Identification of Mucin Depleted Foci in the Human Colon

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Abstract Aberrant crypt foci (ACF) originally described in rodents treated with colon-specific carcinogens have been identified also in humans at high risk of colon cancer (CRC) and are extensively used as cancer biomarkers. However, studies documenting the heterogeneity of ACF have questioned their precancerous nature. Recently, we described dysplastic foci depleted of mucins (MDF) in the colon of rats treated with colon-specific carcinogens. Like colon tumors, MDFs show activation of Wnt signaling driven by mutations in the *β-catenin* gene and *Apc*, a key gene in colorectal carcinogenesis. Because MDFs have been identified thus far only in rodents, we wanted to search for similar lesions in humans. Familial adenomatous polyposis (FAP) subjects, carrying germ-line mutations in the *APC* gene, are at high risk of CRC. Therefore, we first searched for MDF-like lesions in unsectioned colon samples from FAP patients and then in patients with sporadic CRC. MDFs were present in the colon of FAP patients (average of 0.0577 lesions/cm^2) and at a much lower density in CRC patients (average of 0.0006 lesions/cm^2). ACFs were also observed in all patients. Histologic preparations of all the MDFs identified in FAP and CRC consisted of microadenomas at variable grades of dysplasia. The occurrence of MDF-like lesions in high-risk patients provides evidence that these lesions have a counterpart in human pathology and, as observed in rodents, may represent the very early stages of CRC.

Foci of aberrant crypts (ACF), microscopically visible in the unsectioned colon of carcinogen-treated mice, were originally described by Ranjana Bird in 1987 as being related to the early steps of colon carcinogenesis (1). The results of many studies characterizing ACF (2–4) and the demonstration that ACF-like lesions are also present in humans (5, 6) have reinforced the hypothesis that ACFs are precursors of colon cancer (CRC) and have led to their widespread use as biomarkers of colon carcinogenesis (7, 8). However, reports documenting the heterogeneity of ACF and their relationship with cancer not always straightforward (9–12) opened a debate on the validity of ACF as surrogate end points, stressing the need for additional biomarkers more robustly correlated with cancer (12–15).

Recently, mucin depleted foci (MDF), formed by dysplastic crypts with scant or absent mucin production, were identified by our group in the colon of rodents treated with azoxymethane or 1,2-dimethylhydrazine (16), which induces CRC through histologic and molecular alterations similar to human carcinogenesis (17). Like ACFs, MDFs are easily identified in unsectioned colon. Moreover, studies carried out by us and others indicate that MDFs are correlated with carcinogenesis and can thus serve as cancer biomarkers in chemoprevention studies (18–21). We documented that MDFs share pathologic and molecular alterations with more advanced lesions, such as Wnt pathway activation, caused in part by mutations in the *β-catenin* gene (19). Moreover, the fact that MDFs carry mutations in the *Apc* gene at a frequency similar to tumors (22) reinforces the hypothesis that MDFs are precursors of CRC in rodents.

MDFs have been identified thus far only in rodents treated with azoxymethane/1,2-dimethylhydrazine; therefore, we thought it important to show that lesions similar to MDF are also present in humans.

Familial adenomatous polyposis (FAP) subjects, carrying germ-line mutations in the *APC* gene, are at high risk of developing CRC (23). Therefore, we began by searching for MDF-like lesions in FAP patients. We also studied patients with sporadic CRC because they are also at risk of developing a second CRC (24). In the same samples in which we searched for MDF-like lesions, we also studied ACF. Histologic analysis of the various lesions identified was then done.

Materials and Methods

Clinical material

Colonic resections from 23 patients were obtained immediately after surgery at the University of Modena and Reggio Emilia (Modena, Italy;

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Table 1. Characteristics of the patients studied

Group	Sex (M/F)	Mean age, years (range)	Diagnosis	Source of tissue for MDF and ACF evaluation	Area examined (cm^2)/patient* (range)
FAP (2)†	1/1	39 (38-40)	FAP	1 Ascending colon 1 Transverse colon	86.6 ± 23.5 (70-103)
CRC (21)†	10/11	65.1±10.0 (44-81)	AdK‡ in the right colon (9): A§ (4), C (1), T (4) AdK‡ in the Left Colon (12): D(3), S(6), R(3)	Right colon (9): A (5), T (4) Left Colon (12): D(8), S(4)	84.1±37.9 (30-157)

Abbreviations: AdK, adenocarcinoma; A, ascending colon; C, cecum; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.
*Values are means ± SD.
†In parenthesis, the number of patients in each category.
‡In parenthesis, the number of samples in each category.
§In parenthesis, the number of tumors in each category.

4 patients) and at the University of Florence, Careggi Hospital (Florence, Italy; 19 patients). Two samples were from patients with FAP identified based on family history and clinical manifestations. The first FAP patient was a male aged 38 y with ~840 adenomatous polyps in the colon (size, 0.8-6 mm) and extracolonic manifestations (fundic gastric polyps and duodenal adenomatous polyps); his mother died of ovary cancer and was also affected with FAP. The second FAP patient was a female (40 y old) presenting ~320 adenomatous polyps in the colon (size, 3-10 mm) and duodenal polyps; her brother died of CRC (38 y old). The additional colonic samples studied (n = 21) were from patients with sporadic CRC with no familial risk for this disease based on clinical manifestations, age of onset, and family history. A summary of clinical data of patients in the study is presented in Table 1. All cancers in the CRC patients were adenocarcinomas: 9 were located in the right colon and 12 in the left colon (Table 1). Informed consent for the use of the colonic samples was obtained from patients.

Identification of ACF and MDF in unsectioned human colons

Immediately after surgery, macroscopically normal mucosa was gently peeled from the submucosa and muscularis propria layers

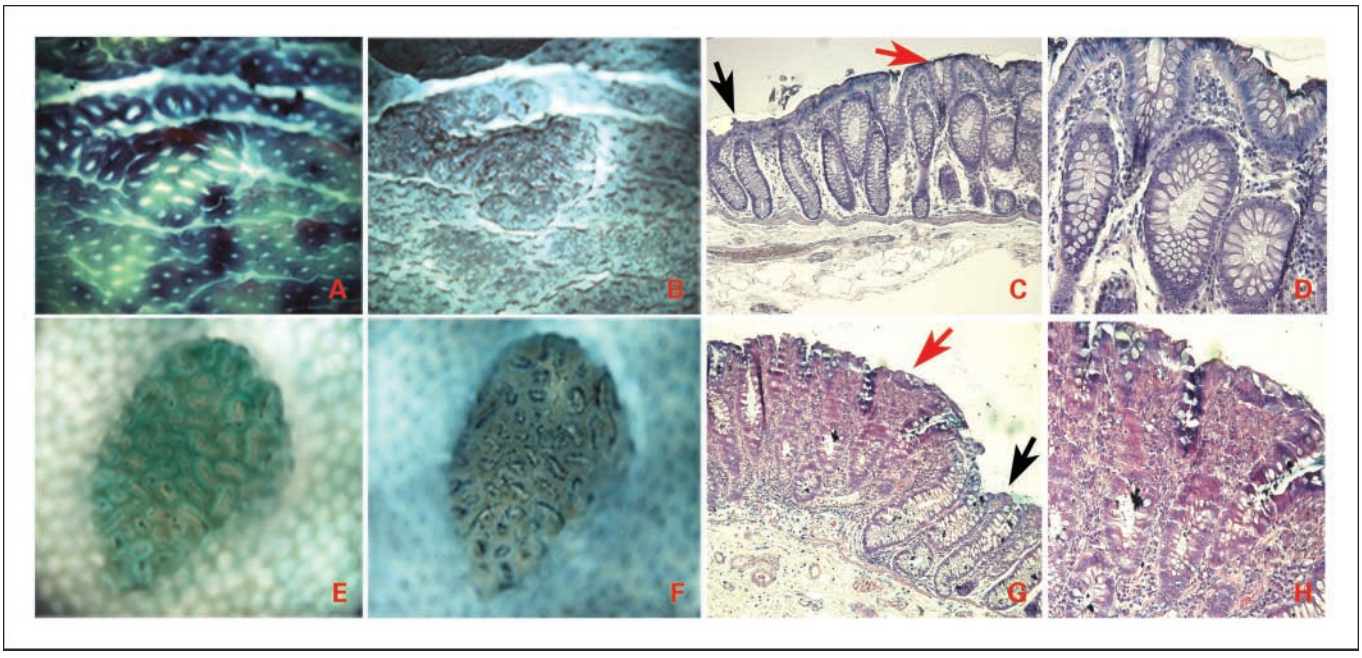

Fig. 1. Topographical and histologic features of the observed ACF. *A* and *E,* ACF of CRC patients in MB-stained colon. *A,* ACF formed by 20 crypts. *E,* ACF formed by 65 crypts. Original magnification, ×4. *B* and *F,* the same ACF as the first column (*A* and *E*) observed after HID-AB staining. Original magnification, ×4. *C* and *G,* histologic features of ACF as observed in H&E-stained slides. Original magnification, ×10. *Red arrows*, ACF; *black arrows*, normal adjacent crypts. *D* and *H,* histology of ACF indicated by *red arrows* in *C* and *G*, respectively, shown at higher magnification (original, ×20). *C* and *D,* nonhyperplastic/nondysplastic lesion (*A* and *B* at topography). *G* and *H,* microadenoma with a low grade of dysplasia (*E* and *F* at topography).

using scissors or a scalpel and pinned flat on a polystyrene board as previously described (19). After fixation in buffered formalin (for at least 24 h), each sample was cut into small segments (~3 × 5 cm) to facilitate microscopic observation.

Colons were stained with high-iron diamine Alcian blue (HID-AB) to identify MDF, but because this procedure precludes subsequent staining with methylene blue (MB) for ACF determination, the colon samples were first stained with MB (0.1%) for 5 to 10 min (1). After ACF determination, colons were kept in formalin and then stained with HID-AB to visualize MDF as described for rats (16), with the following modifications. Briefly, colons were rinsed in distilled water and stained for 1 h at room temperature with HID solution obtained by dissolving simultaneously 120 mg of *N*-*N*′-dimethyl-*m*-phenylene diamine and 20 mg of *N*-*N*′-dimethyl-*p*-phenylene diamine in 50 mL of distilled water and then adding 1.4 mL of 60% ferric chloride. The colons were rinsed thrice in distilled water and stained for 30 min with AB solution (1% Alcian blue in 3% acetic acid). The colons were then rinsed thrice with 80% ethanol followed by distilled water and then observed under a microscope (mucosal side up) to determine MDF. As reported in rodents, MDFs were visible as focal lesions (i.e., there was a clear distinction between normal surrounding crypts and MDF) characterized by the absence, or very limited production, of mucins when compared with surrounding crypts. Elevation of the lesion above the surface of the colon is a frequent feature of MDF. Single crypts without mucus were never considered as an MDF.

Histology of the observed lesions

After identification of MDF and ACF in the unsectioned colons, 37 lesions (11 MDFs and 26 ACFs as defined at the topographic observation at the microscope) were marked with permanent ink (The Davidson Marking System, Bradley Products) as described (19), dissected, and embedded in paraffin in such a way that the crypts could be sectioned longitudinally. Histologic sections (4 μm)

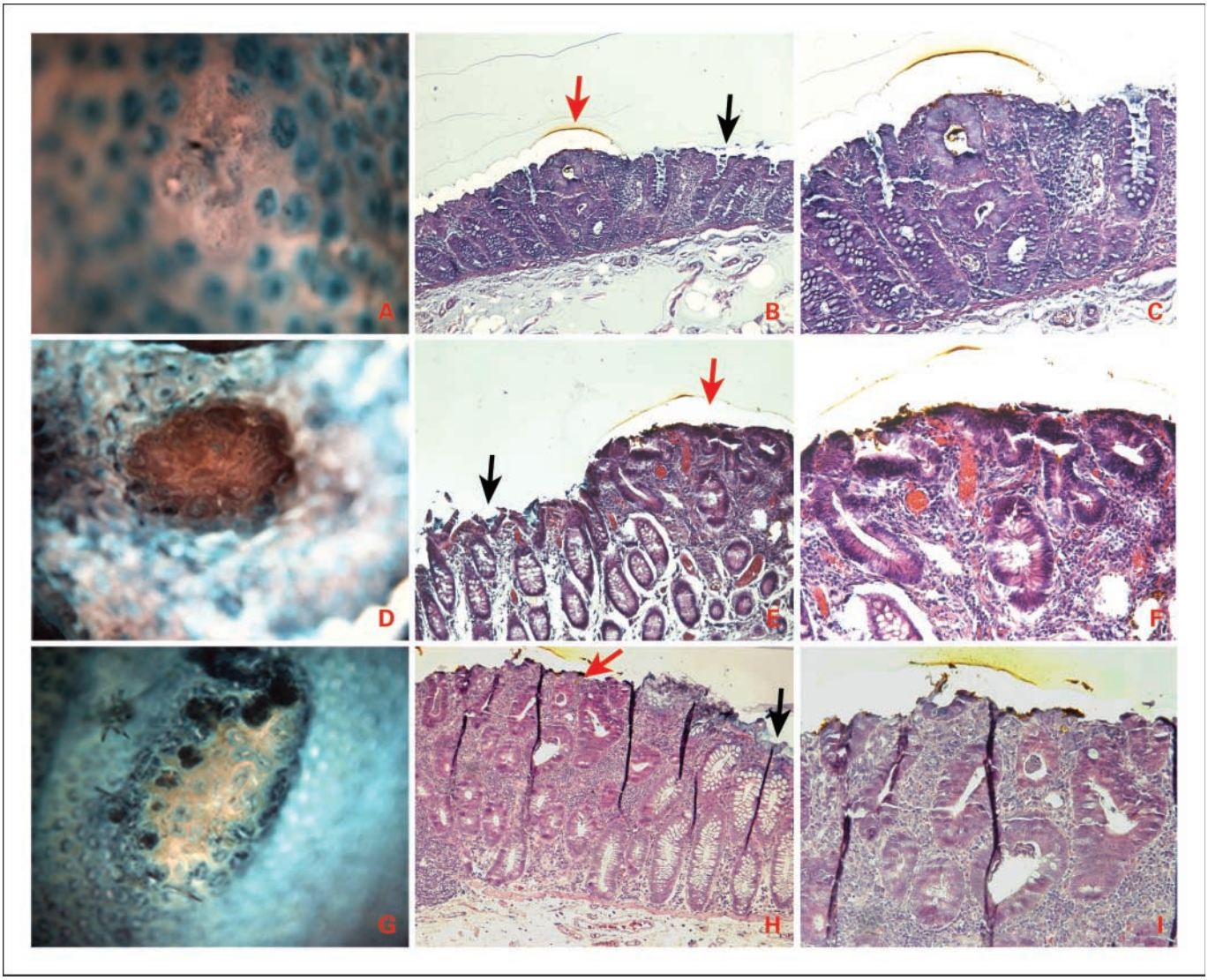

Fig. 2. Topographical and histologic features of the observed MDF. *A, D,* and *G,* topographical appearance of MDF in HID-AB–stained colon. *A,* MDF from a FAP patient formed by six crypts. Original magnification, ×10. *D,* MDF from a FAP patient formed by 45 crypts. Original magnification, ×4. *G,* MDF from a CRC patient formed by 60 crypts. Original magnification, ×4. *B, E,* and *H,* histologic features of MDF depicted in the first column (*A, D*, and *G*) as observed in H&E-stained slides. Original magnification, ×10. *Red arrows*, MDF; *black arrows*, normal adjacent crypts. *C, F,* and *I,* histology of MDF indicated by *red arrows* in *B, E*, and *H*, respectively, shown at higher magnification (original, ×20). *B* and *C,* microadenomas with a low grade of dysplasia (*A* at topography). *E* and *F,* microadenomas with a low grade of dysplasia (*D* at topography). *H* and *I,* microadenoma with a moderate grade of dysplasia (*G* at topography).

Table 2. ACF density in FAP and CRC patients

Group	ACF/cm^2 [total number of ACF in each category]		
FAP (2)*	1.585 ± 0.261 (274)		
CRC (21)*	0.07± 0.073† (134)	Right colon (9)*	0.031 ± 0.022 (24)
			Ascending 0.021 ± 0.021 (11)
			Transverse 0.043 ± 0.019 (13)
		Left colon (12)*	0.109 ± 0.081‡ (110)
			Descending 0.122 ± 0.088 (93)
			Sigmoid 0.083 ± 0.067 (17)

*In parenthesis, the number of patients in each category.
†$P < 0.001$ versus FAP patients.
‡$P < 0.05$ versus right colon (ascending and transverse) with t test for unpaired samples.

were stained with H&E and evaluated by a pathologist (A. Giannini) unaware of the topographical classification of the lesion. Lesions showing dysplastic features were classified as microadenomas (25, 26) with low or moderate grade of dysplasia (27, 28). Lesions showing no dysplasia were classified as hyperplastic lesions (25, 26) or nondysplastic/nonhyperplastic lesions according to previously described criteria (28–30).

Results

Determination of ACF in colon of FAP and CRC patients

We first stained the colon samples with MB and determined the presence of ACF. As expected, ACFs were easily visualized in FAP and CRC patients (Fig. 1A and E) and were more numerous in FAP than in CRC patients (Table 2). When considering only CRC patients, the density of ACF was significantly higher in the left colon (sigmoid and descending colon) than in the right colon (ascending and transverse colon; Table 2). After ACF determination with MB, the samples were stained with HID-AB to highlight mucin production and identify any MDF. As reported in rodents (16–19), we found that the HID-AB staining allowed a good visualization of the ACF (in Fig. 1, the first and second columns show the same ACF observed in MB-stained and HID-AB–stained colon, respectively). Moreover, MB did not alter the subsequent HID-AB staining.

Determination of MDF in the colon of FAP and CRC patients

In HID-AB–stained samples from FAP patients, it was possible to identify MDF-like lesions (Fig. 2A and D). In the 2 FAP samples, we found a total of 10 MDFs, with a mean multiplicity of 34 crypts per lesions (Table 3). Some of these MDFs were small (i.e., formed by only three to six crypts) and some had gone undetected at the previous observation of the MB-stained colons (4 of 10 lesions, such as the lesion in Fig. 2A). The remaining MDFs were identified as ACFs during the observation with MB (6 of 10 lesions, such as the lesion in Fig. 2D). In FAP patients, the mean density of MDF was 0.0577 lesions/cm^2, a value ~30 times lower than the ACF density.

Contrary to what was observed in FAP patients, the density of MDF in CRC samples was extremely low (Table 3), only one MDF being observed in the 21 colon samples examined (total area examined, 1,766 cm^2). This MDF (Fig. 2G) was observed in a sample of left colon (sigmoid) from a male patient (age, 54 years) operated for adenocarcinoma of the rectum, with no familial risk for CRC. The MDF was formed by ~60 crypts, and had been classified as an ACF in the previous observation with MB. The density of ACF in this colon sample was low (only 2 ACFs being present in ~82 cm^2 analyzed).

Histologic analysis of the observed ACF and MDF

After identification of ACF and MDF in the unsectioned colon, all observed MDFs (11 lesions) and a representative sample of the ACF (26 lesions) were marked with permanent ink and sectioned for histopathology. The results indicated (Table 4) that all the MDFs in FAP patients were microadenomas with low-grade dysplasia (Fig. 2B and E), whereas the only MDF found in the CRC sample was a microadenoma of moderate dysplasia (Fig. 2H). The histologic analysis of the 26 ACFs (Table 4) showed that all the ACFs from FAP patients (17 samples) were microadenomas with low grade of dysplasia. On the contrary, of the nine ACFs from CRC patients, only one was a microadenoma with a low degree of dysplasia (Fig. 1G), the others being either nondysplastic/

Table 3. Characteristics of MDF-like lesions in FAP and CRC patients

Patients	Total MDF	Crypts/ lesion	Crypts/MDF (mean ± SD)	MDF/ cm^2
FAP #1	6	3*	33.4 ± 33.5	0.0577
		6*		
		6*		
		38		
		50		
		52		
FAP #2	4	4*		
		20		
		45		
		110		
CRC	1	60	60	0.0006

*Not seen as ACF in MB-stained colon.

Table 4. Histology of MDF and ACF identified at the topographical observation

Lesion	Group	Diagnosis
MDF	FAP (10)	Microadenomas with a low grade of dysplasia (10)
	CRC (1)	Microadenoma with a moderate grade of dysplasia (1)
ACF	FAP (17)	Microadenomas with a low grade of dysplasia (17)
	CRC (9)	Microadenoma with a low grade of dysplasia (1)
		Hyperplastic lesion (1)
		Non-hyperplastic/non-dysplastic lesions (7)

*Numbers in parenthesis are the number of samples analyzed for histology in each category.

nonhyperplastic (Fig. 1C) or hyperplastic lesions. Interestingly, the only ACF classified as a microadenoma (Fig. 1E-H) was identified in the same CRC patient in whom we identified the MDF.

Discussion

This is the first report showing that MDF-like lesions are present in humans, notably in subjects at high risk of developing CRC, such as FAP patients, and at a much lower density in CRC patients. All the MDFs identified, either in FAP or CRC patients, were dysplastic at histology. We also found that some of the MDFs observed in FAP patients were formed by few crypts (three to six crypts), an observation in line with previous reports on the presence of minute adenomas formed by only a few crypts in FAP (31).

We also determined ACF in all the samples examined in our study. ACFs are monoclonal proliferations considered putative precursors of colorectal cancer in both humans and experimental animals because they show molecular abnormalities that occur in and are characteristic of more advanced lesions (32, 33). However, several studies have also documented that ACFs are a heterogeneous population of lesions, the majority of ACFs in CRC patients and carcinogen-treated rodents being not dysplastic (8, 28–31, 34–37). In line with these last observations, we found that only one ACF among those analyzed in CRC patients was dysplastic, whereas the others were hyperplastic or nonhyperplastic/nondysplastic lesions. On the other hand, we also found that all the ACFs examined were dysplastic in FAP patients, in agreement with previous reports (8, 35, 38). As reported by other authors (8, 37, 39, 40), we confirm that ACFs are more common in the left than in the right colon.

We previously reported that the same dosage of colon carcinogens, which induces about one to two colonic tumors after 8 to 9 months in rats, induces ~10 MDFs and >100 ACFs per animal at an early time (16–19). We also observed many more ACFs than MDFs in FAP and CRC patients. We found that some MDFs, notably those formed by a few crypts, passed unnoticed during the first observation with MB, whereas a fraction of MDFs in our two FAP patients (~60%) were classified as ACFs with MB. The only MDF observed in CRC patients was also identified as an ACF at the previous observation with MB and was classified as a microadenoma with a moderate degree of dysplasia at histology. Similar findings were reported by us in rodents (19), suggesting that at least some of the MDFs identified in an HID-AB–stained colon are dysplastic ACFs with defective production of mucins. On the other hand, it can also be observed that in both CRC and FAP patients, MDFs represent only a part of the dysplastic lesions present. The CRC patient with the MDF (Fig. 2G-I) had a second lesion identified as an ACF (Fig. 1E-H) that was also dysplastic (i.e., in this CRC patient, MDF identified 50% of the lesions found to be dysplastic). In FAP patients, <10% of the dysplastic lesions are identified by the HID-AB staining because in these patients there are many dysplastic ACFs missed by the HID-AB staining. Although we do not know whether dysplastic lesions identified as MDFs with HID-AB are more likely to progress to cancer than dysplastic lesions that retain mucin staining, previous studies in rats showed that MDFs are closer to cancer in terms of genetic and molecular alterations than ACFs identified in HID-AB (16, 19, 22). However, in the present study, we did not evaluate the molecular profile of these lesions because the main goal of our work was the demonstration that MDF-like lesions were present in humans.

At present, the methodology available for identifying MDF requires fixation and a staining of the tissue, which unfortunately is unsuitable for identification of MDF *in vivo*; moreover, the paucity of MDF in CRC patients makes them probably unsuitable as cancer biomarkers. However, the fact that all the MDFs identified, even if classified as ACFs with MB, were dysplastic raises the possibility of development of this technique for detecting some early dysplastic lesions also *in vivo*, although these lesions might be rare.

In conclusion, the occurrence of MDF-like lesions in FAP patients at high risk of CRC and their rare occurrence in sporadic CRC patients prove that these lesions, identified thus far only in rodents treated with colon carcinogens, have a counterpart in human pathology. Therefore, MDF may represent in humans, as in rodents, an early step in the process of carcinogenesis. Their characterization and identification *in vivo* may be relevant as early pathologic alterations in CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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