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Microsatellite Status of Primary Colorectal Cancer Predicts the Incidence of Postoperative Colorectal Neoplasms

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Abstract. *Background/Aim: Few studies have evaluated the risk of postoperative colorectal neoplasms stratified by the nature of primary colorectal cancer (CRC). In this study, we revealed it on the basis of the microsatellite (MS) status of primary CRC. Materials and Methods: We retrospectively reviewed 338 patients with CRC and calculated the risk of neoplasms during postoperative surveillance colonoscopy in association with the MS status of primary CRC. A propensity score method was applied. Results: We identified a higher incidence of metachronous rectal neoplasms after the resection of MS stable CRC than MS instable CRC (adjusted HR 5.74, p=0.04). We also observed a higher incidence of colorectal tubular adenoma in patients with MSS CRC (adjusted hazard ratio 7.09, p<0.01) and a higher incidence of postoperative tubulovillous/villous adenoma in patients with MS instable CRC (adjusted HR=8.50, p=0.03). Conclusion: The MS status of primary colorectal cancer influenced the risk of postoperative colorectal neoplasms.*

Colorectal cancer (CRC) is one of the most common cancers and one of leading causes of death in Japan. Patients with a history of CRC are known to be at a higher risk of developing secondary CRC (1-7). Therefore, postoperative surveillance colonoscopy is important to detect premalignant colorectal neoplasms, resulting in the prevention of secondary CRC (8). The high rate of postoperative colorectal neoplasms (17-35%) also reinforces the necessity of postoperative surveillance (9-11).

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In addition to the history of CRC, other clinical features such as smoking, gender, age, and the history of synchronous colorectal polyp together with primary CRC have been reported to affect the incidence of postoperative colorectal polyps (6, 12-14). On the other hand, only few studies evaluated its risk in association with the genetic nature of primary CRC. For example, Balleste *et al*. (15) and Kang *et al*. (16) observed a similar incidence of adenomas among patients with microsatellite-instable (MSI) cancer and those with microsatellite-stable (MSS) cancer. Although these reports were novel, they did not eliminate the unfair bias influenced by the genetic nature of primary CRC. For instance, MSI cancers tend to locate in the proximal colon and MSS cancers in distal, and this feature results in the different type of colorectal resection. In addition, MSI cancer tends to develop at an earlier age compared to MSS cancer. The favorable survival outcome of MSI cancer may have an impact on the duration of postoperative observation. These features possibly affect the accuracy of evaluation and should be minimized.

In this study, we evaluated the incidence of postoperative colorectal neoplasms in association with the MS status of primary CRC. To minimize the heterogeneous features among groups, we introduced propensity score methods for the first time. Furthermore, we assessed detailed pathological features of postoperative neoplasms because different types of colorectal polyps have different types of genetic alteration during polyp development. By means of these methods, we revealed the different behavior of postoperative colorectal neoplasms among different genetic types of primary CRC.

Materials and Methods

Patient recruitment. Patients who were diagnosed with primary CRC and underwent complete surgical resection of the primary lesion at the University of Tokyo Hospital from January 2008 to December 2013 were retrospectively reviewed. Patients generally underwent a total colonoscopy prior to surgery, and all neoplasms other than the primary tumor were endoscopically removed prior to

the operation. Those patients manifesting an occlusive CRC or those who underwent emergency colorectal resection underwent total colonoscopy after operation as soon as possible. In this study, patients with a clean colon were defined as those with no neoplasm other than hyperplastic polyps in the remaining colorectum. All the patients who achieved clean colon within a year after primary CRC resection were included in this study, except for those with a history of familial adenomatous polyposis (FAP) or inflammatory bowel disease (IBD) and who underwent a total or sub-total proctocolectomy. Neoplasms resected within a year from surgery were regarded as synchronous neoplasms and not counted as metachronous ones. Follow-up colonoscopies were performed every 1 or 2 years by expert colorectal gastroenterologists.

Patient characteristics. We also collected data on age, gender, body mass index, smoking history, alcohol intake history, qualification of Amsterdam II criteria and revised Bethesda criteria, history of other malignancies or Lynch syndrome-associated tumors, preoperative carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), synchronous polyp, and the location of the primary tumor by reviewing patients' medical records. All CRC were histopathologically examined, and the pathological TNM classification and staging was determined using the 7th edition of Union for International Cancer Control (UICC) staging system.

Assessment of colonic neoplasms. All colorectal metachronous neoplasms larger than 5 mm were removed during surveillance colonoscopy. Even if the polyps were smaller than 5mm, those suspicious as adenomatous lesions were also removed. We recorded the size, location, pathological grade of the metachronous neoplasms as well as the period between the time achieving clean colon and the detection of metachronous neoplasms for further analysis. Histopathology of the neoplasms was classified as follows: tubular adenomas, tubulovillous/villous adenomas, hyperplastic polyps, and serrated adenomas. An advanced adenoma was defined as being ≥1 cm in diameter, or containing villous features, or high-grade adenoma, or carcinoma in situ.

Analysis of microsatellite instability (MSI) of primary CRC. We investigated 338 pairs of matched normal colonic mucosa and tumor specimens. All specimens obtained were snap frozen in liquid nitrogen immediately after resection and stored at −80˚C until use. DNA was extracted from each specimen with a QIAamp DNA mini kit (Qiagen, Tokyo, Japan) and then analyzed by PCR at two microsatellite loci: BAT25 and BAT26. The following sets of primers were used: BAT25, 5'-NED-TCGCCTCCAAGAATG TAAGT-3' and 5'-TCTGCATTTTAACTATGGCTC-3'; BAT26, 5'-FAM-TGACTACTTTTGACTTCAGCC-3' and 5'-AACCATTCA ACATTTTTAACCC-3'. PCR was performed in 10 μl reaction volumes containing 1× PCR Gold Buffer, 1.5 mM $MgCl_2$, 0.9 mM dNTP Mix, 0.15 μM of each primer, 20 ng of extracted DNA, and 0.4 units of AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific, Waltham, MA). DNA was amplified in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, Foster City, CA, USA) using the following protocol: 10 min at 95˚C for polymerase activation; 35 cycles at 95˚C for 30 sec, 60˚C for 1 min and 72˚C for 1 min; then an additional 30 min at 70˚C. After denaturation by heating at 95˚C for 5 min, PCR products were electrophoresed using a ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and the fluorescent signals were analyzed using GeneScan version 3.7 and Genotyper version 2.1 software according to the manufacturer's instructions.

To confirm that screening with the 2 designated mononucleotide markers accurately reflected MS status, we conducted a further analysis using 3 additional dinucleotide markers (D2S123, D5S346, and D17S250) on 61 randomly selected specimens that were negative for the 2 mononucleotide markers. The following primer sequences were used: D2S123, 5'-HEX-GCCAGAGAAAT TAGACACAGTG-3' and 5'-CTGACTTGGATACCATCTATCTA-3'; D5S346, 5'-FAM-TACTCACTCTAGTGATAAATCGG-3' and 5'-TTCAGGGAATTGAGAGTTACAG-3'; D17S250, 5'-NED-AATAGACAATAAAAATATGTGTGTG-3' and 5'-TATATATTTA AACCATTTGAAAGTG-3'.

We classified tumors as MSI positive when the PCR product of tumor DNA exhibited allelic shifts with two markers. High-frequency MSI (MSI-H) was defined by the instability of both markers, and low-frequency MSI (MSI-L) was defined by instability in one marker. MSS was defined as a lack of marker instability. MSI cancer was defined as a MSI-L or MSI-H tumor, and MSS cancer was defined as a MSS tumor.

Statistical analysis. The differences between patient characteristics were analyzed using a Chi-square test or Fisher's exact test for categorical variables and a Student's *t*-test for normally distributed continuous variables. A Mann-Whitney test was performed for non-normally distributed continuous variables. The analysis of correlation between the clinicopathological variables and the duration until the development of each metachronous neoplasm was completed using the Kaplan-Meier estimator, log-rank test, and Cox proportional hazard model. To minimize the differences in background features of patient groups between MSI cases and MSS cases, a propensity score analysis was performed using an inverse probability of treatment weighting (IPTW) method. Covariates in the models for propensity scores included four variables with significant differences between two groups.

All analyses were performed with the JMP 11 (SAS Institute Inc., Cary, NC, USA) statistical software package and statistical program R version 3.3.0. Differences with a p-value<0.05 were considered statistically significant.

Results

Patient characteristics. A total of 338 patients were included in this study. Patient characteristics are shown in Table I. Twenty-six patients were positive for two markers (BAT25 and BAT26). No specimen met the criteria for MSI-L; thus, all MSI tumors were defined as MSI-H. To validate our method judging the MS status using two markers, we randomly selected 61 specimens negative for BAT25 and BAT26 and confirmed the MS status using three additional dinucleotide markers. All 61 specimens were negative for these additional markers, which justified our method (17-23).

Patients with MSI cancer had a higher tendency to meet the Revised Bethesda criteria (63.0% *vs*. 34.7%, MSI *vs*. MSS; p<0.01) and lower serum CEA levels (3.5 *vs*. 4.7, MSI *vs*. MSS; p=0.04). The locus of the primary CRC was divided into three groups: proximal colon as cecum to transverse colon, distal colon as descending to sigmoid colon, and

Table I. *Patient characteristics*.

	MSI (n=27)	MSS (n=311)	*p*-Value
Age, mean±SD	67.2±15.1	65.7±10.6	0.63
Female (%)	15 (55.6)	130 (41.8)	0.17
Stage (%)			
0 (Tis)	0 (0.0)	3 (1.0)	
I	9 (33.3)	59 (19.0)	
II	10 (37.1)	111 (35.7)	0.26
III	8 (29.6)	112 (36.0)	
IV	0 (0.0)	26 (8.3)	
Amsterdam II (%)	1 (3.7)	4 (1.3)	0.34
Revised Bethesda (%)	17 (63.0)	108 (34.7)	<0.01
History of other malignancies (%)	9 (33.3)	53 (17.0)	0.06
History of other Lynch syndrome associated tumors (%)	3 (11.1)	21 (6.8)	0.42
BMI, mean ±SD	22.3±3.4	23.1±3.7	0.23
CEA (ng/ml), median (range)	3.5 (1.2-27.3)	4.7 (0.9-1207)	0.04
CA19-9 (ng/ml), median (range)	9 (1-169)	12 (1-2432)	0.49
Smoking history (%)	12 (44.4)	167 (53.7)	0.36
Alcohol history (%)	16 (59.3)	211 (67.9)	0.36
Adjuvant chemo therapy (%)	9 (33.3)	121 (38.9)	0.68
Synchronous polyp (%)	15 (55.6)	157 (50.5)	0.61
Locus of cancer (%)			
Proximal colon	22 (81.5)	78 (25.1)	
Distal colon	3 (11.1)	106 (34.1)	<0.0001
Rectum	2 (7.4)	127 (40.8)	
Histopathology (%)			
Grade 1	10 (37.0)	159 (51.1)	
Grade 2	12 (44.5)	144 (46.3)	<0.01
Grade 3	5 (18.5)	8 (2.6)	

BMI: Body Mass Index; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

rectum. With respect to the nature of primary CRC, MSI cancer tended to be located in the proximal colon ($p<0.0001$) and showed a higher pathological grade ($p<0.01$).

The rate of smoking and synchronous polyp were similar among the two groups, which were reported as risk factors for metachronous polyps (6).

Postoperative colonoscopy surveillance and metachronous neoplasms. The mean follow-up period of patients was 3.0 (0.3-7.4) years, and the cumulative total follow-up time of 338 cases was 1109 years. Colonoscopy surveillance was performed an average of 2.9 times from surgery and 2.7 times from the date of a clean colon diagnosis. During the follow-up period, 126 (37.3%) patients had more than one metachronous neoplasm removed. Table II shows the clinicopathological features of the postoperative metachronous neoplasms analyzed by Cox proportional hazard model. No statistically significant difference was observed between MSS and MSI tumors.

Table II. *Hazard ratio (HR) of each postoperative neoplasm for MSI and MSS CRC patients using the Cox Proportional Hazard Model*.

	MSI (n=27)	MSS (n=311)	HR (MSI/MSS)	95%CI	*p*-Value
All neoplasms	7	119	0.83	0.35-1.66	0.63
Locus					
Proximal colon	5	76	0.90	0.32-2.02	0.82
Distal colon	3	60	0.68	0.16-1.83	0.48
Rectum	2	21	1.19	0.19-4.06	0.82
Histology					
Tubular adenoma	5	100	0.64	0.23-1.43	0.30
Tubulovillous/villous adenoma	1	6	2.04	0.11-12.0	0.55
Hyperplastic polyp	2	19	1.43	0.23-4.97	0.65
Serrated adenoma	0	12	4.94E-09	2.17-2.17	0.17
Advanced adenoma	2	25	1.00	0.16-3.37	1.00
Metachronous Tis lesion	1	1	11.30	0.45-286	0.12
Metachronous CRC	1	0	3.56E+09	1.89-1.89	0.03

CRC: Colorectal cancer; CI: confidence interval.

A propensity score analysis was performed using the IPTW method. Covariates in the models for propensity scores included four statistically different variables (the Revised Bethesda criteria, CEA, locus and histopathology of the primary CRC). Figure 1 shows the curves of polyp-free rate of patients with MSI/MSS cancer. The results are shown in Figure 2. We identified a higher incidence of metachronous rectal neoplasms and tubular adenoma in the MSS group than in the MSI group (adjusted hazard ratio of rectal neoplasm, 5.74; $p=0.04$ and adjusted hazard ratio of tubular adenoma, 7.09; $p<0.01$). The incidence of metachronous tubulovillous/villous adenoma was higher in the MSI group compared to the MSS group (adjusted hazard ratio=8.50, $p=0.03$).

Discussion

This is the first report to clarify the incidence of postoperative neoplasms based on the MS status of the primary cancer using propensity score method. The incidence of metachronous rectal neoplasms and tubular adenoma was significantly higher in the MSS group, and the incidence of metachronous tubulovillous/villous adenoma was significantly higher in the MSI group. We introduced four covariants that presented different ratios among the two MS status (Revised Bethesda criteria, serum CEA level, locus of primary CRC, and histopathological grade). Although the history of smoking and existence of

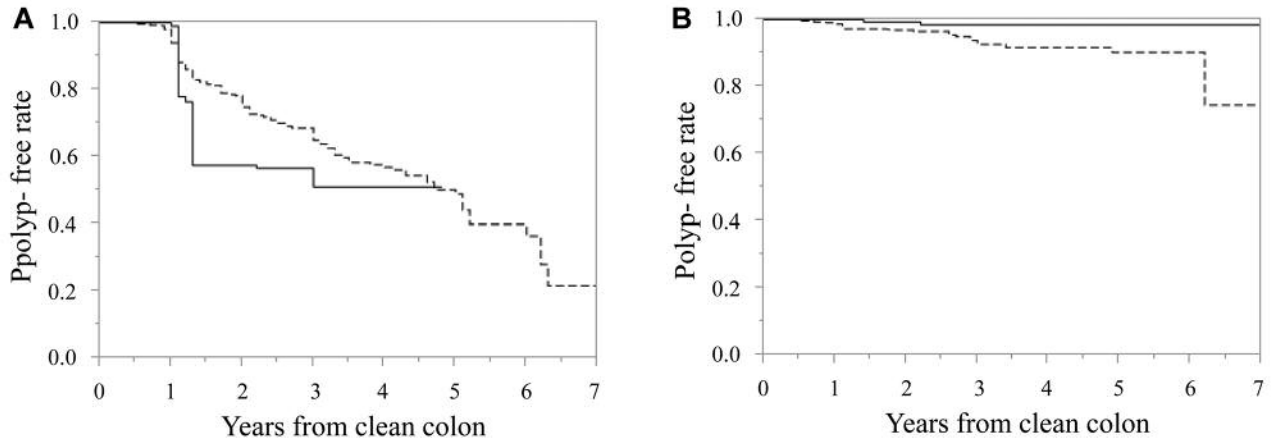
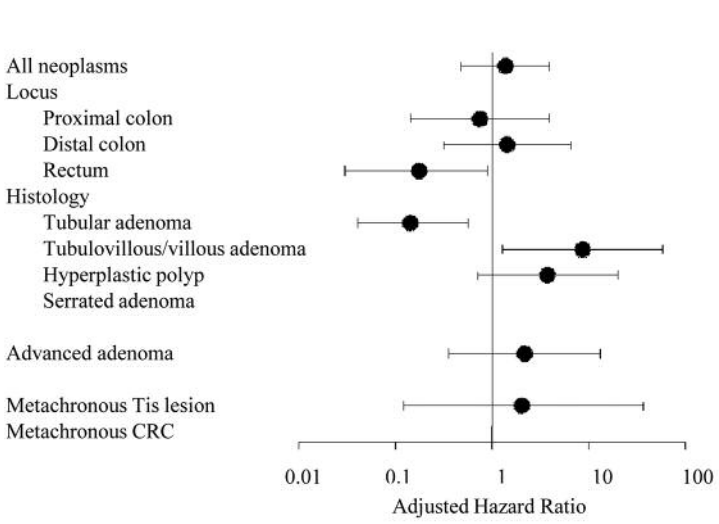


Figure 1. *Polyp-free rate curve of postoperative all neoplasms (A; p=0.56) and rectal neoplasms (B; p=0.04). Black line shows MSI CRC patients and Dotted line shows MSS CRC patients.*



	Adjusted Hazard Ratio MSI/MSS	95% CI	*p*-Value
All neoplasms	1.36	0.48-3.91	0.56
Locus			
Proximal colon	0.75	0.14-3.90	0.73
Distal colon	1.41	0.31-6.41	0.66
Rectum	0.17	0.03-0.90	0.04
Histology			
Tubular adenoma	0.14	0.04-0.56	<0.01
Tubulovillous/villous adenoma	8.5	1.25-57.5	0.03
Hyperplastic polyp	3.71	0.70-19.6	0.12
Serrated adenoma	1.44E-09	5.15E-10 - 4.00E-09	2.00E-16
Advanced adenoma	2.14	0.35-13.0	0.41
Metachronous Tis lesion	2.03	0.12-36.0	0.63
Metachronous CRC	5.59E+08	6.97E+07 - 4.48E+09	2.00E-16

Figure 2. *Forest plot of the adjusted hazard ratio for each postoperative neoplasm between MSI with MSS CRC patients using a propensity score (IPTW method).*

synchronous polyp were reported as risk factors of metachronous colorectal polyps (6), we observed similar ratios among two groups and did not employ them as covariants in the IPTW method. This result means that these two risk factors were equally distributed in the two groups.

We revealed that the incidence of metachronous rectal neoplasms was significantly higher in the MSS group. Although several studies have reported the number of metachronous neoplasms after CRC resection stratified by the locus of neoplasms (8, 15, 24-26), only few have reported a risk based on the location of neoplasms by statistical analysis. Jin H.P. *et al.* (24) reported that postoperative neoplastic polyps were commonly located in the right colon and the proximal part of the anastomosis. They surveyed the registry only for first surveillance colonoscopy after radical surgery (mean=7.1 months, range=3-15 months); thus, their definition of metachronous neoplasms was different from that used in our study. In addition, they did not analyze the MS status of the primary cancer, though distal colon and rectum are known to be

favorite sites for primary MSS cancer (26). More than 40% patients with MSS cancer underwent resection of the large part of the rectum in our cohort, which means that adjusting by the propensity score (IPTW) to minimize the form of resection and consideration of the MS status, which influences the location of primary CRC, were essential.

Although often regarded as a single disease, CRC represents a family of diseases with different precursor lesions (27-30). Conventional (tubular and tubulovillous/villous) adenomas are the most common neoplasms resulting from APC mutations, and these adenomas follow the traditional chromosomal instability pathway, which results in sporadic MSS tumors (24, 29). There are several reports on epigenetic mutations in the background mucosa of the large bowel. Luo *et al*. (31) compared the status of DNA methylation in the background colon mucosa in patients with and without a history of sporadic CRC. The authors determined that the frequency of DNA methylation was higher in the background mucosa of patients with a history of CRC. Ahearn *et al*. (32) reported that patients with colorectal polyps had a decreased APC/beta-catenin score in the background mucosa when compared with patients without polyps (MAP-II study, 2002), suggesting that the epigenetic alterations in the background mucosa influences the development of polyps. On the other hand, Rosa *et al*. (26) investigated *APC* gene mutations in the background mucosa of 11 patients that had at least one *APC* mutation at exon 15 in the CRC. No APC alterations were found in the background mucosa of these patients. Based on our search on Pubmed, this is the only study that compared *APC* gene mutations in the background mucosa of CRC stratified by the MS status. However, the sample size was too small to assure the reliability of this study. Thus, we considered that a higher incidence of tubular adenoma in the MSS group possibly be influenced by the epigenetic alteration in the background mucosa.

We also identified a higher incidence of tubulovillous/villous adenoma in the MSI cancer group. Although this result is novel, the small sample size may affect the reliability of our study. Therefore, we should conduct further analysis including larger sample sizes.

In this study, 12 serrated adenomas were observed in the MSS group. On the other hand, serrated adenoma was not observed in the remnant large bowel of patients in the MSI group. Therefore, we could not perform a propensity score analysis for serrated adenomas, and this is a limitation of the study. According to Leggett *et al*. (29), a sessile serrated adenoma is a precancerous lesion of sporadic MSI cancer which grows from a microvesicular hyperplastic polyp caused by a *BRAF* mutation in the background mucosa. There is no consensus on the epigenetic risks of precursor lesions on the background mucosa in MSS/MSI cancer, and additional studies are needed to validate our results.

In this study, the incidence of postoperative neoplasms was different between the MSI and MSS groups. The incidence of metachronous rectal neoplasms and tubular adenoma was significantly higher in the MSS group, and the incidence of tubulovillous/villous adenoma was significantly higher in the MSI group. Further investigation, including epigenetic DNA mutations in the background mucosa of MSS/MSI cases, is needed to clarify the mechanism behind our findings.

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