT-11/mitomycin C (CPT-11 150 mg/m^2 div and mitomycin C 5 mg/m^2 bolus, biweekly).

2.2. Clinical evaluation and response criteria

Clinical response was evaluated every 6–8 weeks by CT imaging. Responders to treatment were classified as those patients whose tumours shrank by 50% or more, as estimated on two observations not less than 6 weeks apart. More precisely, a complete response (CR) was defined as the complete disappearance of all evidence of tumour, while a partial response (PR) was defined as a greater than 50% decrease in the sum of the products of the largest perpendicular diameters of all measurable lesions, without the occurrence of new lesions. Amongst those classified as non-responders, stable disease (SD) was defined as a change of less than 25% in tumour size, and progressive disease (PD) was defined as an increase of greater than 25% in the area of the measurable tumour deposits or the appearance of new lesions. Time to progression (TTP) during first-line or second-line chemotherapy was defined as the period from the starting date of chemotherapy to the date on which progression was confirmed.

2.3. Laboratory methods

Ten-micrometre-thick sections of resected primary CRC tumours were obtained from identified areas with the highest tumour concentration and were then mounted on uncoated glass slides. For histologic diagnosis, representative sections were stained with haematoxylin and eosin by standard methods. Before microdissection, sections were stained with nuclear fast red (NFR, American MasterTech Scientific, Lodi, CA). The sections of interest were selectively isolated by laser capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany), according to standard procedures.[12] The dissected particles of tissue were transferred to a reaction tube containing 400 μl of RNA lysis buffer.

The samples were homogenised and heated at 92 °C for 30 min. Fifty microlitres of 2 M sodium acetate was added at pH 4.0, followed by 600 μl of freshly prepared phenol/chloroform/isoamyl alcohol (250:50:1). The tubes were vortexed for 15 s, placed on ice for 15 min, and then centrifuged at 13,000 rpm for 8 min in a chilled (8 °C) centrifuge. The upper aqueous phase was carefully removed and placed in a 1.5-mL centrifuge tube. Glycogen (10 μl) and 300–400 μl of isopropanol were added and the samples were vortexed for 10–15 s. The tubes were chilled at −20 °C for 30–45 min to precipitate the RNA. The samples were then washed in 500 μl of 75% v/v ethanol and air-dried for 15 min. The pellet was

resuspended in 50 μl of 5 mM Tris. Finally, cDNA was prepared as described by Lord and colleagues.[13]

Quantification of the 12 genes of interest and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System, TaqMan®, Perkin–Elmer [PE] Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 1200 nM of each primer, 200 nM of probe, 0.4 U of AmpliTaq gold polymerase, 200 nM each of dATP, dCTP, dGTP and dTTP, 3.5 mM of $MgCl_2$ and 1 × Taqman buffer A containing a reference dye. The final volume of the reaction mixture was 20 μl (all reagents from PE Applied Biosystems, Foster City, CA). Cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 46 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and probes used are listed in Table 1. Gene expression values (relative mRNA levels) are expressed as ratios (differences between Ct values) between the gene of interest and the internal reference gene (β-actin).

2.4. *Statistical analysis*

To evaluate the association of gene expressions with response and TTP, gene expression levels were categorised into low and high values. To determine cutoff values, the maximally selected χ^2 method was employed.[14–16] For each observed value, patients were classified as falling below or equal to that value, or above that value. The maximally selected χ^2-test statistic was used to compare the response rates of the two resulting groups of patients (below or equal to the value versus above the value). The value that yielded the largest χ^2-test statistic (the maximal χ^2 statistic) was selected as the optimal cutoff point. To determine the *P*-value associated with the maximal χ^2 statistic, we performed 2000 bootstrap-like simulations. For each simulation, a randomly selected value was drawn (with replacement) from the set of observed values and assigned to each of the observed responses; the maximal χ^2 statistic was calculated based on this set of randomly matched values and responses. The corrected *P*-value was calculated as the proportion of the 2000 simulated maximal statistics that was larger than the original maximal χ^2 statistic. This analysis was repeated using the log-rank test to compare TTP. If promising significant predictive variables were found on this analysis, multivariate logistic regression analysis was preformed for the response to fluoropyrimidines. Stepwise variable selection was done using a significance level of 0.01 for entering into or remaining in the model.

All reported *P*-values are two-sided, and the level of significance was set at $P < 0.05$, except for stepwise variable selection. All analyses were performed using the statistical software package R, version 2.4.1 and the SAS statistical package, version 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Demographics and patients assessed for response and TTP*

A total of 92 Japanese patients (54 men and 38 women; median age, 61 years; range 27–77 years) were evaluated (Table 2). Seventy of these patients (43 men and 27 women; median age, 62 years; range 27–77 years) had received 5-FU/LV regimens, and 63 had received CPT-11 as second-line chemotherapy; 43 patients with CPT-11 and 20 patients with CPT-11/ mitomycin C. Gene expression levels of TS, DPD, FPGS, GGH, RFC1, TOPO I, ERCC1, MGMT, GSTpi, EGFR, VEGF and cyclin E were assessed in all patients, and the relations of these levels to response and TTP were examined.

The response to first-line fluoropyrimidine-based chemotherapy was CR in 2 patients (2%), PR in 39 (42%), NC in 30 (33%) and PD in 21 (23%). The median TTP was 5.1 months. The response to first-line 5-FU/LV treatment was CR in 2 patients (3%), PR in 31 (44%), NC in 21 (30%) and PD in 16 (23%). The median TTP was 5.0 months. For second-line CPT-11-based chemotherapy, the response was PR in 9 patients (14%), NC in 32 (50%) and PD in 22 (36%). The median TTP was 3.5 months.

Table 1 – Primers and probes

Gene	GenBank Accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Taqman probe (5′–3′)
β-Actin	NM_001101.2	GAGCGCGGCTACAGCTT	TCCTTAATGTCACGCACGATTT	ACCACCACGGCCGAGCGG
TS	NM_001071.1	GCCTCGGTGTGCCTTTCA	CCCGTGATGTGCGCAAT	TCGCCAGCTACGCCCTGCTCA
DPD	NM_000110.2	AGGACGCAAGGAGGGTTTG	GTCCGCCGAGTCCTTACTGA	CAGTGCCTACAGTCTCGAGTCTGCCAGT
FPGS	M98045	GGCTGGAGGAGACCAAGGAT	CATGAGTGTCAGGAAGCGGA	CAGCTGTGTCTCCATGCCCCCCTAC
GGH	NM_003878	GCGAGCCTCGAGCTGTCTA	AATATTCCGATGATGGGCTTCTT	ACCCCACGGCGACACCGC
RFC1	NM_194255.1	CATCGCCACCTTTCAGATT	TGGCAAAGAACGTGTTGAC	CCCGAAGACCAGGGCACAGA
TOPO I	NM_003286	TGTAGCAAAGATGCCAAGGT	TGTTATCATGCCGGACTTCT	CCTTCTCCTCCTCCAGGACATAAGTGGA
ERCC1	NM_001983.2	GGGAATTTGGCGACGTAATTC	GCGGAGGCTGAGGAACAG	CACAGGTGCTCTGGCCCAGCACATA
MGMT	NM_002412	CGTTTTCCAGCAAGAGTCGTT	GAAATCACTTCTCCGAATTTCACA	TCAGCAGCTTCCATAACACCTGTCTGG
GSTpi	X06547	CCTGTACCAGTCCAATACCATCCT	TCCTGCTGGTCCTTCCCATA	TCACCTGGGCCGCACCCTTG
EGFR	X00588	TGCGTCTCTTGCCGGAAT	GGCTCACCCTCCAGAAGGTT	ACGCATTCCCTGCCTCGGCTG
VEGF	NM_003376.4	AGTGGTCCCAGGCTGCAC	TCCATGAACTTCACCACTTCGT	TGATTCTGCCCTCCTCCTTCTGCCAT
Cyclin E	NM_001238	CAGCTTATTGGGATTTCATCTTT	ATACGCAAACTGGTGCAACT	TGCAGCCAAACTTGAGGAAATCTATCC

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione *S*-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

Table 2 – Characteristics of 92 patients treated with first-line fluoropyrimidine

Characteristic	Frequency
Median age, years (range)	61 (27–77)
Gender	
Male	54
Female	38
PS	
0	65
1	26
2	1
Metastatic site	
Liver	64
Lung	44
Lymph node	27
Peritoneum	19
Ovary	2
Bone	1
Regimens	
5-FU/l- LV	70
5-FU continuous infusion	10
UFT/LV	9
UFT	1
TS-1	2
Clinical response	
Complete response	2
Partial response	39
Stable disease	30
Progressive disease	21

PS: Performance status of Eastern Cooperative Oncology Group; 5-FU: 5-fluorouracil; it l-LV: l-leucovorin; UFT, uracil-tegafur; LV: leucovorin.

3.2. *Gene expression levels and clinical outcome of patients receiving first-line fluoropyrimidine-based treatment*

Median gene expression levels relative to the level of the house-keeping gene β-actin, used as an internal reference, are shown in Table 3. For descriptive purposes, we call gene expressions below the designated cut-point 'low' while those above the designated cut-point are called 'high'. The results of univariate analysis for response and TTP are shown in Table 3. Low TS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to fluoropyrimidines on univariate analysis ($P = 0.0030$, 0.0250, 0.0120, 0.0030 and 0.0020, respectively). Low TS, low GGH, high RFC1, low TOPO I, high MGMT, low GSTpi and low cyclin E expression levels significantly correlated with a long TTP in patients given fluoropyrimidine on univariate analysis ($P = 0.027$, 0.023, 0.045, 0.025, 0.039, 0.002 and 0.009, respectively).

Seventy of the 92 patients given fluoropyrimidines had received 5-FU/LV regimens. Low TS, high FPGS, low GGH, high RFC1, high MGMT and low cyclin E expression levels were significantly associated with a good response to 5-FU/LV on univariate analysis ($P = 0.0060$, 0.0350, 0.0355, 0.0415, 0.0030 and 0.0015, respectively). Low GGH, low GSTpi, high VEGF and low cyclin E expression levels significantly correlated with a long TTP in patients given 5-FU/LV on univariate analysis ($P = 0.016$, 0.045, 0.032 and 0.003, respectively).

3.3. *Multiple logistic regression analysis of clinical response in patients receiving first-line fluoropyrimidines*

Among the expressions of TS, GGH, RFC1, MGMT and cyclin E, which were significantly associated with response as well as TTP in patients given fluoropyrimidines, TS and MGMT expressions continued to be independent predictors of the response to fluoropyrimidines on multiple logistic regression analysis. The clinical responses of patient groups divided according to the cutoff values of TS and MGMT expressions are shown in Table 4. The sensitivity of low TS or high MGMT for the response to fluoropyrimidines (responding patients in the low TS and high MGMT groups/responding patients in all groups) was 0.88, and the specificity of high TS and low MGMT for the response to fluoropyrimidines (non-responding patients in the high TS and low MGMT groups/non-responding patients in all groups) was 0.59. The positive predictive value of low TS or high MGMT for the response to fluoropyrimidines was 0.63, and the negative predictive value of high TS and low MGMT was 0.86. The shortest TTP was observed in the group of patients with TS above and MGMT below the respective cutoff values ($P = 0.083$) (Fig. 1). The median TTP was 5.7 months in patients with low TS or high MGMT and 3.3 months in those with both high TS and low MGMT.

3.4. *Gene expression levels and clinical outcome of patients receiving second-line CPT-11-based treatment*

The results of univariate analyses for response and TTP are shown in Table 5. High TS, high FPGS, low ERCC1, high MGMT, high GSTpi and low VEGF expressions significantly correlated with a good clinical response to second-line CPT-11 treatment ($P = 0.0085$, 0.0145, 0.0015, 0.0215, 0.0155 and 0.0165, respectively). No significant correlation was demonstrated between any gene expression and TTP for second-line chemotherapy.

4. Discussion

Our primary end-point was to clarify the gene expression levels of enzymes involved in the targeting, metabolism or catabolism of fluoropyrimidines, the metabolism or transport of folinic acid, DNA-repair systems and drug detoxification systems and thereby identify predictors of clinical outcomes in patients with CRC who receive fluoropyrimidines. The presence of both high TS and low MGMT expression levels was found to be a significant predictor of a poor response to fluoropyrimidine therapy. Clinically, this combination would have an important role in the selection of first-line treatment for CRC with regimens such as FORFIRI (CPT-11/5-FU/LV) or CPT-11 alone.

MGMT is a DNA-repair enzyme that removes alkyl adducts from O^6-methylguanine. Since the MGMT gene is usually not mutated or deleted in human cancers, loss of MGMT function is probably due mostly to epigenetic changes.[17] Abnormal MGMT activity causes O^6-methylguanine to accumulate in cellular DNA, potentially resulting in the activation of oncogenes or inactivation of tumour suppressor genes, followed

Table 3 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 92 patients treated with first-line fluoropyrimidine-based regimens

Gene	Number of patients	mRNA expression levels relative to β-actin × 10^{-3}, Median (range)	Cut-point	Bootstrap P-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression						
TS	92	1.4 (0–10.9)	1.37	0.0030	60.0	29.8
DPD	87	0.22 (0–1.16)	0.48	0.0995	41.3	66.7
FPGS	92	0.49 (0.04–1.69)	0.49	0.1035	36.0	54.8
GGH	91	2.17 (0–9.94)	1.21	0.0250	68.4	38.9
RFC1	92	1.67 (0–8.79)	0.87	0.0120	18.8	50.0
TOPO I	92	1.71 (0–4.93)	1.59	0.1845	53.5	36.7
ERCC1	92	0.41 (0–2.95)	0.38	0.1680	36.4	52.1
MGMT	92	1.75 (0–64.73)	2.59	0.0030	34.3	72.0
GSTpi	92	2.19 (0.48–7.6)	3.71	0.0815	48.2	18.2
EGFR	92	0.86 (0–5.31)	0.48	0.2140	57.9	41.1
VEGF	92	3.86 (0.88–24.3)	4.83	0.2890	40.0	55.6
Cyclin E	92	0.51 (0–2.28)	0.99	0.0020	50.7	13.3

Gene	Number of patients	Cut-point	Bootstrap P-value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	92	1	0.027	230	132
DPD	87	0.31	0.128	141	232
FPGS	92	0.75	0.293	141	165
GGH	91	4.87	0.023	151	64
RFC1	92	0.87	0.045	68	155
TOPO I	92	2.68	0.025	155	105
ERCC1	92	0.6	0.296	137	165
MGMT	92	3.22	0.039	134	253
GSTpi	92	2.47	0.002	169	86
EGFR	92	0.6	0.161	148	141
VEGF	92	7.06	0.108	141	232
Cyclin E	92	1.09	0.009	155	57

Table 4 – Predictive value of TS, MGMT and their combination for the response to fluoropyimidne (TS and MGMT were selected as independent variables in multiple logistic regression analysis)

Gene expression status	Number of non-responding patients	Number of responding patients	RR (%)	P-value (χ^2-test)
All	51	41	44.6	–
Low MGMT	44	23	34.3	0.0012
High MGMT	7	18	72.0	
Low TS	18	27	60.0	0.0036
High TS	33	14	29.8	
High MGMT or low TS	21	36	63.2	<.0001
Low MGMT and high TS	30	5	14.3	

TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

by carcinogenesis.[17–20] In previous studies, the significance of the correlation between MGMT promoter hypermethylation or loss of MGMT expression and patients' prognosis was controversial,[21–25] and its prognostic value for patients treated with specific regimens of anticancer agents remains a matter of debate.

As for CRC, abnormal MGMT expression has been examined in many studies in connection with microsatellite instability (MSI) or CpG island methylator phenotype (CIMP).[25–27] Kohonen-Corish and colleagues reported that low-MSI characterised a distinct subgroup of patients with stage C colon cancer who had poor outcomes.[25] They also found that loss or reduced MGMT protein expression was associated with the low-MSI phenotype, but was not a prognostic factor for overall survival in colon cancer.[25] Recent studies have shown that 5-FU-based adjuvant chemotherapy improves overall

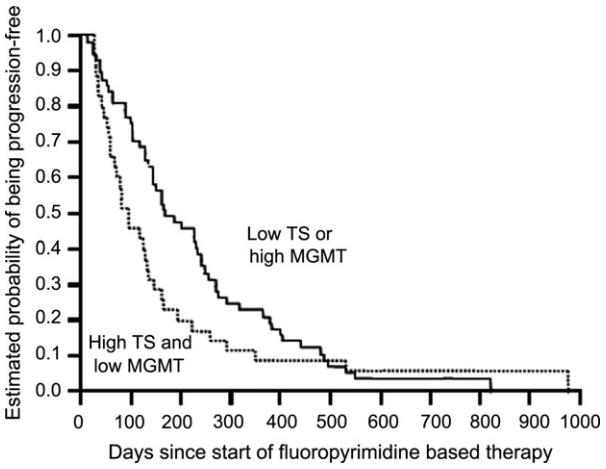

Fig. 1 – Time to progression in patients given fluoropyrimidines according to the cutoff levels of TS and MGMT expression ($p = 0.083$). TS: thymidylate synthase, MGMT: O^6-methylguanine-DNA methyltransferase.

survival in patients who have non-high-MSI CRC as compared with those who have high-MSI CRC.[28,29] On the other hand, Nagasaka and colleagues reported that the later the tumour stage at diagnosis, the less likely MGMT promoter will be methylated; in addition, the recurrence rate associated with oral fluoropyrimidine-based adjuvant chemotherapy was significantly higher in patients with unmethylated MGMT than in those with methylated MGMT (not adjusted for MSI status).[27] To date, the value of MGMT as a prognostic or predictive marker for fluoropyrimidines remains controversial. In this study, MGMT expression was found to be the most significant biomarker for the response to fluoropyrimidines. Furthermore, MGMT combined with TS, one of the most promising enzymes for predicting clinical outcomes of fluoropyrimidine treatment, was shown to be a powerful predictor of the response to fluoropyrimidines.[1,30]

In the subset analysis of 70 patients who received 5-FU/LV as first-line chemotherapy, three enzymes involved in the formation, degradation and transfer into cells of folates, i.e. FPGS, GGH and RFC1, were significantly related to response and TTP. It suggests that high FPGS, low GGH and high RFC1 activity promotes optimal modulation of 5-FU by LV, probably by augmenting the retention of high levels of reduced polyglutamated folates in tumours. We also investigated the predictive value of gene expression levels in patients who received second-line CPT-11-based chemotherapy. Ideally, predictive markers should be evaluated by large prospective randomised trials in patients receiving first-line chemotherapy, and our results suggested that TS, FPGS, ERCC1, MGMT,

Table 5 – Univariate analysis of gene expression levels and clinical outcome (A: response, B: time to progression) in 63 patients treated with second-line CPT-11-based regimens

Gene	Number of patients	Cut-point	Bootstrap *P*-value	RR (%) in low group	RR (%) in high group
A: Correlation between response and gene expression					
TS	63	1.43	0.0085	5.7	25.0
DPD	60	0.42	0.4735	11.5	25.0
FPGS	63	0.83	0.0145	10.9	37.5
GGH	62	1.1	0.2390	0.0	15.4
RFC1	63	0.77	0.3420	28.6	12.5
TOPO I	63	2.13	0.1630	10.6	25.0
ERCC1	63	0.11	0.0015	44.4	9.3
MGMT	63	1.74	0.0215	6.3	22.6
GSTpi	63	2.43	0.0155	5.3	28.0
EGFR	63	0.39	0.0810	33.3	11.1
VEGF	63	1.97	0.0165	37.5	10.9
Cyclin E	63	1.02	0.056	11.1	33.3
Gene	Number of patients	Cut-point	Bootstrap *P*- value	Median TTP (day) in low group	Median TTP (day) in high group
B: Correlation between time to progression (TTP) and gene expression					
TS	63	1.38	0.089	97	103
DPD	60	0.14	0.231	99	102
FPGS	63	0.35	0.096	151	98
GGH	62	1.04	0.058	57	102
RFC1	63	1.32	0.221	103	98
TOPO I	63	2.71	0.100	98	113
ERCC1	63	0.11	0.465	119	99
MGMT	63	1.64	0.086	87	110
GSTpi	63	2.89	0.060	92	155
EGFR	63	0.39	0.119	118	98
VEGF	63	2.78	0.243	151	92
Cyclin E	63	0.78	0.321	87	113

TS: thymidylate synthase, DPD: dihydropyrimidine dehydrogenase, FPGS: folylpolyglutamate synthetase, GGH: γ-glutamyl hydrolase, RFC1: reduced folate carrier 1, TOPO I: DNA topoisomerase I, ERCC1: excision repair cross-complementing 1, MGMT: O^6-methylguanine-DNA methyltransferase, GSTpi: glutathione *S*-transferase pi, EGFR: epidermal growth factor receptor, VEGF: vascular endothelial growth factor.

GSTpi and VEGF are candidate genes for the prediction of the response to CPT-11.

On the other hand, the methodologies in this study to evaluate mRNA levels in the primary tumours with real time RT-PCR offered several advantages. It can easily overcome problems associated with sample volume and tumour heterogeneity by obtaining specimens from primary tumours by laser-captured microdissection. Furthermore, formalin-fixed, paraffin-embedded specimens of the primary tumours are obtained from nearly all patients with CRC. However, only a few studies have examined the relation between levels of molecular markers in primary colorectal tumours and associated metastases.[10,31,32] Since we analysed samples from primary tumours to predict the response of metastatic lesions to chemotherapy, the clinical value of our technique must be validated in larger prospective studies. In addition, we should bear in mind that all patients in our study were Japanese. The potential importance of ethnicity in studies of gene expressions should be taken into account in prospective clinical trials in the future.

Our findings suggest that the presence of both high TS and low MGMT expression is a significant predictor of a poor response to fluoropyrimidine treatment, and the diagnostic value of these predictive markers should be validated in larger cohorts of patients. Furthermore, future studies should also evaluate predictive markers for chemotherapy in patients who receive oxaliplatin-based or CPT-11-based regimens as first-line treatment. A combined analysis of these results might provide new insights into the optimal design for randomised clinical trials.

Conflicts of interest statement

Yoshihiro Okayama and Toshinori Oka are employees of Optimal Medication Research Laboratory, Taiho Pharmaceutical Co., Ltd., Tokushima, Japan.

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