

Microsatellite Instability in Double Primary Cancers of the Colorectum and Stomach

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Little is known about genetic alterations of patients who present multiple primary cancers. We hypothesized that microsatellite instability (MSI) is one of the underlying genetic factors in the development of double primary cancers in colorectal cancer patients. We examined for MSI in 41 colorectal cancer patients who presented with extra-colonic primary cancers consisted of 17 gastric and 24 non-gastric cancers. Coincident MSI+ in tumors of two organs were observed in 3 (17.7%) of 17 patients with colon and stomach cancers and 0 of 24 patients with colon and non-gastric cancers ($P = .03$). In 17 patients with colon and stomach cancers, 6 (31.6%) of 19 colon cancers and 3 (17.7%) of 17 gastric cancers exhibited MSI+. Among four patients with metachronous colon cancers who were identified within the 41 double primary cancer patients, two patients were associated with the MSI+ phenotype. In summary, the prevalent coincidence of MSI suggests that genetic defect of mismatch repair deficiency may be responsible for a small subset of double primary cancers of the colorectum and stomach.

KEY WORDS: Colon, Double primary cancer, Microsatellite instability, Stomach.

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The incidence of multiple primary cancers is increasing significantly. Nearly 10% of cancer patients develop another primary cancer within 10 years of their first operation (1). Stomach cancer is the most prevalent extracolonic malignancy in multiple primary cancers with colon cancer in both Koreans (2)

and Japanese (3). One of the tumors with significantly elevated risk is colon cancer after the diagnosis of stomach cancer (4).

The development of multiple primary cancers is known to be a feature of hereditary nonpolyposis colorectal cancer (HNPCC), and significantly increasing numbers of cancers of the stomach, small intestine, upper urologic tract (renal pelvis and ureter), and endometrium have been observed in HNPCC (5). Over 90% of the HNPCC tumors are MSI+ (6). However, the MSI+ phenotype has also been observed in approximately 10 to 15% of apparently sporadic colorectal cancers (7), and in about 10% of sporadic gastric cancers (8). Previously, significant associations have been noted between the MSI+ phenotype, and cancer multiplicity, cancers in other organs in the HNPCC patients (9, 10), and sporadic metachronous multiple colorectal carcinomas (11). Patients with multiple primary cancers are likely to be a part of a group with an underlying inherited predisposition.

Individuals who carry germline mutations in *hMSH2* or *hMLH1* probably have a higher risk of accumulating mutations in cancer-associated genes and of developing multiple primary cancers. There have been a few reports on the contribution of replication error in double cancers of the colorectum and stomach. Genetic instability may play an important role in the development of multiple primary cancers, and testing for MSI in a primary cancer may be an appropriate approach to the detection of patients at high risk for developing multiple primary cancers. Although it would be laborious to examine cancer patients for germline mutations in various mismatch repair genes, the presence or absence of MSI in tumors may constitute a useful marker for screening patients and identifying those at high risk for additional primary cancers. If the MSI+ tumor phenotype is an indicator of increased cancer susceptibility in these multiple primary cancer patients, then MSI detection may constitute a useful screening tool.

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To investigate the above possibility, the MSI phenotype and frameshift mutation were analyzed in 17 patients with double primary cancers of the colorectum and stomach and 24 patients with double primary colorectal cancer and non-gastric malignancy. Additional studies for loss of heterozygosity (LOH) of the chromosomal arm 17p, and p53 immunohistochemical staining, were performed.

MATERIALS AND METHODS

Patients

Nineteen colon cancers and 17 gastric cancers from 17 patients with double primary cancers who underwent surgical resection between 1988 and 1997 at Seoul National University Hospital, Seoul, Korea, were analyzed. Double cancers of the colorectum and stomach were found synchronously in 5 patients and metachronously in 12 patients. The majority of the colon cancers were Dukes' stage B or C (73.7% and 26.3%, respectively). The stomach cancers consisted of six early gastric cancers and 11 advanced gastric cancers. Nine were of the intestinal type and eight were of the diffuse type. The average age of 17 patients was 56.3 years, and the male to female ratio was 7.5:1.

As a control group, twenty-six colorectal cancers and 24 cancers of non-gastric origin from 24 patients of double primary cancers were analyzed for MSI. They were composed of malignant tumors from the prostate in four cases, the breast in three, the kidney in three, the urinary bladder in three, the uterine cervix in three, the liver in two, the skin in two, the small intestine in one, the endometrium in one, the hematopoietic system (multiple myeloma) in one, and sarcoma (malignant fibrous histiocytoma) in one. The average age of 24 patients was 58.6 years, and the male to female ratio was 1.7:1.

Among 41 patients with double cancers analyzed, 4 patients of metachronous colorectal cancers were identified. Two patients were of colon and stomach cancer pairs (cases 23 and 25), and remaining two were of colon and non-gastric origin cancer pairs (renal cell carcinoma and prostatic adenocarcinoma, respectively).

For comparison, 247 single primary colorectal cancers and 152 single primary gastric cancers were analyzed for MSI.

Information concerning cancer in first- and second-degree relatives has been obtained for all patients investigated in the present study. Patients with family history of the Amsterdam criteria for HNPCC were excluded. Family history of cancers in colorectum or stomach were identified in two (11.8%) of 17 patients with double primary cancers of the colorectum and stomach and 18 (7.3%) of 247 patients with single primary colorectal cancer.

DNA Extraction

DNA from 41 patients was obtained from formalin-fixed, paraffin embedded surgical sections. The microdissection procedure was performed with 26 gauge needle under light microscopy. To reduce the possibility that the genetic abnormalities in the tumor cells would be compromised by the presence of normal cells, the neoplastic areas were microdissected from hematoxylin-eosin stained slides. This microdissection procedure harvests more than 60% of tumor cell populations (12).

Microsatellite Markers

For the evaluation of MSI status in the 17 patients who had double cancers of the colorectum and stomach, five primers (BAT26, D5S346, D17S520, D17S786, and TP53) were purchased from Research Genetics (MapPair, Huntsville, AL). In the 24 patients with pairs of colorectal cancer and non-gastric malignancy, the MSI-H tumor was detected using the sensitive and specific marker, BAT26 (Fig. 1, *left*; 7, 12–14). The primers for the mononucleotide repeat microsatellite sequences were as follows: BAT26, located within Intron 5 of the *hMSH2* gene; poly(A)₁₀ tract of *TGF-β RII*; poly(G)₈ tract of *BAX*; poly(G)₈ tract of *IGFIIR*; and poly(A)₈ tract of *hMSH3*. We used the same primers as described in a previously published paper (12).

PCR Amplification and Microsatellite Analysis

PCR amplification with MapPair primers was performed with 5 pmol/μL of each primer, 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 0.5 unit *Taq* polymerase, 0.1 μL [α -³²P] dCTP (3,000 Ci mmol⁻¹, NEN, Dupont, Boston, MA), and 1–2 μL of DNA in a total volume of 10 μL. The PCR conditions

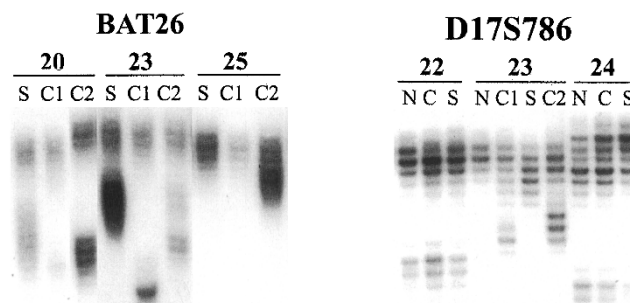


FIGURE 1. *Left*, examples of BAT26 alteration. Carcinoma with size variation in the BAT26 allele is defined as MSI+. C1 and C2, metachronous colorectal cancers. Case 20 and Case 23 were MSI+ in two metachronous colon cancers and stomach cancer. Case 25 was MSI+ in one colon cancer but was MSI- in the other colon cancer and stomach cancer. *Right*, examples of microsatellite alteration. N, normal mucosa; C, colorectal cancer; S, stomach cancer. At marker D17S786, Case 22 exhibited prominent decrease of upper-band density in DNA of the colon cancer (*Lane C*) and stomach cancer (*Lane S*). Case 23 showed MSI in the stomach cancer (*Lane S*) and two colon cancers (*Lanes C1* and *C2*).

were 95°C for 5 minutes, followed by 33 cycles (94°C for 30 s, 45–58°C for 30 s, 72°C for 40 s), and a final elongation step at 72°C for 10 minutes. The PCR products were diluted to a concentration of 1:4 with a loading buffer, heated at 100°C for 5 minutes, and stored on ice until analysis. Then 1.5- μ L aliquots of each sample were separated on denatured 6% polyacrylamide gel. The gel was dried on a vacuum slab gel dryer at 80°C for 1–1.5 hours, and exposed to X-ray film at –70°C for 12–72 hours.

MSI of any marker was defined as appearance of additional band in the PCR product in a tumor compared to normal counterpart. MSI+ denoted cases with MSI in two or more markers. MSI- included cases with no instability in any of the markers (MSS) and MSI in one marker (MSI-L; 7). LOH was scored when the band intensity of one allele was less than 50% in the tumor DNA than in normal DNA (Fig. 1, *right*).

To detect frameshift mutations in the coding regions, the reaction involved 32 cycles at 94°C for 1 minutes, at 53–60°C for 1 minutes, and at 72°C for 1 minutes using 3.7×10^4 Bq of 32 P-dCTP in 10 μ L of reaction mixture. The subsequent procedures were the same as for the MSI analysis.

p53 Immunostaining

Paraffin-embedded tissue sections of 4 μ M thickness were stained using murine monoclonal antibody against p53 (Dakopatts, Copenhagen, Denmark, 1:100) by the avidin-biotin complex (ABC) method. Biotinylated rabbit anti-IgG and avidin-biotin-peroxidase conjugate were used for labeling and 3-diaminobenzidine was used for coloring.

More than 10% of nuclear staining was considered to be positive.

Statistical Analysis

Either chi-test or Fisher's exact test was used to analyze the differences in MSI status between the tumor groups. Statistical significance was defined as $P < .05$.

RESULTS

Six (31.6%) of 19 colon cancers and 3 (17.7%) of 17 gastric cancers were MSI+ in 17 patients with double cancers of the colorectum and stomach (Table 1). Three (17.7%) of 17 patients were MSI+ in both colon and gastric cancers. Twelve (70.6%) patients were MSI- in both the colorectal and gastric cancers. The remaining two (11.8%) patients were MSI+ in colon cancer, but MSI- in gastric cancer. We summarized the MSI results in Table 2.

Of 24 patients with double primary pairs of colorectal cancer and non-gastric malignancy, MSI+ was found in three (11.5%) of 26 colorectal cancers and one (4.2%) of 24 extra-colonic cancers (breast cancer). In the patient with MSI+ breast cancer, the colon cancer was MSI-. There were three patients with MSI+ both in colon and stomach pairs, compared to none in colon and non-gastric pairs (3 of 17 vs 0 of 24, $P = .033$, Fisher's exact test).

Of 4 patients with metachronous colorectal cancers included in this study, one patient was MSI+ in both colon and stomach cancers (cases 23), one patient was MSI+ in colon cancer and MSI- in

TABLE 1. Clinicopathologic Profiles of 17 Double Primary Cancers of the Colorectum and Stomach

Case	Age	Sex	MSI ^a		Colorectal Cancer				Stomach Cancer			
			C	S	Site ^b	Stage	WHO ^c	LN ^d	Site ^b	Stage	Lauren ^c	LN ^d
23-1	45	M	●	●	T	T3	MUC	–	C	T1	I	–
23-2			●	●	C	T3	PD	–				
25-1	61	M	○	○	R	T2	MD	–	B	T3	D	+
25-2			●	●	C	T3	MD	–				
18	74	F	●	●	C	T3	PD	+	A	T3	I	–
67	43	M	●	●	R	T3	MD	–	A	T3	I	+
65	71	M	●	○	T	T3	MD	–	A	T1	I	–
28	61	M	○	○	R	T3	WD	–	B	T2	D	+
22	61	M	○	○	A	T3	MD	–	B	T3	D	+
24	68	M	○	○	R	T2	MD	–	A	T3	I	–
21	53	M	○	○	R	T3	MD	–	B	T3	D	+
26	50	F	○	○	T	T3	WD	+	A	T3	I	+
27	57	M	○	○	R	T3	MD	–	A	T1	I	+
29	54	M	○	○	S	T3	MD	–	A	T3	D	+
30	53	M	○	○	D	T3	PD	+	B	T1	D	–
32	52	M	○	○	S	T3	MD	+	A	T2	D	–
62	46	M	○	○	D	T3	MD	+	A	T1	I	+
64	56	M	○	○	R	T2	MD	–	A	T3	D	–
66	53	M	○	○	R	T2	MD	–	B	T1	I	–

^a MSI, microsatellite instability status; C, colorectal cancer; S, stomach cancer; ●, MSI+; ○, MSI–.

^b C, cecum; A, ascending; T, transverse; D, descending; S, sigmoid; R, rectum; A, antrum; C, cardia; B, body.

^c WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; MUC, mucinous carcinoma; I, intestinal type; D, diffuse type.

^d LN, lymph node metastasis; +, present; –, absent.

TABLE 2. Results of MSI in Double Primary Colonic and Extracolonic Cancers

Group	Double Primary Cancers (n = 41)		Metachronous Colon Cancers ^a (n = 4)	Single Primary Colorectal Cancers (n = 247)
	Stomach (n = 17)	Nongastric (n = 24)		
No. of colon cancers	19	26	8	
No. of extracolonic cancers	17	24	4	
MSI+ rates in cancers, n (%)				24/247 (9.7) ^e
Colorectum cancers	6/19 (31.6) ^b	3/26 (11.5)	3/6 (50)	
Extracolonic cancers	3/17 (17.7)	1/24 (4.2)	1/4 (25)	
Patients with concurrent MSI+, n (%)	3/17 (17.7) ^c	0/24 (0) ^d	2/5 (40)	
Patients with discordant MSI status, n (%)	2/17 (11.8)	0/24 (0)	1/5 (20)	

^a Patients identified within the group of double primary cancers.

^b Significantly different from ^e, $P = .004$, χ^2 test.

^c Significantly different from ^d, $P = .033$, Fisher's exact test.

gastric cancer (case 25), and the remaining two patients with non-gastric cancer were MSI- in all of the detected cancers.

In our experimental study for MSI in single primary cancers, MSI+ rate was 9.7% in colorectal cancers, and 9.2% in stomach cancers. MSI+ rate in stomach cancers of the double cancer patients was not greater than expected one (3 of 17 vs 14 of 152, $P = .27$), however, it was significantly higher than expected in colorectal cancers (6 of 19 vs 24 of 247, $P = .004$; Table 2).

Five (83.3%) of 6 MSI+ colon cancers showed a frameshift mutation in the poly(A)₁₀ tract of the *TGF-β RII* gene. All three MSI+ gastric cancers showed mutation in that gene. In contrast, mutations of *hMSH3*, *IGFIIR* or *BAX* in both cancers were found in less than half of the cases (Table 3).

The MSI+ colorectal cancers showed characteristics of localization in the proximal or right side of the colon, grossly ulcerofungating type, mucinous or poorly differentiation histologically, rare lymph node metastasis, and larger average tumor size (7.1

versus 4.8 cm). Three MSI+ stomach cancers showed exclusively intestinal type, no lymph node metastasis, and smaller average tumor size (2.5 versus 4.3 cm). In the colorectal cancers, 3 of 6 MSI+ cancers and one of thirteen MSI- cancers showed high numbers of intraepithelial lymphocytes (IELs; $P = .036$). However, no significant association between the degree of IELs and MSI status was found in the stomach cancers (1 of 3 vs 6 of 14, $P = .76$).

LOH at 17p was noted in 13 (81.3%) of 16 colorectal cancers and nine (64.3%) of 14 gastric cancers. In 10 (76.9%) of 13 MSI- colon cancers and 9 (64.3%) of 14 MSI- gastric cancers, 17p allelic loss was identified. Twelve (63.2%) of 19 colorectal cancers and 6 (35.3%) of 17 stomach cancers were positive in p53 immunostaining. Nine (69.2%) of 13 colorectal cancers and 6 (85.7%) of 7 gastric cancers with p53 overexpression were MSI-. The p53-positive cancers tended to be MSI-. 17p allelic loss was observed in 8 (66.7%) of 12 colorectal cancers and 4 (66.7%) of 6 gastric cancers with p53 overex-

TABLE 3. Genetic Profiles of 17 Double Primary Cancers of the Colorectum and Stomach

Case	Colorectal Cancer					Stomach Cancer				
	MSI ^a	<i>TGF-βRII</i>	<i>HMSH3</i>	<i>IGFIIR</i>	<i>BAX</i>	MSI	<i>TGF-βRII</i>	<i>hMSH3</i>	<i>IGFIIR</i>	<i>BAX</i>
23-1	●	-1/wt	wt	wt	wt	●	-1/wt	wt	wt	-1/wt
23-2	●	+1/wt	wt	wt	-1/wt					
25-1	○	wt	wt	wt	wt	○	wt	wt	wt	wt
25-2	●	-1/wt	-1/wt	wt	wt					
18	●	wt	wt	wt	-1/wt	●	-2/wt	wt	wt	-1/wt
67	●	-1/wt	wt	-1/wt	-1/wt	●	-1/wt	wt	wt	wt
65	●	-1/wt	wt	+1/wt	wt	○	wt	wt	wt	wt
28	○	wt	wt	wt	wt	○	wt	wt	wt	wt
22	○	wt	wt	wt	wt	○	wt	wt	wt	wt
24	○	wt	wt	wt	wt	○	wt	wt	wt	wt
21	○	wt	wt	wt	wt	○	wt	wt	wt	wt
26	○	wt	wt	wt	wt	○	wt	wt	wt	wt
27	○	wt	wt	wt	wt	○	wt	wt	wt	wt
29	○	wt	wt	wt	wt	○	wt	wt	wt	wt
30	○	wt	wt	wt	wt	○	wt	wt	wt	wt
32	○	wt	wt	wt	wt	○	wt	wt	wt	wt
62	○	wt	wt	wt	wt	○	wt	wt	wt	wt
64	○	wt	wt	wt	wt	○	wt	wt	wt	wt
66	○	wt	wt	wt	wt	○	wt	wt	wt	wt

MSI, microsatellite instability status; *TGF-βRII*, transforming growth factor β receptor II; *IGFIIR*, insulin growth factor II receptor.

^a ●, MSI+; ○, MSI-.

pression. No correlation was demonstrated between p53 overexpression and 17p allelic loss.

DISCUSSION

In our study, 3 (17.7%) of 17 patients with double primary cancers of the colorectum and stomach showed the MSI+ in both cancers. It is suggested that there is a genetic component in the coincidence of double primary cancers of the colorectum and stomach, and that a defect in the mismatch repair system might be the causative mechanism in a small subset of double primary cancers.

Frequent MSI in multiple primary cancers of the colorectum or stomach have been reported (15, 16). In one series (15), MSI+ rate was reported in 17 (27.4%) of 62 cases of the multiple primary cancers excluding the HNPCC patients compared to 7 (8.1%) of 86 of the single primary colon cancers. These MSI+ rates are similar to our study. However, concordant MSI+ in double primary cancers was not analyzed in their study. In another series (16), 34 (89.5%) of 38 patients with multiple primary cancers were determined as MSI+, however, they had a limitation that MSI-L and MSI-H were not distinguished. To apply the recent consensus about MSI+ (7), they misclassified low frequency MSI (MSI-L) cases that were instable in one of 5 markers as MSI+. The 13 cases that were instable in one marker should be classified as MSI-.

Multiple cancers occurred in 5–10% (17, 18) of ordinary colorectal cancer patients, and in 11.5 (19)–24% (20) of HNPCC patients. In HNPCC, 10-year cumulative incidence of metachromous cancer is 40% if the first cancer is treated with less than a subtotal colectomy (5). Overall MSI+ rate in multiple colorectal cancers including HNPCC and sporadic tumors has been reported to be 28% (9).

In our study, most MSI+ tumors exhibited a *TGF-β RII* frameshift mutation and half of the MSI+ tumors showed *BAX* mutation. It is well known that the *TGF-β RII* gene is the main target of MSI+ sporadic colorectal cancer as well as of HNPCC (21, 22).

We demonstrated that the MSI+ tumors tended to show a lower rate of p53 overexpression as previously reported (10). The observed mismatch repair deficiency of double primary colorectal and gastric cancers suggests that there is an independent pathway involved in their carcinogenesis, which differs from the p53 pathway.

The MSI+ colorectal cancers in our study showed gross features of the fungating type, larger size, microscopically poorly differentiated or mucinous type histology, and rarely metastasized into the regional lymph nodes. It has been assumed that MSI+ colorectal cancers show similar histopatho-

logic findings to HNPCC (23). All three cases of MSI+ gastric cancer in our study were of the intestinal type, and the size of the tumors was smaller than in MSI- stomach cancer. In addition, MSI+ stomach cancer seldom spread to the lymph nodes. The histological features of MSI+ colorectal cancer showed a large size and poor differentiation. In contrast, MSI+ stomach cancer showed a smaller size and good differentiation. The reason for the above discrepancy between these two different types of cancers should be investigated in future studies.

Considering the strong association of MSI+ with double primary colorectal-gastric cancers, we suggest that the carcinogenic background is related to genetic factors. The cancer susceptibility of non-HNPCC MSI+ colorectal-gastric primary cancer patients in this study is more likely to be the result of unrecognized genetic predisposition to mismatch repair defect.

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Book Review

Henson DE, Albores-Saavedra J: Pathology of Incipient Neoplasia, Third Edition, 856 pp, New York, Oxford University Press, 2001 (\$165.00).

In the “era” of early cancer detection, a book to guide pathologists in the recognition of lesions that have been associated with early neoplastic development is welcome. Because the fundamental basis of knowledge in cancer pathology was obtained from invasive forms of cancer, the histologic interpretation of incipient neoplastic or preneoplastic lesions still remains controversial in some fields. This book covers in several chapters divided by different organs/systems, like a traditional book of tumor pathology, the histological criteria for diagnosis of several preneoplastic or early neoplastic lesions.

The chapters are written in a comprehensive way by several experts in the different fields of pathology. Most of them incorporate to the morphological descriptions the more relevant molecular alterations that can be recognized in the incipient neoplasias. This is an important approach because in past years pathologists have used information such as oncogene amplification, mutations in tumor suppressor genes, aneuploidy, among others, more frequently in cancer pathology for the diagnosis and definition of

outcome. It could be debatable if topics covered in some chapters represent true “incipient” neoplasias and not advanced ones, but it reflects the natural difficulty in the present status of knowledge to define precisely how incipient or advanced a lesion is in some organs. There are black and white and color pictures, most of them illustrative and of good quality. However, some (rare, fortunately!) figures are out of focus and have green filters that compromise their quality. In future editions these figures should be changed. The references provide extensive coverage of all conditions related but could be more updated, specially in areas of fast-growing knowledge. The index is complete and user-friendly.

In summary, this text is a forward step in the future of tumor pathology, when we are requested not to recognize advanced cancers but to diagnose and predict outcomes in early lesions based on a blending of the information derived from morphology and molecular biology.

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