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Colorectal cancer with high-frequency microsatellite instability expresses high-level thymidine phosphorylase but not dihydropyrimidine dehydrogenase

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Recent clinical studies have reported that microsatellite instability (MSI) colorectal cancers show a high sensitivity to 5-FU, but these reports are contradictory to findings from *in vitro* analyses. In this study, we analyzed the relationship between MSI phenotypes and the expression of 5-FU metabolic enzymes in human colorectal cancer specimens. MSI phenotypes in 174 sporadic colorectal carcinomas were determined and grouped into the following three categories based on the Bethesda guidelines: high-frequency MSI (MSI-H), low-frequency MSI (MSI-L), and stable microsatellite (MSS). The expressions of dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP) in tumor specimens were measured by enzyme-linked immunosorbent assays. The ratio of TP to DPD expression (TP/DPD ratio) was calculated for each tumor. These three factors were compared with regard to MSI phenotypes by non-parametric and logistic regression analyses using cut-off values at their medians. MSI-L tumors were excluded from statistical analyses. Thirteen tumors were classified as MSI-H, 8 tumors as MSI-L, and 153 tumors as MSS. DPD expression did not differ between MSI-H tumors and MSS tumors. TP expression and the TP/DPD ratio were significantly higher in MSI-H tumors than in MSS tumors [TP, 160.1± 104.0 vs 97.3 ± 53.7 (Units/mg protein) (P=0.009); TP/DPD ratio, 3.04 ± 1.62 vs 2.07 ± 1.08, (P=0.016)]. These differences were also significant in multivariate analysis. In conclusion, these data suggest that 5-FU catabolic activity in cancer tissue does not differ between MSI-H and MSS tumors. However, 5-FU anabolic activity in cancer tissue is higher in MSI-H than in MSS colorectal carcinomas.

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Introduction

Two independent genetic pathways have been elucidated for colorectal carcinoma to date. One is multistep carcinogenesis in which accumulation of aberrations in *APC, K-ras, p53*, and *DPC4* genes contributes to tumorigenesis[1)]. The other pathway is the mutator phenotype pathway, which results from dysfunction of DNA mismatch-repair machinery[2)]. In recent clinical studies, survival of colorectal cancer patients who received 5-fluorouracil (5-FU) based-chemotherapy is significantly better in tumors with MSI than in those without MSI[3-6)]. However, *in vitro* studies have shown that tumor cells with MSI do not have a high susceptibility to 5-FU[7-8)]. Therefore, it is unclear why colorectal carcinomas with MSI have revealed a better response to 5-FU based-chemotherapy.

Tumor sensitivity to 5-FU is associated with activity or expression of its metabolic enzymes. Dihydropyrimidine dehydrogenase (DPD) catalyzes 5-FU to 5-fluoro-dihydrouracil. Greater than 80 % of an administered dose of 5-FU is

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eliminated by catabolism via DPD[9]. Previous *in vitro* and *in vivo* analyses have revealed an inverse correlation between 5-FU efficacy and tumor DPD activity or expression[10-11]. In a clinical study for colorectal cancer patients who received 5-FU based-chemotherapy, tumor DPD expression was lower in responding patients than in non-responding patients[12]. Thymidine phosphorylase (TP) is one of the first step enzymes for 5-FU anabolism [9]. Especially, TP is the rate-limiting enzyme for 5-FU conversion of capecitabine[13], which is recognized as one of standard chemotherapy in colorectal cancer patients[14-15].

TP overexpression in cell culture and xenograft models has been shown to increase sensitivity to 5-FU[16]. Furthermore, the efficacy of capecitabine has been correlated with the expression of TP in tumor[17] and with the ratio of TP to DPD in a tumor by a clinical trial[18].

In this study, we analyzed the MSI phenotype and tumor expression of DPD and TP in resected human colorectal cancer specimens to estimate the relationship between MSI status and 5-FU sensitivity.

Materials and methods

Patients

We studied 174 patients with colorectal cancer who underwent surgery at the Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences from July 1991 to October 2001. There were no patients with familial adenomatous polyposis, or patients who fulfilled Amsterdam criteria II for hereditary nonpolyposis colorectal cancer[19]. The mean age of patients was 65 years (range, 29-90) and 88 patients were male and 86 were female. None of the patients received preoperative chemotherapy or radiotherapy. One hundred-twenty tumors were localized in the colon and 54 tumors were localized in the rectum. Each tumor was histopathologically classified in accordance with World Health Organization criteria[20]. Twenty-eight tumors were classified as well-differentiated adenocarcinomas, 134 tumors as moderately differentiated adenocarcinomas, and 12 tumors as poorly differentiated adenocarcinomas. The American Joint Committee on Cancer Classification and stage grouping was used to classify the tumors[21]. The one hundred seventy-four patients included 16 patients with stage I disease, 70 with stage II, 60 with stage III, and 28 with stage IV. Written informed consent was obtained from every patient in this study.

Microsatellite analysis

Genomic DNA was extracted from tumors and normal colonic tissues that stored at -80 ℃ in accordance with standard protocol. Matched DNA from carcinomas and normal mucosae was used for microsatellite analysis. We examined microsatellite instabilities at five loci: *BAT 25*, *BAT 26*[22], *D2S123*[23], *D5S346*[24], and *D17S250*[25]. Polymerase chain reaction product was electrophoresed on a 7M urea 6% polyacrylamide gel, and length of the product was detected by autoradiography. Positive MSI was determined as a length change at the microsatellite loci within the tumor when compared to its normal tissue. According to international criteria[26], tumors were classified into three groups: microsatellite stable (MSS), no microsatellite instability at any of the loci examined; low-frequency microsatellite instability (MSI-L), only one locus demonstrating instability; and high-frequency microsatellite instability (MSI-H), two or more loci demonstrating instability.

Enzyme-linked immunosorbent assay for TP and DPD Expression

Total protein extraction from frozen tissues was performed as follows. Frozen materials from primary tumors stored at -80 ℃ were minced by scissors in a micro tube with extraction buffer containing a cocktail of three protease inhibitors (1 μg/ml of aprotinin and leupeptin, and 0.1mM PMSF). The tissue in extraction buffer was then homogenized by Kontes tubes with a pestle for 1 minute. The solution was centrifuged at 55000g for 45 minutes at 4 ℃. The supernatant was used for further analysis. Total protein concentration was analyzed with the Bio-Rad protein assay kit (Bio-Rad, Tokyo, Japan). The DPD and TP expression was measured by enzyme-linked immunosorbent assay (ELISA). The amount of DPD sandwiched with two anti-DPD monoclonal antibodies (clone 4B9 and 3A5) and the amount of TP sandwiched with two anti-TP monoclonal antibodies (clone 104B and 232-2) were estimated by measuring its absorbency at 450 nm. The amount of TP and DPD were calibrated with values measured for standard solutions[27-28]. In this study, we also calculated the ratio of tumor TP expression to tumor DPD expression (TP/DPD ratio) in each tumor.

Statistical analysis

Statistical analyses were performed using the computer program STATISTICA™ (StatSoft, Tulsa, OK, USA). Fisher's exact test or the Chi-squared test was used for univariate

analysis of categorical data. The Mann-Whitney U test or Kruskal-Wallis test was applied for consecutive data. Logistic regression analyses were performed step-wise to evaluate the co-variables that affected tumor DPD expression, tumor TP expression, and the TP/DPD ratio. The cut-off values of these three factors were defined at their median values (tumor DPD, 45.9 Units/mg protein; tumor TP, 86.9 Units/mg protein; and TP/DPD ratio, 1.89). Two variables with continuous data - age and maximum tumor diameter- were classified into two groups based upon the medians of those respective variables (66 years, 4.8 cm, respectively). Tumor locations were classified into two groups. The tumors located proximal to the splenic flexure were classified as right-sided and the tumors located distal to the splenic flexure were classified as left-sided. All tests were two-tailed and a P value of less than 0.05 was considered to be statistically significant.

Results

MSI phenotypes

Among the 174 colorectal cancers, 13 tumors (7.5%) were classified as MSI-H, 8 tumors (4.6%) as MSI-L, and 153 tumors (87.9%) as MSS. All 13 MSI-H tumors showed microsatellite instability at the BAT-26 locus (Figure 1). MSI-L tumors were excluded from further analysis because the genetic and clinicopathological characteristics of MSI-L colorectal cancers have not been clarified. Furthermore, the frequency of MSI-L tumors was low in this study. Table 1 summarizes the clinicopathological features of MSS tumors and MSI-H tumors. MSI-H tumors were more likely to be right-sided, and poorly differentiated adenocarcinomas were more frequent in MSI-H tumors.

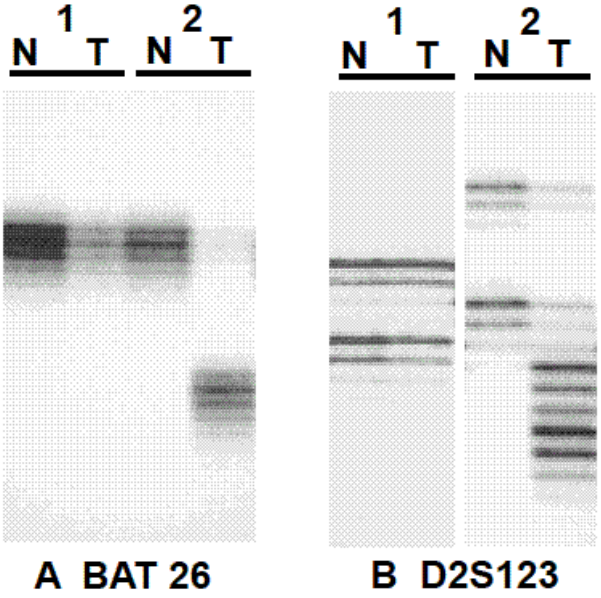

Figure 1. Microsatelite analysis with the BAT 26 (A) and D2S123 (B) markers. Patient 1 displayed no evidence of MSI at any loci examined, and judged as MSS. Patient 2 showed MSI at four of five loci examined, and judged as MSI-H. N, DNA from normal mucosa; T, DNA from tumor.

Table 1. Comparison of clinicopathological features according to MSI Phenotypes

Variables	MSS	MSI-H	*P*
Age (mean ± SD)	64±11	67±13	0.32[a]
Tumor location[b]			0.0004
Right-sided (n=54)	44	10	
Left-sided (n=112)	109	3	
Histological grade			0.0005
Well[c] (n=28)	26	2	
Mod (n=128)	121	7	
Poor (n=10)	6	4	
Stage			0.99
I (n=15)	14	1	
II (n=68)	63	5	
III (n=56)	51	5	
IV (n=27)	25	2	

[a]Mann-Whitney U test. [b]Classification into two groups according to proximal or distal to splenic flexure. [c]Well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma.

MSI phenotypes and tumor DPD expression

Tumor DPD expression was significantly higher in right-sided tumors than in left-sided tumors. Tumor DPD expression was also significantly higher in moderately or poorly differentiated adenocarcinomas versus well-differentiated adenocarcinomas (Table 2). Tumor DPD expression did not differ with respect to presence or absence of lymphatic invasion or venous invasion, T- and N-stage, and the presence or absence of distant metastases. Tumor DPD expression did not differ between MSI-H tumors and MSS tumors (Table 2). We designated the cut-off value of tumor DPD expression at 45.9 (Units/mg protein) (median), and divided the tumors into two groups: high-DPD and low-DPD groups. In logistic regression analysis, only moderately or poorly differentiated adenocarcinomas significantly correlated with high-DPD (Table 3).

Table 2. DPD and TP expressions and TP/DPD ratio, and their relation to clinicopathological features

Variables	Number of cases	DPD expression (Units/mg protein)	*P*	TP expression (Units/mg protein)	*P*	TP/DPD ratio	*P*
All cases	166	56.3±40.2		102.2±611.1		2.19±1.28	
Range		3.3−275.3		16.3−432.6		0.42−9.00	
Median value		45.9		86.9		1.89	
Age			0.19[c]		0.58[c]		0.02[c]
< 66	80	60.4±45.3		99.9±57.7		1.95±1.11	
≥ 66	86	52.6±34.7		104.3±64.3		2.32±1.17	
Gender			0.54[c]		0.73[c]		0.24[c]
Male	86	59.0±42.8		101.9±55.2		1.98±0.81	
Female	80	53.5±37.3		102.5±67.1		2.32±1.42	
Tumor location[a]			0.04[c]		0.009[c]		0.70[c]
Right-sided	54	64.6±42.3		118.6±69.1		2.16±1.04	
Left-sided	112	52.3±38.7		94.2±55.4		2.14±1.21	
Maximum tumor diameter			0.26[c]		0.60[c]		0.009[c]
< 4.8 cm	79	60.9±46.3		97.1±52.4		1.97±1.20	
≥ 4.8	87	52.2±33.5		106.8±67.9		2.30±1.09	
Histological grade			0.04[d]		0.004[d]		0.26[d]
Well[b]	28	46.0±37.7		86.1±52.2		2.30±1.31	
Mod	128	58.3±41.4		103.9±63.9		2.09±1.14	
Poor	10	59.2±28.2		125.3±34.5		2.40±0.80	
MSI phenotype			0.53[c]		0.009[c]		0.016[c]
MSI-H	13	59.9±31.1		160.1±104.0		3.04±1.62	
MSS	153	56.0±41.0		97.3±53.7		2.07±1.08	

Data presented as mean value ± standard deviations. [a]Classification into two groups according to proximal or distal to splenic flexure. [b]well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma. [c]Mann-Whitney U test. [d]Kruskal-Wallis test.

Table 3. Logistic regression analyses with respect to high-DPD, high-TP, and high-TP/DPD ratio

Variables	Odds ratio (95% CI)	*P*
With respect to high-DPD		
Well[a] vs mod, poor	2.98 (1.22−7.28)	0.02
MSS vs MSI-H	0.83 (0.26−2.65)	0.75
With respect to high-TP		
Well vs mod, poor	1.41 (0.61−3.29)	0.42
MSS vs MSI-H	6.19 (1.30−29.27)	0.02
With respect to high-TP/DPD ratio		
< 4.8[b] vs ≥ 4.8 cm	1.78 (0.95−3.36)	0.07
MSS vs MSI-H	5.72 (1.20−27.26)	0.03

[a]Well, well-differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma. [b]Maximum tumor diameter.

MSI phenotypes and tumor TP expression

Tumor TP expression was significantly higher in right-sided tumors than in left-sided tumors. Tumor TP expression was also significantly higher in poorly differentiated adenocarcinomas than in moderately or well-differentiated adenocarcinomas (Table 2). Tumor TP expression did not differ with respect to the presence or absence of lymphatic or venous invasion, T- and N-stage, or the presence or absence of distant metastases. Tumor TP expression was significantly higher in MSI-H tumors than in MSS tumors (Table 2). We designated the cut-off value of tumor TP expression at 86.9 (Units/mg protein) (median), and divided the tumors into two groups: high-TP and low-TP groups. Logistic regression analysis revealed that MSI-H was a significant and independent factor for high-TP (Table 3). Multivariate analysis could not include these two factors together in the same model because of the problem of colinearity between the tumor location and the MSI phenotype. Thus, we compared tumor TP expression between MSS

tumors and MSI-H tumors in right-sided tumors and in left-sided tumors, independently. Tumor TP expression was significantly higher in MSI-H tumors than in MSS tumors in both right-sided and left-sided tumor groups (Table 4).

Table 4. Comparison of tumor TP expression between MSI-H and MSS tumors in right-sided tumors and in left-sided tumors

	TP (Units/mg protein)		
	MSS	MSI-H	*P*
Tumor location[a]			
Right-sided	107.6±55.7	153.7±107.6	0.05[b]
Left-sided	91.5±52.0	181.4±109.2	0.005[b]

Data presented as mean value ± standard deviation. [a]Classification into two groups according to proximal or distal to splenic flexure. [b]Mann-Whitney U test.

MSI phenotypes and the ratio of TP expression to DPD expression

The TP/DPD ratio was significantly higher in patients older than 66 years than in patients younger than 66 years. The TP/DPD ratio was also significantly higher in tumors with a maximum diameter greater than 4.8cm than in tumors with a maximum diameter less than 4.8cm (Table 2). The TP/DPD ratio did not differ with respect to the presence or absence of lymphatic invasion or venous invasion, T- and N-stage, or the presence or absence of distant metastases. The TP/DPD ratio was significantly higher in MSI-H tumors than in MSS tumors (Table 2). We designated the cut-off value of TP/DPD ratio at 1.89 (median), and divided the tumors into two groups: high-TP/DPD ratio and low-TP/DPD ratio groups. Multivariate analysis revealed that only MSI-H was significantly associated with a high-TP/DPD ratio (Table 3).

Discussion

In this study, the incidence of MSI-H was slightly lower (7.5 %) than values previously reported in un-selected sporadic colorectal cancer patients (range, 8.2 % to 14%)[29-31]. However, the clinicopathological features of MSI-H tumors in this study were consistent with features reported in the literature, which is a high-incidence in right-sided tumors and in poorly differentiated adenocarcinomas. Moreover, all MSI-H tumors in this study showed instability at the BAT-26 locus, a robust marker for MSI-H detection[32]. These findings suggest that the sensitivity of MSI-H detection in this study is acceptable.

To date, it is well known that tumor sensitivity to 5-FU is associated with expression of its catabolic and/or anabolic enzymes in tumor cells. To our knowledge, there have been no studies that have investigated the expression of 5-FU metabolic enzymes with regard to tumor MSI phenotypes in colorectal cancer.

Although the regulatory mechanism of DPD expression in cancer cells has not been clarified, tumor DPD expression correlates with tumor sensitivity to 5-FU[10-12]. In this study with univariate and multivariate analyses, tumor DPD expression did not differ between MSI-H and MSS tumors. Because DPD is a rate-limiting enzyme for catabolism of 5-FU[9], these findings suggested that the catabolic activity of 5-FU in tumor tissues did not differ between MSI-H and MSS tumors. In this study, low-level expression of tumor DPD correlated with well-differentiated adenocarcinomas. However, because the TP/DPD ratio did not differ among histological grades, it is impossible to deduce 5-FU metabolic activities in tumor cells from its histological grade.

TP catalyzes the reversible phosphorolysis of thymidine and deoxyuridine to their respective bases (i.e., thymine and uridine) and 2-deoxyribose 1-phosphatase. TP is also one of the first step enzymes in the 5-FU anabolic pathway[9]. Its high-level expression in cancer cell increases 5-FU sensitivity[16]. Furthermore, the balance between 5-FU catabolic activity and anabolic activity in tumor cells will determine the efficacy of 5-FU. *In vitro* and clinical studies have shown that a high expression of TP and a high TP/ DPD ratio contribute to high sensitivity to fluoropyrimidines[13,17-18]. In this study, tumor TP expression and the TP/DPD ratio were significantly higher in MSI-H tumors than in MSS tumors. Furthermore, MSI-H was significantly and independently correlated with high-TP and a high-TP/DPD ratio by multivariate analyses. Moreover, tumor TP expression was compared between MSS and MSI-H tumors in right-sided tumors and in left-sided tumors, independently. We found tumor TP expression to be significantly higher in MSI-H tumors than in MSS tumors in both right-sided and left-sided tumor groups. These findings suggest that 5-FU anabolic activity in cancer tissue is higher in MSI-H colorectal carcinomas than in MSS colorectal carcinomas.

It has been revealed that host lymphoid reactions, such as Crohn's-like lymphoid reaction, peritumoral lymphocyte infiltration, and intraepithelial lymphocyte infiltration within tumor epithelium, are higher in MSI-H colorectal cancers than in MSS colorectal cancers[30,33]. Tumor-infiltrating lymphocytes in human colorectal carcinoma specimen produce interferon gamma[34] and tumor necrosis factor-alpha[35]. These

cytokines can induce TP expression in human colorectal cancer cells[36-37]. One possible mechanism of high TP expression in MSI-H tumors is that cytokines that originate from tumor-infiltrating lymphocytes induce TP expression in tumor cells. Previous in vitro and animal experiments have not included the influence of human tumor-infiltrating lymphocytes. This also may be a reason for contradictory findings with respect to tumor sensitivity to 5-FU and MSI phenotype between *in vitro* analyses and clinical studies. Recently, Ishikawa *et al*. analyzed immune responses against altered peptides generated by frameshift mutations through MSI using serological identification of tumor antigens by cDNA expression cloning[38]. The authors suggested that tumor-specific peptides generated by MSI may be involved in host lymphoid reactions of MSI colorectal cancer patients.

In conclusion, our data revealed that MSI-H colorectal carcinoma expresses high-level TP, but tumor DPD expression does not differ in humans between MSI-H tumors and MSS tumors *in vivo*. In future studies, thymidylate synthase, a target enzyme of 5-FU, and orotate phosphoribosyl transferase, one of the anabolic enzymes of 5-FU, must be analyzed with respect to MSI phenotype in colorectal carcinoma.

Conflict of interest statement

None declared.

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