Advances in Brief

Microsatellite Instability and Mismatch-Repair Protein Expression in Hereditary and Sporadic Colorectal Carcinogenesis[1]

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Abstract

Aberrant crypt foci (ACF) are microscopic clusters of altered colonic crypts considered premalignant lesions in the large bowel. Genomic instability at short tandem repeats in the DNA, referred to as microsatellite instability (MSI) is the hallmark of hereditary nonpolyposis colorectal carcinoma (HNPCC) caused by mutations in DNA mismatch-repair genes, mostly *hMLH1* and *hMSH2*. In this study, we evaluated for MSI ACF ($n = 16$), adenomas ($n = 18$), carcinomas ($n = 22$), and lymph node metastases ($n = 3$) from 17 patients with colorectal cancer positive for MSI. Ten patients were members of HNPCC families; 7 patients had no family history of cancer. MSI was found in 7 of 7 (100%) ACF and 11 of 12 (91%) adenomas from patients with HNPCC. MSI was not related to histology and size of ACF. A progressive increase in instability as estimated by the number of shifted bands was observed along the ACF-adenoma-carcinoma sequence. In contrast, two of nine (22%) ACF and none of six adenomas from patients with MSI sporadic carcinoma were unstable at microsatellite loci. hMLH1 or hMSH2 protein expression was altered only in MSI-positive premalignant lesions (ACF and/or adenomas), but not in all MSI-positive lesions in patients with HNPCC. These observations provide evidence of the premalignant nature of ACF in HNPCC and suggest that MSI is a very early event both in HNPCC and in sporadic colorectal carcinogenesis, although in the latter it seems infrequent.

Introduction

Colorectal carcinogenesis is a stepwise process that from normal colonic mucosa leads to carcinoma through premalignant lesions known as adenomas. Recently, a large body of evidence has suggested that early changes, referred to as ACF,[3] identified on the colonic mucosal surface of experimental animals treated with colon carcinogens (1, 2) and of humans (3, 4) can be considered premalignant (5–7). Some genetic alterations underlying colorectal cancer development have been demonstrated in human ACF, including K-*ras* and *APC* mutations (8, 9), which seem early events in the tumorigenic process. However, hereditary and sporadic colorectal cancer show distinct genetic alterations, suggesting that the key events leading to neoplastic growth are different along the two pathways. Recently, variability in the length of short tandem repeats in the DNA, referred

to as MSI, has been demonstrated in some human ACF (10, 11). MSI is the molecular feature of HNPCC, a genetic syndrome caused by mutations in DNA mismatch-repair genes. Previous studies have indicated that up to 57% of colonic adenomas from patients with HNPCC and 2–3% from patients with no family history of colon cancer show MSI (12, 13). These early changes would imply a distinct mismatch repair-deficient pathway for colorectal cancer, as is frequently the case with HNPCC, in which most adenomas are unstable (12), thus suggesting that mismatch-repair gene mutations occur early in the progression of HNPCC.

In this study, we examined MSI and hMLH1 and hMSH2 protein expression along the ACF-adenoma-carcinoma sequence in patients with HNPCC and sporadic colon cancer with the purpose of evaluating the timing of onset of MSI in HNPCC and sporadic colorectal carcinogenesis. We selected patients with carcinomas positive for MSI, both HNPCC and sporadic, and evaluated MSI in premalignant lesions. This study provides evidence that ACF are premalignant lesions in HNPCC and that MSI is a very early event both in HNPCC and in sporadic colorectal carcinogenesis, although in the latter it seems infrequent.

Materials and Methods

Patients. The eligibility criterion for recruitment of patients was being affected by a MSI(+) colorectal cancer with at least one synchronous premalignant lesion (ACF or adenoma). Seventeen patients were enrolled: 10 with a diagnosis of HNPCC according to the standard clinical criteria (14) and 7 with a negative cancer family history. Five of 10 HNPCC patients were carriers of a germline mutation in one of the two major mismatch-repair genes, *hMLH1* or *hMSH2*, 2 patients were negative for mutations in the latter two genes, and for 3 patients the analysis was not complete at the time of the study.

Lesions and DNA Extraction. The collected material included 16 ACF, 7 in patients with HNPCC and 9 in patients with sporadic colon cancer (Table 1); 18 adenomas (12 HNPCC and 6 sporadic); 22 carcinomas (15 HNPCC and 7 sporadic); and 3 lymph node metastases. For the detection of ACF, after operation for colorectal cancer, normal colonic mucosa was fixed flat in 10% buffered formalin for no longer than 72 h. The colonic mucosa was then stained with 0.2% methylene blue in saline solution for 10–15 min and examined under a dissecting microscope at ×20–30. ACF were removed, embedded in paraffin, and cut into 5-μm sections. Ten to 20 horizontal sections were prepared for DNA extraction and at least 3, taken at different height along the crypts axis, were stained with H&E to evaluate histological alterations. Sections (5-μm thick) were also cut from paraffin blocks of carcinomas, adenomas, lymph node metastases, and normal mucosa. Ten sections from each lesion were collected on microscopic slides and microdissected with sterile scalpels into polypropylene tubes. The DNA was then extracted following a previously described method (15). Samples used for DNA extraction contained at least 50% of cells of the colorectal lesion, as evaluated by histological sections, to minimize contamination by normal and necrotic tissue areas.

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Table 1 *Features of ACF in patients with HNPCC and in patients with sporadic colorectal cancer*

	Patient no.	Patient age (years)	ACF no.	Site	Histology	Multiplicity (no. of crypts per focus)	MSI
HNPCC	1	51	1	R[a]	H	52	+
			2	R	H	25	+
	2	58	3	R	H	12	+
			4	R	H	35	+
			5	R	H	300	+
	3	34	6	R	D	300	+
	4	46	7	R	D	200	+
Sporadic CRC	1	65	1	L	H	50	−
			2	L	H	25	−
	2	94	3	R	D	100	−
			4	R	D	250	−
			5	R	D	400	−
	3	78	6	R	H	8	−
	4	59	7	R	H	70	−
	5	77	8	R	D	200	+
			9	R	D	13	+

[a] R, right colon (cecum, ascending colon, hepatic flexure, transverse colon, and splenic flexure); H, hyperplastic ACF; D, dysplastic ACF; L, left colon (descending and sigmoid colon, rectum); CRC, colorectal cancer.

Analysis of MSI. PCRs were performed to amplify colorectal lesions and corresponding normal DNAs at four simple repeated sequences. The loci examined were *BAT26*, *BAT40*, *D2S123*, and *D18S57*. The reaction volume for PCR was 10 μl, which contained 50–100 ng of DNA; 10 ng of unlabeled primers; 200 μM dGTP, dTTP, and dATP; 2 μM dCTP; 0.7 μCi (α33P) of dCTP; 1.5 mM MgCl$_2$; 50 mM KCl; 10 mM Tris (pH 8.3); and 0.3 units of Taq polymerase. The PCR reaction was run as follows: initial denaturation step of 4 min at 94°C; 27 cycles consisting of 30 s at 94°C, 75 s at 55°C, and 15 s at 72°C; and a final extension step of 4 min at 72°C. PCR products from the colorectal lesions and corresponding normal DNA of the same patient were loaded in adjacent lanes on a standard 6% denaturing polyacrylamide gel and visualized by autoradiography. Lesions were scored as positive [MSI(+)] when instability was detected in at least two microsatellite loci (50%; Ref. 16).

Analysis of hMLH1 and hMSH2 Protein Expression. Formalin-fixed, paraffin-embedded samples of ACF, adenomas, and carcinomas were sectioned at 6 μm. After deparaffination and rehydration, slides were submitted to microwave antigen retrieval [30 min at 350 W in 10 mM citrate buffer (pH 6.0)]. Mouse monoclonal antibodies to full-length hMLH1 and hMSH2 proteins (G168-15 and G129-1129, PharMingen, San Diego, CA) were used at a 1:40 dilution. Immunoperoxidase staining using diaminobenzidine as chromogen was carried out with the Nexes Automatic Staining System (Ventana, Strasbourg, France). Lesions were considered positive for protein inactivation when a complete absence of detectable nuclear staining was evident in epithelial cells of the lesions. Definite nuclear staining of adjacent nonneoplastic epithelial and stromal cells or lymphocytes served as internal positive controls.

Results

ACF were found in all samples of mucosa examined. The density of ACF (number of ACF per cm^2 of mucosa examined) was lower in patients with HNPCC than in those with sporadic colorectal cancer [median of 0.02 (range, 0.01–0.04) *versus* median of 0.10 (range, 0.07–0.15); $P < 0.01$, Mann-Whitney U test].

All MSI(+) lesions showed high levels of MSI (16), most of them ≥75%. No sample had instability at only one locus. MSI was detected in all seven ACF from HNPCC patients and in two of nine ACF from patients with sporadic tumors (Table 1). Seven ACF showed features of dysplastic epithelium (two in patients with HNPCC and five in patients with sporadic carcinoma), and nine were classified as hyperplastic (five HNPCC and four sporadic; Ref. 17). MSI was detected in either dysplastic or hyperplastic ACF (Table 1). Moreover, MSI was independent of crypt multiplicity, *i.e.,* the number of crypt per focus, which is related to the size of the focus. Indeed, MSI was present even in small foci in patients with either HNPCC or sporadic colorectal cancer.

The presence of microsatellite alterations was observed in 11 of 12 HNPCC adenomas, whereas 6 sporadic adenomas were all MSI negative. MSI was evident even in the three lymph node metastases from two patients with HNPCC and one patient with sporadic cancer. Thus, MSI was present along the HNPCC colorectal carcinogenesis sequence from ACF to carcinoma and lymph node metastasis (Fig. 1*A*). On the other hand, most premalignant lesions from patients with MSI(+) sporadic tumors were stable (Fig. 1*B*). In addition, MSI(+) ACF showed shifts of a few bands only, whereas adenomas and carcinomas had more evident instability, in particular at the *BAT26* locus (Fig. 1*A*). In HNPCC colorectal carcinogenesis, MSI(+) adenomas seemed to have a pattern of bandshift intermediate between ACF and carcinoma (Fig. 1*A*). The number of shifted bands was not related to the degree of dysplasia in adenomas, at least with *BAT26*.

hMLH1 and hMSH2 protein expression was evaluated in lesions from seven HNPCC patients with known germline mutations (Table 2), and from four patients with sporadic colorectal cancer (Table 3). The other patients were excluded because sufficient material, *i.e.,* at least one premalignant lesion (ACF or adenoma) other than the synchronous carcinoma for the analysis, was not available. Of two ACF examined from patients with HNPCC, one had normal expression of both proteins (Table 2, *patient 3*), whereas the other showed inactivation of hMLH1 protein (Table 2, *patient 4*). In the latter case, loss of hMLH1 expression was also evident in the synchronous adenoma and carcinoma. Among the 11 MSI(+) adenomas from HNPCC patients, hMLH1 protein was not expressed in 5 adenomas from 3 patients, hMSH2 was not expressed in 2 adenomas from 2 patients, and hMLH1 and hMSH2 were expressed normally in 4 adenomas from 2 patients. In the two patients harboring the four adenomas showing no loss of protein expression (Table 2, *patients 3 and 6*), no germline mutations of the *hMLH1* and *hMSH2* genes were found. Protein expression in premalignant lesions was always con-
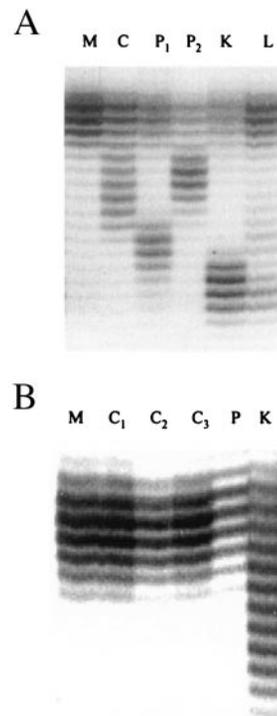

Fig. 1. *A*, MSI along the sequence normal mucosa (*M*), dysplastic ACF (*C*), adenomas (*P$_1$* and *P$_2$*), carcinoma (*K*), and lymph node metastasis (*L*) at the *BAT26* locus from one patient with HNPCC (Table 2, *patient 3*). *B*, MSI along sporadic colorectal carcinogenesis sequence normal mucosa (*M*), dysplastic ACF (*C$_1$*, *C$_2$*, and *C$_3$*), adenoma (*P*), and carcinoma (*K*) at the *BAT26* locus from one patient with sporadic colon cancer (Table 3, *patient 2*).

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Table 2. *Germline mutation, microsatellite status, and immunohistochemical analysis of hMLH1 and hMSH2 protein expression in ACF, adenomas (P), and carcinomas (K) of seven patients with HNPCC.*

Patient no.	Germline mutation	Type of lesion	MSI	hMLH1	hMSH2
3	Negative	ACF	+	+	+
		P1	+	+	+
		P2	+	+	+
		K	+	+	+
4	hMLH1	ACF	+	−	+
		P	+	−	+
		K	+	−	+
5	hMLH1	P1	+	−	+
		P2	+	−	+
		P3	+	−	+
		K1	+	−	+
		K2	+	−	+
6	Negative	P1	+	+	+
		P2	−	+	+
		K1	+	+	+
		K2	+	+	+
		K3	+	NT[a]	NT
7	hMSH2	P	+	+	−
		K	+	+	−
8	hMSH2	P	+	+	−
		K	+	+	−
9	hMLH1	P	+	−	+
		K	+	−	+

[a] NT, not tested.

cordant with that of the corresponding carcinomas in HNPCC patients (Table 2), and it was in agreement with the results of the mutational analysis of *hMLH1* and *hMSH2* genes in patients examined. Both unstable (Table 3, *patient 5*) and stable (Table 3, *patient 2*) ACF examined from patients with sporadic colorectal cancer showed normal expression of both proteins. It should be noted that the carcinoma as well as the ACF from patient 5 retained normal expression of these proteins. The six adenomas tested from these patients were MSI negative and retained normal expression of both proteins (Table 3). Finally, three of four sporadic carcinomas showed loss of hMLH1 protein expression.

Discussion

Colorectal cancer develops through a series of distinct histological steps, from normal mucosa to invasive cancer. ACF are microscopic lesions that have been postulated to precede the development of adenomas and are considered the earliest premalignant lesions in colon carcinogenesis. The presence of MSI in ACF suggests that the MSI(+) phenotype may occur early in colorectal carcinogenesis (10, 11). To clarify the role of mismatch-repair defects and when such changes occur during the ACF-adenoma-carcinoma sequence, we analyzed ACF and/or adenomas from patients with HNPCC and sporadic unstable colorectal carcinomas for the presence of MSI. MSI was found in 100% and 91% of ACF and adenomas, respectively, in patients with HNPCC, suggesting that MSI represents an early event in HNPCC colorectal carcinogenesis. Indeed, the results of the analysis of hMLH1 and hMSH2 protein expression confirmed that in HNPCC patients DNA mismatch-repair genes seem altered beginning with the early phases of the carcinogenic pathway: 8 of 13 (61%) premalignant lesions examined (1 ACF and 7 adenomas) showed inactivation of hMLH1 or hMSH2 protein. The five premalignant lesions that retained expression of these proteins resembled the carcinomas seen in these patients and agreed with the genetic data (Table 2). This implies that homozygous or inactivating mutations in mismatch-repair genes occur at an early stage of HNPCC colorectal carcinogenesis. These observations provide for the first time strong evidence of the premalignant nature of ACF in HNPCC. Moreover, they may explain the lower density of ACF and the rapid growth of

premalignant lesions in HNPCC patients. In fact, early loss of mismatch-repair gene function could make ACF and adenomas progress faster than in sporadic colorectal carcinogenesis.

On the other hand, 22% of ACF and no adenomas from patients with sporadic carcinoma were unstable. Augenlicht *et al.* (10) found that 3 of 27 ACF (11.1%) from 2 patients with apparently sporadic carcinoma showed MSI. Heinen *et al.* (11) reported 2 MSI(+) ACF of 19 (10.5%). In the present study, two sporadic ACF from the same patient (patient 5) showed MSI, which could be caused by inactivation of secondary mutators (*i.e.,* MSH6). It is possible that the higher rate of MSI(+) sporadic ACF found in this study (22%) could be explained by the selection criteria adopted for the study. Indeed, we chose MSI(+) carcinomas to select patients. Moreover, six of seven sporadic carcinomas examined for MSI were localized in the proximal colon, and right-sided colon carcinomas show higher rates of MSI than left-sided (18), although this does not seem the case for adenomas (13). This study, however, cannot be definite on that point because only two ACF were taken from left-sided colonic mucosa. Finally, the loss of hMLH1 protein expression in three of four sporadic carcinomas could be caused by epigenetic mechanisms of inactivation of *hMLH1* gene (*i.e.,* hypermethylation of the promoter; Ref. 19).

ACF are histologically heterogeneous, showing features ranging from mild histological alterations to severe dysplasia, sometimes coexisting in the same lesion (17, 20, 21). Interestingly, we observed that MSI occurs either in dysplastic or in hyperplastic ACF from HNPCC patients. This suggests that MSI is not associated with histological features of ACF, as occurs in colorectal polyps from HNPCC patients (22), but is associated with a defective mismatch-repair system and underlies the very early steps of HNPCC colorectal carcinogenesis, being present even in small ACF. In addition, MSI in sporadic ACF also seems independent of ACF size.

In the present study, 91% of adenomas in patients with HNPCC showed MSI. This proportion is higher than that reported previously (12). The most probable reason for the difference is the selection of patients with MSI(+) carcinoma. Moreover, seven MSI(+) adenomas were found in the five patient carriers of a constitutional mutation in one of the two most frequently mutated mismatch-repair genes.

Another intriguing result is the progressive accumulation of mutations (bandshifts) at the locus *BAT26*, indicative of a stepwise worsening of the defect from normal mucosa to carcinoma, as suggested previously (11). Indeed, alterations in allele length seem to increase and accumulate with tumor growth in colorectal carcinogenesis, involving target genes with a specific timing of mutation (23).

In conclusion, the results of the present study provide evidence that ACF may be considered the earliest lesions in HNPCC colorectal

Table 3 *Microsatellite status and immunohistochemical analysis of hMLH1 and hMSH2 protein expression in ACF, adenomas (P), and carcinomas (K) from four patients with sporadic colorectal cancer*

Patient no.	Type of lesion	MSI	hMLH1	hMSH2
2	ACF1	−	+	+
	ACF2	−	+	+
	ACF3	−	+	+
	P	−	+	+
	K	+	−	+
5	ACF1	+	+	+
	ACF2	+	+	+
	K	+	+	+
6	P	−	+	+
	K	+	−	+
7	P1	−	+	+
	P2	−	+	+
	P3	−	+	+
	P4	−	+	+
	K	+	−	+

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carcinogenesis as is the case for sporadic carcinogenesis. MSI is a very early event in both HNPCC and sporadic colorectal tumorigenesis, although in the latter it seems rather infrequent, in accordance with the low rates of MSI(+) tumors. Whereas most unstable carcinomas probably occur outside the context of HNPCC, unstable adenomas and ACF might be a useful marker for this syndrome. In particular, MSI analysis in ACF could be an appropriate tool to identify suspected hereditary forms of colorectal cancer.

Acknowledgments

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