



OPEN More subtle microsatellite instability better predicts fluorouracil insensitivity in colorectal cancer patients

Kaname Miyashita^{1,2,3,9}, Seiji Shioi^{1,2,9}, Tatsuhiro Kajitani^{4,9}, Yumiko Koi⁵, Mototsugu Shimokawa^{2,6}, Akitaka Makiyama⁷, Shinya Oda^{1,2}✉ & Taito Esaki^{2,8}

Microsatellite instability (MSI) is now widely used as an indispensable biomarker. However, the relationship between MSI-H (high) and defective DNA mismatch repair (MMR) is not as straightforward as has been expected. Genome-edited cells carrying Lynch syndrome mutations do not exhibit drastic MSI typical in MSI-H (*i.e.* Type B) but more subtle MSI (*i.e.* Type A). In this study, we explored a connection between Type A MSI and 5-fluorouracil (5-FU) resistance in colorectal cancer patients. Using our precision and high-resolution MSI assay technique, tumour microsatellites were analysed in 30 colorectal cancer patients treated with FOLFOX or CAPOX. Among 30 tumours, eleven (37%) were judged as Type A MSI-positive. In Type A MSI⁺ tumours, the patient response to fluoropyrimidine and oxaliplatin was significantly poor (Fisher's exact test, $p = 0.021$). Accordingly, median PFS and OS were significantly poor in Type A⁺ patients (log-rank test, $p < 0.001/p = 0.009$). Type A MSI was an independent predictor of patient prognosis in this pilot cohort (Cox regression analysis, $p = 0.003$). Thus, more subtle Type A MSI better predicts fluoropyrimidine insensitivity in colorectal cancer patients, which may shed light on a hitherto overlooked connection between the MSI phenotypes and drug resistance in human cancer.

Keywords Microsatellite instability (MSI), DNA mismatch repair (MMR), Drug resistance, 5-fluorouracil (5-FU), Fluoropyrimidine, Colorectal cancer

Microsatellite instability (MSI) is now widely used as an indispensable biomarker in the field of clinical oncology, where it has been established that MSI straightforwardly reflects cellular defects in DNA mismatch repair (MMR). Defective MMR confers on tumour cells both insensitivity to fluoropyrimidines such as 5-fluorouracil (5-FU)¹ and sensitivity to immune checkpoint inhibitors (ICIs)^{2–4}. Therefore, MSI-positive tumours are expected to be resistant to the former and sensitive to the latter. 5-FU is one of the most frequently and widely used cytotoxic anticancer agents, and ICIs being regarded as a currently most promising target-based drug. Thus, the testing of MSI in tumour cells is now prerequisite in current cancer treatment strategies. For this purpose, five selected mononucleotide microsatellites are widely analysed using PCR and capillary electrophoresis^{5–7}, although next generation sequencing (NGS) is also partly used. However, this PCR and capillary electrophoresis-based assay used worldwide is, in fact, not free of methodological problems⁸. In addition, molecular mechanisms of microsatellite destabilisation in eukaryotic cells are not limited to MMR but include polymerase proofreading⁹, 5' flap processing^{10,11} and recombination^{12,13}. It is thus expected that the microsatellite-unstable phenotypes in human cancer cells are not uniform but rather complex, and that the relationship of them to MMR deficiency is not straightforward. The established guidelines for MSI classification, however, simply utilise the frequency of instability in a set of microsatellites, *i.e.* MSI-H (high) and -L (low)¹⁴, and MSI-H has been established as a

¹Cancer Genetics Laboratory, Clinical Research Institute, NHO Kyushu Cancer Center, Fukuoka 811-1395, Japan.

²Clinical Research Institute, NHO Kyushu Cancer Center, Fukuoka, Japan. ³Department of Hematology, NHO Kyushu Cancer Center, Fukuoka, Japan. ⁴Department of Medicine and Biosystemic Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. ⁵Department of Breast Oncology, NHO Kyushu Cancer Center, Fukuoka, Japan. ⁶Cancer Biostatistics Laboratory, Clinical Research Institute, NHO Kyushu Cancer Center, Fukuoka, Japan. ⁷Cancer Center, Gifu University Hospital, Gifu, Japan. ⁸Department of Gastrointestinal and Medical Oncology, NHO Kyushu Cancer Center, Fukuoka, Japan. ⁹These authors contributed equally: Kaname Miyashita, Seiji Shioi and Tatsuhiro Kajitani. ✉email: oda.shinya.zq@mail.hosp.go.jp

genuine phenotype reflecting defective MMR. These underlying complexities may cast a shadow on the accuracy and reliability of this biomarker in predicting the utility of fluoropyrimidines and ICIs.

Microsatellites are a class of short tandem repeat (*alias* simple sequence repeat (SSR)¹⁵) and comprised of short repeat units ranging from one to six-bp. Numerous microsatellites are distributed throughout the eukaryotic genome, and more than one million are mapped on the human genome. However, their physiological functions are unknown. Since DNA polymerases are highly prone to slip on the repetitive sequences such as microsatellites, strand misalignments leading to repeat length changes are frequently formed and repaired by MMR. However, microsatellites may be a hotspot for DNA replication errors including not only strand misalignments but also other modes of DNA strand damage. This may implicate the potential instability of eukaryotic microsatellites. Indeed, as mentioned above, several different mechanisms destabilise the repeat sequences, and not only MMR but also other repair systems stabilise them. We have previously shown the role of DNA polymerase proofreading in mononucleotide microsatellite stability⁹. More importantly, we have observed at least two qualitatively distinct modes of microsatellite alterations, *i.e.* Type A and Type B, in human tumours¹⁶. In case of dinucleotide microsatellites, Type A is defined as length changes within 6-bp (three repeat units), and Type B as more large-scale length expansions or contractions¹⁶ (see more detailed definitions in Materials and methods and Fig. 1). Type B alterations are remarkable and, therefore, readily detectable using common assay

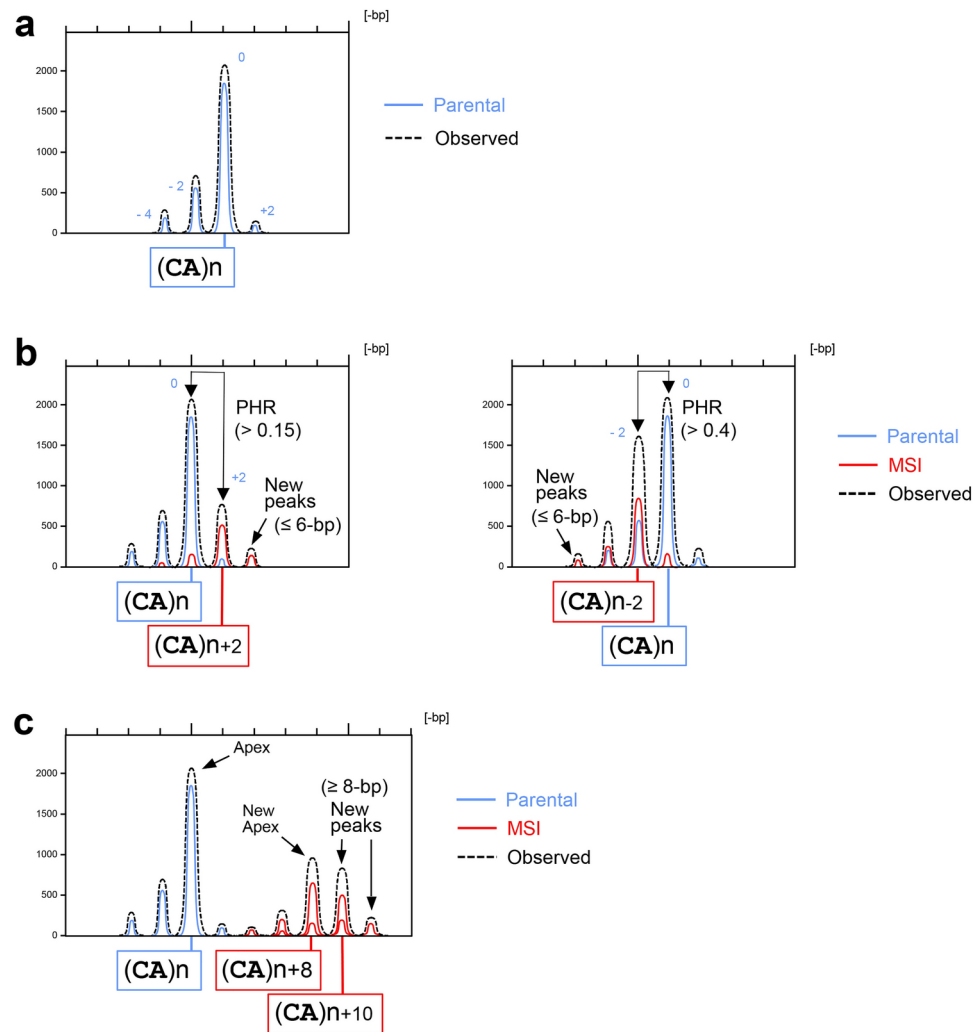


Fig. 1. The definitions of Type A and B MSI. The electrophoretic profiles of PCR amplified dinucleotide microsatellites are comprised of four peaks: + 2, 0, - 2 and - 4, and peak 0 corresponds to the correct size of template microsatellite sequence (Ref. 19) (a). When MSI occurs, there are several new minor microsatellite alleles with altered number of dinucleotide repeats, and the electrophoretic profiles are modified by them. Type A MSI is defined as an (i) appearance of new peaks within 6-bp (three repeats) (b, right and left) or a change in (ii) peak height ratio (PHR) above 0.15 between peak 0 and + 2 (b, left) or (iii) over 0.40 between peak 0 and - 2 (b, right). On the other hand, Type B is defined as an (i) appearance of new peaks over the length range of 6-bp (four or more repeat units) and/or an (ii) appearance of a new apex (c). Blue lines indicate parental (homozygous) alleles, and red lines altered MSI alleles. Observed electrophoretic profiles are shown by black interrupted lines.

platforms, whereas Type A is more subtle and minor and, therefore, obviously prone to be overlooked by less sensitive assay techniques. Since Type B alterations are typically observed in MSI-H tumours, MSI-H/Type B has been directly connected to MMR deficiency. However, in a line of our observations using MMR-gene knock-out mice¹⁶ and genome-edited human cells¹⁷, we found that MSI directly caused by defective MMR is not Type B but Type A. In other words, MMR deficiency is sufficient for Type A, but not for Type B. The relationship between MSI-H/Type B and defective MMR is obviously not straightforward^{16,17}. These findings strongly prompted us to explore a connection between Type A MSI and 5-FU insensitivity or ICI sensitivity in real cancer patients. In this study, we tested the former hypothesis. In order to sensitively detect Type A alterations in tumour DNA, we employed our precision and high-resolution assay techniques^{18,19}. The results obtained strongly indicate that tumours exhibiting Type A MSI are significantly more resistant to 5-FU-based chemotherapy, although the cohort size was limited. Here, we report a thus far overlooked connection between 5-FU resistance and more subtle MSI.

Materials and methods

Patients

Thirty colorectal cancer patients with surgically unresectable and objectively measurable metastatic lesions in the liver, who were consecutively diagnosed and treated in the Department of Gastrointestinal and Medical Oncology, NHO Kyushu Cancer Center from 2009 to 2015, were enrolled in this study. Patients received one or more cycles of fluoropyrimidine (5-FU or capecitabine) and oxaliplatin chemotherapy, *i.e.* mFOLFOX6^{20,21} or CAPOX²², as a first-line treatment. These patients were ineligible for bevacizumab²³, cetuximab²⁴ or panitumumab²⁵ because of the following medical conditions: bleeding, coagulopathy, history of cardiovascular diseases, ascites, unhealed wound and *KRAS* mutation *etc.* Ethical approval for this study was obtained from the institutional review board.

Microsatellite instability

Precision and high-resolution multi-fluorescence microsatellite analysis has been described in detail elsewhere^{18,19}. Briefly, human dinucleotide microsatellite sequences, D2S123, D5S107, D10S194, D13S175 and D17S250, were amplified by PCR using *TaKaRa Taq* (Takara Bio Inc., Kusatsu, Japan) and primers labelled with a fluorescent dye, 6-FAM™ (6-carboxyfluorescein) or HEX™ (6-carboxy-2', 4', 7', 4', 7, hexachloro-fluorescein) (Applied Biosystems, Thermo Fisher Scientific, Foster city, CA, USA). In addition to genomic DNA, artificially synthesised single-stranded oligodeoxynucleotides (ssODNs), the sequences of which are completely identical to the reference sequences except the repeat number, were also used as a template for PCR (see Sup. Figs. 1, 2). PCR products were electrophoresed in the ABI PRISM™ 3500 or 310 Genetic Analyzer (Applied Biosystems), and the data were processed using GeneScan™ and GeneMapper™ softwares (Applied Biosystems). Microsatellite repeat alterations are judged by the deviations from the basic electrophoretic profiles¹⁹. The most remarkable and readily scorable mode of microsatellite alterations is (I) Type B, which is defined as an (i) appearance of new peaks over the length range of 6-bp (insertions or deletions of four or more repeat units in dinucleotide microsatellites)¹⁶ and/or an (ii) appearance of a new apex (a highest peak in a peak cluster) (Fig. 1c). The Type B mode is universally observed in MSI-H tumours. On the other hand, the more subtle mode is (II) Type A, which was initially defined as an (i) appearance of new peaks within 6-bp (three repeats)¹⁶ (Fig. 1b). However, according to the results of the simulations in this study (see Sup. Figs. 1, 2), we have expanded the scoring criteria to include (ii) significant alterations in peak height ratio (PHR) between two peaks: above 0.15 between peak 0 and +2 (Fig. 1b left) or over 0.40 between peak 0 and -2 (Fig. 1b right). In this form of microsatellite assays, loss of heterozygosity (LOH) is also observable, because it causes a decrement of peak heights. However, some patterns of Type A alterations are theoretically indistinguishable from LOH, as discussed elsewhere²⁶, and, therefore, not included as Type A in this study (see Sup. Table 1). Type A MSI positivity in tumours was defined as Type A alterations in more than one ($\geq 40\%$) of five markers, according to the Bethesda guideline¹⁴.

Statistical analyses

Clinical characteristics were compared between patients with and without MSI by Fisher's exact test or Bonferroni correction for categorical variables and Wilcoxon rank sum test for continuous variables. Tumour response to fluoropyrimidine and oxaliplatin chemotherapy was assessed according to the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1²⁷. One patient, patient code F06, was unable to be evaluated on response due to an unexpected early severe adverse event (intestinal perforation). Patient response, *i.e.* 'complete response' or 'partial response'²⁷, was compared between Type A MSI-positive and -negative patients by Fisher's exact test. The diameters of five or less metastatic lesions were measured in each patient, and the maximal changes from the initial size were calculated, compared between patients with and without Type A MSI using the Wilcoxon rank-sum test and expressed as a waterfall plot (see Fig. 2). In this study, progression-free survival (PFS) was defined as the time from the start of first-line treatment to disease progression without or after response or death for any reason, overall survival (OS) as the time from the initial treatment to death. Survival time was recorded from the initiation of treatment until progression, last visit or death for PFS and last visit or death for OS. Data of patients were censored at the date of the last visit or the date on which the patient was last known to be alive unless disease progression and/or patient death occurred. As for four patients, F07, F13, F21 and F30, who underwent liver resection after fluoropyrimidine and oxaliplatin, data were censored at the day before the subsequent surgery and subjected to the PFS analyses. In one patient, F05, data were censored at the date of the last follow-up and subjected to PFS analysis. Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. In order to determine the significance of each clinicopathological variable as a prognostic factor, the Cox proportional hazards model was used. Cox regression analyses were performed using the eight variables: age, sex, ECOG PS, histopathology, tumour location, *KRAS* mutation,

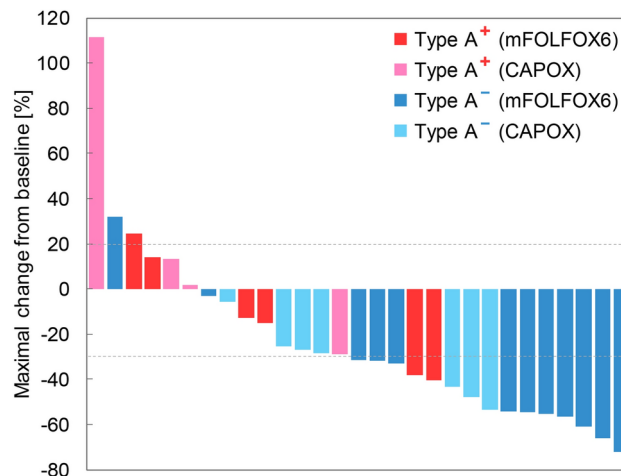


Fig. 2. Tumour response to fluorouracil and oxalipatin in colorectal cancer patients with Type A MSI^{+/-} tumours. The diameters of metastatic liver lesions were measured in each patient who underwent first-line mFOLFOX6 or CAPOX, and the maximal changes from the initial size were compared and shown as a waterfall plot. Each bar represents the maximal relative change in the tumour size in each patient and is coloured according to the MSI status (Type A MSI⁺, red; Type A MSI⁻, blue) and chemotherapy regimen (mFOLFOX6, dark; CAPOX, light). The cut-offs for significant response and disease progression, indicated by dashed horizontal lines, are $\geq 30\%$ decrease and $\geq 20\%$ increase, respectively, which are based on the RECIST version 1.1. Data of Patient F06, who had an unexpected early severe adverse event, is not included in this figure.

first-line chemotherapy and MSI, provided that the proportional hazards assumption holds. Multivariate Cox regression analyses were performed on the assumption that all the eight variables may be confounding factors, using a backward selection process, which excludes the variable with the largest p value in each round of analysis and is continued until only variables with $p < 0.05$ are obtained. Two-sided p values of < 0.05 were considered statistical significance. The above analyses were performed using JMP version 14.3.0 (SAS Institute, Inc., Cary, NC, USA) and/or R version 4.2.1 (The R Foundation for Statistical Computing, Vienna, Austria).

Results

More subtle microsatellite destabilisation in colorectal cancer

Before proceeding to the analysis of microsatellites on the genome in real patients, we re-verified the sensitivity and the quantitativity of our assay system. For this purpose, we artificially synthesised several single-stranded oligodeoxynucleotides (ssODNs) carrying the base sequences completely identical to those of human microsatellites with a variety in the repeat number and then used them as PCR templates¹⁹. Theoretically, MSI is in fact defined as an appearance of new alleles with different repeat numbers, and, therefore, most simply classified into two patterns: an appearance of alleles with longer or shorter repeats, which are finally expressed either as an appearance of new peaks or peak height alterations in capillary electrophoresis. We simulated the latter two patterns *in vitro* using the above ssODNs (Sup. Figs. 1, 2). The results clearly indicate that our system detects microsatellite length changes sensitively and quantitatively, and that not only small new peaks but also subtle changes in the relative peak height are indeed significant. According to these findings, we re-defined Type A MSI as an (i) appearance of new peaks within 6-bp (three repeats) or (ii) significant relative peak height changes (Fig. 1, Sup. Figs. 1, 2). Using this sensitive and quantitative system, we analysed tumour microsatellites in patients.

As a subject cohort, we selected colorectal cancer patients who had quantitatively assessable liver metastases and were treated with FOLFOX or CAPOX. The patient characteristics are summarised in Table 1. In these patients, tumour tissue specimens were collected and archived in the biobank in our hospital, and high molecular weight genomic DNA were extracted from them. Then, five dinucleotide microsatellites were analysed in tumour DNA, using the above assay technique. Microsatellites were indeed unstable in several tumours, and various alterations were observed. As expected, not only obvious new peak appearance (see examples in Sup. Fig. 3a) but also significant alterations in peak height ratio (PHR) (Sup. Fig. 3b) were observed. In addition to Type A alterations, Type B MSI (Sup. Fig. 3c) was also observed in one tumour (F19). The results are summarised in Table 2. In Type A MSI, there are some patterns of alterations that are not theoretically distinguishable from LOH (see Materials and methods). Therefore, these alterations are not scored as Type A in this study (see 'A/LOH' in Sup. Table 1). Finally, according to the Bethesda guideline¹⁴, Type A MSI positivity in each tumour was determined, *i.e.* tumours with Type A in two or more of five markers were judged as positive. Among 30 tumours, 11 (37%) were designated Type A MSI-positive. The tumour, F19, which exhibited both Type A and B alterations, was classified into the Type A-positive subgroup.

Variable	<i>n</i>
Age, median (range)	67 (42–84)
Sex, <i>n</i> (%)	
Male	24 (80)
Female	6 (20)
ECOG PS, <i>n</i> (%)	
0	10 (33)
1	16 (53)
2	2 (7)
3	2 (7)
Histopathological diagnosis, <i>n</i> (%)	
Well differentiated adenocarcinoma	7 (24)
Moderately differentiated adenocarcinoma	19 (63)
Poorly differentiated adenocarcinoma	3 (10)
Unclassifiable	1 (3)
Primary tumour location, <i>n</i> (%)	
Right side	6 (20)
Left side	22 (73)
Unclassifiable	2 (7)
<i>KRAS</i> , <i>n</i> (%)	
Mutant	13 (43)
Wild type	15 (50)
ND	2 (7)
<i>BRAF</i> , <i>n</i> (%)	
Mutant	0 (0)
Wild type	10 (33)
ND	20 (67)
First-line chemotherapy, <i>n</i> (%)	
mFOLFOX6	19 (63)
CAPOX	11 (37)

Table 1. Patient characteristics. CAPOX capecitabine and oxaliplatin, ECOG PS Eastern Cooperative Oncology Group performance status, mFOLFOX6 fluorouracil, oxaliplatin and leucovorin, ND not determined.

Poor response to fluorouracil-based chemotherapy and poor survival in colorectal cancer patients with subtle microsatellite instability

In Type A MSI⁺ patients, seven (7/19, 37%) and four (4/11, 36%) were treated with mFOLFOX6 and CAPOX, respectively (Table 2). There were no significant differences in clinicopathological characteristics between patients positive and negative for Type A MSI (Sup. Table 2). According to the RECIST version 1.1, objective response after the first-line fluoropyrimidine and oxaliplatin treatment was analysed in Type A MSI-positive and -negative tumours, the rates of which being 20% (2/10 tumours) and 68% (13/19), respectively. Thus, the response to fluoropyrimidine and oxaliplatin was significantly poor in Type A MSI⁺ tumours ($p=0.021$). Furthermore, we compared the maximal changes in the diameters of measurable metastatic lesions between Type A MSI-positive/negative tumours, and found that the Type A⁺ diseases were significantly stable in size after the first-line 5-FU-based chemotherapy (median maximal change -5.6% [interquartile range (IQR) -31.3 to +16.6] in Type A⁺ tumour vs. -43.4% [IQR -55.4 to -27.0] in Type A⁻ tumour; $p=0.006$) (Fig. 2).

As expected, median PFS in patients with Type A MSI⁻ tumours was 7.1 months (95% confidence interval [CI] 5.4–9.2), whereas being 3.3 months (95% CI 0.7–4.6) in those with Type A⁺ tumours, the difference of which was statistically significant ($p<0.001$) (Fig. 3a). Moreover, in addition to PFS, OS was significantly poor in Type A⁺ patients (median OS, 9.0 months [95% CI 1.3–24.4]; 25.0 months [95% CI 17.0–28.9] in Type A⁻ patients; $p=0.009$) (Fig. 3b).

We also performed Cox proportional hazards regression analyses, in order to conclude whether or not Type A MSI is an independent predictor of patient survival (Table 3). Univariate analyses revealed that Type A MSI positivity is associated with OS (hazard ratio (HR) 2.79 [95% CI 1.25–6.22], $p=0.012$) (Table 3). ECOG PS tended to be associated with OS, which was however not significant (HR 2.09 [95% CI 0.94–4.64]; $p=0.070$). The other variables, such as age, primary tumour location and chemotherapy regimen *etc.*, did not show significant associations either with PFS or with OS (Table 3). Furthermore, multivariate analyses revealed that Type A MSI is an independent predictor significantly associated with both PFS and OS in this pilot cohort (HR 10.51 [95% CI 3.20–34.50] for PFS, $p<0.001$; HR 3.57 [95% CI 1.52–8.35] for OS, $p=0.003$), although the CIs are broad reflecting the paucity of subjects and thus the statistical power of these analyses may therefore be limited (Table 3).

Patient code	Microsatellite						Treatment
	D2S123	D5S107	D10S197	D13S175	D17S250	MSI	
F01	A			A		A ⁺	mFOLFOX6
F02						-	mFOLFOX6
F03						-	mFOLFOX6
F04	A					-	mFOLFOX6
F05					A	-	mFOLFOX6
F06		A			A	A ⁺	mFOLFOX6
F07						-	mFOLFOX6
F08	A		A		A	A ⁺	mFOLFOX6
F09						-	mFOLFOX6
F10	A				A	A ⁺	mFOLFOX6
F11						-	mFOLFOX6
F12		A				-	mFOLFOX6
F13						-	mFOLFOX6
F14		A			A	A ⁺	mFOLFOX6
F15	A					-	mFOLFOX6
F16						-	mFOLFOX6
F17						-	mFOLFOX6
F18			A		A	A ⁺	mFOLFOX6
F19	(ND)	A	A	B	A	A ⁺	mFOLFOX6
F20			A			-	CAPOX
F21					A	-	CAPOX
F22					A	-	CAPOX
F23						-	CAPOX
F24						-	CAPOX
F25						-	CAPOX
F26	A		A		A	A ⁺	CAPOX
F27			A		A	A ⁺	CAPOX
F28	A		A		A	A ⁺	CAPOX
F29						-	CAPOX
F30	A				A	A ⁺	CAPOX

Table 2. Microsatellite changes in colorectal cancer patients. A Type A MSI, B Type B MSI, CAPOX capecitabine and oxaliplatin, mFOLFOX6 fluorouracil, oxaliplatin and leucovorin, ND not determined.

Discussion

Our precision and high-resolution assay technique detects microsatellite length changes sensitively and quantitatively. This technique has shown the previously unrecognised mode of MSI, Type A, which is more subtle than the widely acknowledged mode, Type B, that is typically observed in MSI-H (high) tumours. In this study, Type A MSI has been defined as an (i) appearance of new peaks within 6-bp (three repeats) or (ii) significant relative peak height changes (see Fig. 1, Sup. Figs. 1, 2). Since our previous observations using MMR-gene knock-out mice¹⁶ and genome-edited human cells¹⁷ suggest that Type A is a primary phenotype of MMR-defective cells, we explored a connection between Type A MSI and 5-FU insensitivity in thirty colorectal cancer patients treated solely with 5-FU-based regimens. The obtained results strongly indicate significant 5-FU resistance in tumours exhibiting Type A MSI. The quantitatively assessed response to the treatment, median PFS and OS were all significantly poorer in patients with Type A-positive tumours. In addition, Type A MSI was an independent predictor of patient prognosis. Although the size of the pilot cohort is extremely limited in this probing study, our findings may strongly suggest that more subtle Type A MSI better predicts 5-FU insensitivity in cancer patients.

Numerous studies of the relationship between the tumour MSI status and the efficacy of 5-FU-based chemotherapy have been done to date. However, the data reported in the literature are not conclusive, which is finely described and typically exemplified by a meta-analysis by Dogliotti E and colleague²⁸. The lack of unity may derive from several problems. One obvious problem is the complexity in chemotherapeutic strategies. Although detailed mechanisms of 5-FU action have not been clarified to date, it has been well established in vitro that cells defective in MMR are resistant to 5-FU^{29–31}. However, in current cancer chemotherapy, 5-FU is not used as a single agent but in combination with several other antineoplastic drugs. Furthermore, it is known that defective MMR modifies cellular sensitivities to some of these agents^{32,33}. In advanced colorectal cancer, FOLFIRI³⁴, FOLFOX³⁵ and CAPOX²² regimens have been chiefly used, the former and the latter two of which include irinotecan and oxaliplatin, respectively. In the both agents, it has been suggested that defective MMR may influence the sensitivity of cancer cells to them^{32,33}. Moreover, these 5-FU-based regimens are

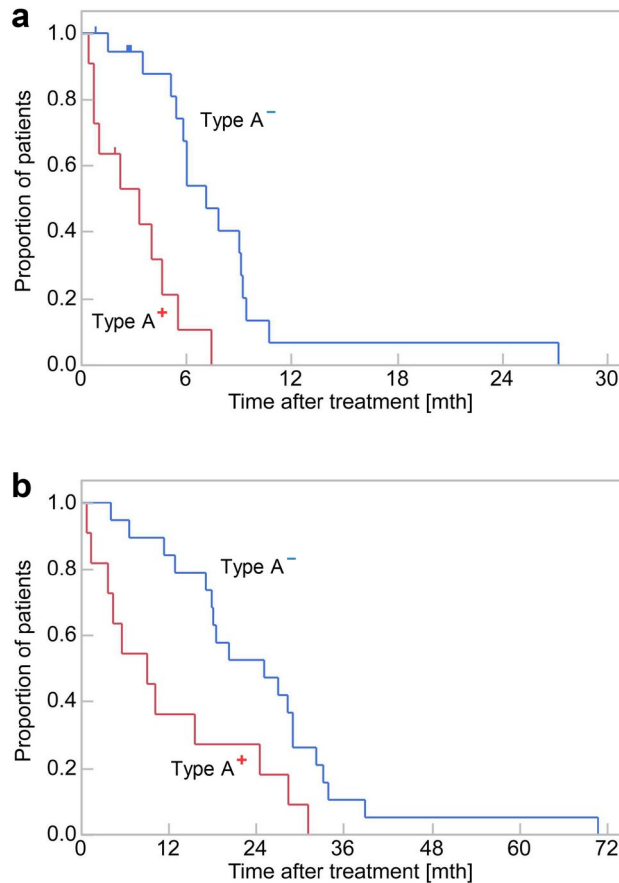


Fig. 3. Patient survival in Type A MSI^{+/-} colorectal cancer after 5-FU-based first-line chemotherapy. In patients with Type A MSI-positive/-negative colorectal carcinomas who received first-line mFOLFOX6 or CAPOX, survival is expressed as a Kaplan–Meier curve and compared: progression-free survival (a) and overall survival (b). Cross symbols indicate censoring. Red line, Type A MSI⁺ patients; blue line, Type A MSI⁻ patients.

Variable	Progression-free survival				Overall survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value	HR (95% CI)	p value
Age ≥ 65	0.83 (0.35–1.95)	0.666			0.90 (0.42–1.91)	0.783		
Female	1.82 (0.50–6.62)	0.364	4.13 (1.01–16.91)	0.049	0.95 (0.37–2.41)	0.912		
ECOG PS ≥ 1	2.39 (0.81–7.05)	0.115	4.11 (1.26–13.38)	0.019	2.09 (0.94–4.64)	0.070	2.67 (1.15–6.18)	0.022
Well differentiated adenocarcinoma	0.96 (0.35–2.60)	0.929			1.03 (0.43–2.46)	0.949		
Right-side tumour location	1.78 (0.63–5.01)	0.275			0.90 (0.36–2.26)	0.826		
KRAS mutant	1.31 (0.55–3.12)	0.547			1.00 (0.46–2.14)	0.996		
First-line chemotherapy CAPOX	1.29 (0.55–2.99)	0.558			0.94 (0.43–2.07)	0.884		
Type A MSI	5.38 (2.04–14.17)	<0.001	10.51 (3.20–34.50)	<0.001	2.79 (1.25–6.22)	0.012	3.57 (1.52–8.35)	0.003

Table 3. Univariate and multivariate analyses using the Cox proportional hazard model for progression-free survival and overall survival. CAPOX capecitabine and oxaliplatin, CI confidence interval, ECOG PS Eastern Cooperative Oncology Group performance status, HR hazard ratio, MSI microsatellite instability.

currently supplemented with several target-based drugs such as bevacizumab, cetuximab and panitumumab. These complex combinations of drugs have obviously made it difficult to observe effects of a single agent in real clinical settings. In this study, the subject cohort comprises colorectal cancer patients treated with mFOLFOX6 or CAPOX but without any other agents. This is the primary reason for the limited cohort size in this study. Needless to say, the subsidiary effects of oxaliplatin may have biased treatment response in the patients. The advantage of oxaliplatin treatment in MSI-positive tumours has indeed been reported³⁶. This effect may, however, diminish or neutralise the observed 5-FU resistance in Type A MSI-positive tumours. On the other hand, effects

context, the DNA fragment analysis using capillary electrophoresis has a limit, although currently regarded as a standard methodology. On the other hand, next generation sequencing (NGS) has started to be used also for microsatellite analysis. The NGS approaches may have some advantages. The sequencing by synthesis (SBS) method is free from polymerase slippage, and, therefore, subtle and minute microsatellite alterations may be precisely detected, although the assay process partly includes PCR. However, the algorithms to call sequence variations have been developed only to pick up large-scale microsatellite length changes such as those in MSI-H/Type B. More subtle and minor Type A alterations have been similarly overlooked as in the common capillary system. New algorithms for SBS-based microsatellite analysis need to be exploited. Nevertheless, considering the costs in NGS, the capillary electrophoresis assays may still have advantages, if being done more precisely, because they are easily accessible, fast and more economical. Further efforts are required to establish MSI as a more reliable biomarker and, consequently, to truly personalise 5-FU-based chemotherapy for more effective treatment of cancer patients.

Conclusions

More sensitive MSI assays reveal the Type A mode, which is more subtle than the widely acknowledged mode typically observed in MSI-H (high) tumours, *i.e.* the Type B mode. We previously observed Type A MSI in MMR-gene knock-out mice¹⁶ and genome-edited human cells¹⁷, which suggests that Type A is a primary phenotype of defective MMR. In this study, we explored a connection between Type A MSI and 5-FU insensitivity in colorectal cancer patients treated with 5-FU-based regimens. The obtained results strongly indicate significant 5-FU resistance in Type A-positive tumours. Although the cohort size of this probing study is extremely limited, our findings may strongly suggest that Type A MSI better predicts 5-FU insensitivity in cancer patients. Our findings warrant future studies of larger cohorts.

Data availability

All the data are available in the manuscript and the supplementary materials.

Received: 18 July 2024; Accepted: 25 October 2024

Published online: 08 November 2024

References

- Heidelberger, C. et al. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature* **179**, 663–666. <https://doi.org/10.1038/179663a0> (1957).
- Brahmer, J. R. et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: Safety, clinical activity, pharmacodynamics, and immunologic correlates. *J. Clin. Oncol.* **28**, 3167–3175. <https://doi.org/10.1200/JCO.2009.26.7609> (2010).
- Topalian, S. L. et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366**, 2443–2454. <https://doi.org/10.1056/NEJMoa1200690> (2012).
- Hamid, O. et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N. Engl. J. Med.* **369**, 134–144. <https://doi.org/10.1056/NEJMoa1305133> (2013).
- Bacher, J. W. et al. Development of a fluorescent multiplex assay for detection of MSI-high tumors. *Dis. Markers* **20**, 237–250. <https://doi.org/10.1155/2004/136734> (2004).
- Le, D. T. et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N. Engl. J. Med.* **372**, 2509–2520. <https://doi.org/10.1056/NEJMoa1500596> (2015).
- Le, D. T. et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* **357**, 409–413. <https://doi.org/10.1126/science.aan6733> (2017).
- Maehara, Y., Oda, S. & Sugimachi, K. The instability within: Problems in current analyses of microsatellite instability. *Mutat. Res.* **461**, 249–263. [https://doi.org/10.1016/s0921-8777\(00\)00061-6](https://doi.org/10.1016/s0921-8777(00)00061-6) (2001).
- Shioi, S. et al. DNA polymerase delta Exo domain stabilizes mononucleotide microsatellites in human cells. *DNA Repair (Amst.)* **108**, 103216. <https://doi.org/10.1016/j.dnarep.2021.103216> (2021).
- Freudenreich, C. H., Kantrow, S. M. & Zakian, V. A. Expansion and length-dependent fragility of CTG repeats in yeast. *Science* **279**, 853–856. <https://doi.org/10.1126/science.279.5352.853> (1998).
- Kucheralapati, M. et al. Haploinsufficiency of Flap endonuclease (Fen1) leads to rapid tumor progression. *Proc. Natl. Acad. Sci. USA* **99**, 9924–9929. <https://doi.org/10.1073/pnas.152321699> (2002).
- Richard, G. F., Dujon, B. & Haber, J. E. Double-strand break repair can lead to high frequencies of deletions within short CAG/CTG trinucleotide repeats. *Mol. Gen. Genet.* **261**, 871–882. <https://doi.org/10.1007/s004380050031> (1999).
- Richard, G. F. & Paques, F. Mini- and microsatellite expansions: The recombination connection. *EMBO Rep.* **1**, 122–126. <https://doi.org/10.1093/embo-reports/kvd031> (2000).
- Boland, C. R. et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res.* **58**, 5248–5257 (1998).
- Li, Y. C., Korol, A. B., Fahima, T., Beiles, A. & Nevo, E. Microsatellites: Genomic distribution, putative functions and mutational mechanisms: A review. *Mol. Ecol.* **11**, 2453–2465. <https://doi.org/10.1046/j.1365-294x.2002.01643.x> (2002).
- Oda, S. et al. Two modes of microsatellite instability in human cancer: Differential connection of defective DNA mismatch repair to dinucleotide repeat instability. *Nucleic Acids Res.* **33**, 1628–1636. <https://doi.org/10.1093/nar/gki303> (2005).
- Hayashida, G. et al. Differential genomic destabilisation in human cells with pathogenic MSH2 mutations introduced by genome editing. *Exp. Cell Res.* **377**, 24–35. <https://doi.org/10.1016/j.yexcr.2019.02.020> (2019).
- Oda, S., Oki, E., Maehara, Y. & Sugimachi, K. Precise assessment of microsatellite instability using high resolution fluorescent microsatellite analysis. *Nucleic Acids Res.* **25**, 3415–3420. <https://doi.org/10.1093/nar/25.17.3415> (1997).
- Shioi, S. et al. Precision length determination and in silico simulation in PCR of microsatellite repeat sequences. *Electrophoresis* **42**, 1323–1332. <https://doi.org/10.1002/elps.202100021> (2021).
- Cheeseman, S. L. et al. A “modified de Gramont” regimen of fluorouracil, alone and with oxaliplatin, for advanced colorectal cancer. *Br. J. Cancer* **87**, 393–399. <https://doi.org/10.1038/sj.bjc.6600467> (2002).
- Allegra, C. J. et al. Initial safety report of NSABP C-08: A randomized phase III study of modified FOLFOX6 with or without bevacizumab for the adjuvant treatment of patients with stage II or III colon cancer. *J. Clin. Oncol.* **27**, 3385–3390. <https://doi.org/10.1200/JCO.2009.21.9220> (2009).

22. Cassidy, J. et al. Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer. *J. Clin. Oncol.* **26**, 2006–2012. <https://doi.org/10.1200/JCO.2007.14.9898> (2008).
23. Saltz, L. B. et al. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: A randomized phase III study. *J. Clin. Oncol.* **26**, 2013–2019. <https://doi.org/10.1200/JCO.2007.14.9930> (2008).
24. Bokemeyer, C. et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J. Clin. Oncol.* **27**, 663–671. <https://doi.org/10.1200/JCO.2008.20.8397> (2009).
25. Douillard, J. Y. et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J. Clin. Oncol.* **28**, 4697–4705. <https://doi.org/10.1200/JCO.2009.27.4860> (2010).
26. Fujii, K. et al. Simulation-based analyses reveal stable microsatellite sequences in human pancreatic cancer. *Cancer Genet. Cytogenet.* **189**, 5–14. <https://doi.org/10.1016/j.cancergencyto.2008.09.008> (2009).
27. Eisenhauer, E. A. et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 11). *Eur. J. Cancer* **45**, 228–247. <https://doi.org/10.1016/j.ejca.2008.10.026> (2009).
28. Guastadisegni, C., Colafranceschi, M., Ottini, L. & Dogliotti, E. Microsatellite instability as a marker of prognosis and response to therapy: A meta-analysis of colorectal cancer survival data. *Eur. J. Cancer* **46**, 2788–2798. <https://doi.org/10.1016/j.ejca.2010.05.009> (2010).
29. Carethers, J. M. et al. Mismatch repair proficiency and in vitro response to 5-fluorouracil. *Gastroenterology* **117**, 123–131. [https://doi.org/10.1016/s0016-5085\(99\)70558-5](https://doi.org/10.1016/s0016-5085(99)70558-5) (1999).
30. Tokunaga, E., Oda, S., Fukushima, M., Maehara, Y. & Sugimachi, K. Differential growth inhibition by 5-fluorouracil in human colorectal carcinoma cell lines. *Eur. J. Cancer* **36**, 1998–2006. [https://doi.org/10.1016/s0959-8049\(00\)00200-8](https://doi.org/10.1016/s0959-8049(00)00200-8) (2000).
31. Meyers, M., Wagner, M. W., Hwang, H. S., Kinsella, T. J. & Boothman, D. A. Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. *Cancer Res.* **61**, 5193–5201 (2001).
32. Aebi, S. et al. Resistance to cytotoxic drugs in DNA mismatch repair-deficient cells. *Clin. Cancer Res.* **3**, 1763–1767 (1997).
33. Fink, D. et al. The effect of different chemotherapeutic agents on the enrichment of DNA mismatch repair-deficient tumour cells. *Br. J. Cancer* **77**, 703–708. <https://doi.org/10.1038/bjc.1998.116> (1998).
34. Fuchs, C. S. et al. Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: Results from the BICC-C Study. *J. Clin. Oncol.* **25**, 4779–4786. <https://doi.org/10.1200/JCO.2007.1.13357> (2007).
35. de Gramont, A. et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J. Clin. Oncol.* **18**, 2938–2947. <https://doi.org/10.1200/JCO.2000.18.16.2938> (2000).
36. Cohen, R. et al. Microsatellite instability in patients with stage III colon cancer receiving fluoropyrimidine with or without oxaliplatin: An ACCENT pooled analysis of 12 adjuvant trials. *J. Clin. Oncol.* **39**, 642–651. <https://doi.org/10.1200/JCO.20.01600> (2021).
37. Aaltonen, L. A. et al. Clues to the pathogenesis of familial colorectal cancer. *Science* **260**, 812–816. <https://doi.org/10.1126/science.8484121> (1993).
38. Fishel, R. et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75**, 1027–1038. [https://doi.org/10.1016/0092-8674\(93\)90546-3](https://doi.org/10.1016/0092-8674(93)90546-3) (1993).
39. Leach, F. S. et al. Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75**, 1215–1225. [https://doi.org/10.1016/0092-8674\(93\)90330-s](https://doi.org/10.1016/0092-8674(93)90330-s) (1993).
40. Tokunaga, E. et al. Frequency of microsatellite instability in breast cancer determined by high-resolution fluorescent microsatellite analysis. *Oncology* **59**, 44–49. <https://doi.org/10.1159/000012136> (2000).
41. Ikeda, Y. et al. Features of microsatellite instability in colorectal cancer: Comparison between colon and rectum. *Oncology* **61**, 168–174. <https://doi.org/10.1159/000055369> (2001).
42. Araki, K. et al. Frequent loss of heterozygosity but rare microsatellite instability in oesophageal cancer in Japanese and Chinese patients. *Oncology* **67**, 151–158. <https://doi.org/10.1159/000081002> (2004).
43. Sakurai, M. et al. High-resolution fluorescent analysis of microsatellite instability in gastric cancer. *Eur. J. Gastroenterol. Hepatol.* **19**, 701–709. <https://doi.org/10.1097/MEG.0b013e3281ac20a8> (2007).
44. Miyashita, K. et al. Frequent microsatellite instability in non-Hodgkin lymphomas irresponsive to chemotherapy. *Leuk. Res.* **32**, 1183–1195. <https://doi.org/10.1016/j.leukres.2007.11.024> (2008).
45. Eto, T. et al. Modal variety of microsatellite instability in human endometrial carcinomas. *J. Cancer Res. Clin. Oncol.* **142**, 353–363. <https://doi.org/10.1007/s00432-015-2030-2> (2016).
46. Miyashita, K. et al. A specific mode of microsatellite instability is a crucial biomarker in adult T-cell leukaemia/lymphoma patients. *J. Cancer Res. Clin. Oncol.* **143**, 399–408. <https://doi.org/10.1007/s00432-016-2294-1> (2017).
47. Miyashita, K. et al. Heterochronous occurrence of microsatellite instability in multiple myeloma: An implication for a role of defective DNA mismatch repair in myelomagenesis. *Leuk. Lymphoma* **59**, 2454–2459. <https://doi.org/10.1080/10428194.2018.1427862> (2018).

Acknowledgements

We all thank Mrs A. Take, N. Hayashi and M. Nakamura for expert assistance.

Author contributions

K.M. and S.S. analysed microsatellites. K.M., T.K., Y.K. and A.M. analysed patients. T.E. commented on the study from oncological and clinical viewpoints. K.M. and M.S. performed statistical analyses. S.O. conceived of and designed the study and wrote the paper.

Funding

This study was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 20K07711.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

All procedures performed in studies involving the patient were in accordance with the ethical standards of

the governmental guidelines and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval for this study was obtained from the institutional review board, NHO Kyushu Cancer Center Ethical Committee (Approval Number: 2017–77).

Informed consent

Written opt-out consent was obtained from each individual participant.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-024-77770-3>.

Correspondence and requests for materials should be addressed to S.O.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2024