Frequent Mutation of *Apc* Gene in Rat Colon Tumors and Mucin-Depleted Foci, Preneoplastic Lesions in Experimental Colon Carcinogenesis

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Abstract

Mucin-depleted foci (MDF) are microscopic dysplastic lesions induced in the colon of rodents by specific colon carcinogens. Most MDF show Wnt pathway activation, whereas only a subset shows mutations in the *Ctnnb1* gene, coding for β-catenin. Because *Apc* is a member of the Wnt pathway and the most frequent mutated gene in human colon cancer, we tested whether MDF harbor *Apc* mutations. F344 rats were treated twice with 150 mg/kg of 1,2-dimethylhydrazine. After 15 or 28 weeks, MDF, aberrant crypt foci (ACF), and tumors were collected. We screened a segment of the *Apc* gene comprising the region homologous to the mutation cluster region (MCR) of human *APC*, which frequently shows mutations in experimental colon tumors. Mutations were identified by PCR amplification and sequencing in 6:24 MDF (25%), 7:23 tumors (30%), 0:24 ACF (0%). Most of the mutations (92%) in MDF and tumors were localized in a region upstream from the MCR. All mutations were single-base substitutions and mainly formed by G:C → A:T and C:G → T:A transitions. The pattern of nucleotide changes was similar in MDF and tumors, and, interestingly, the same mutation in codon 1047 was found in two MDF and in three tumors. Four out of the six mutations found in MDF were nonsense mutations, and two were missense. All mutations in tumors determined a protein truncation. These results show that *Apc* mutations are present in MDF with a frequency similar to that of tumors, strengthening the evidence that they are precancerous lesions in colon carcinogenesis. [Cancer Res 2007;67(2):445–9]

Introduction

Mucin-depleted foci (MDF) are dysplastic lesions originally identified in the colon of azoxymethane-treated rats (1). MDF are dose-dependently induced by colon carcinogens (2), and their occurrence is correlated to the number of tumors after the administration of promoters or chemopreventive agents (3–6), thus suggesting that these lesions are preneoplastic. We previously reported that most MDF, like tumors, show constitutive activation of the Wnt-signaling pathway with cytoplasmic and nuclear accumulation of β-catenin (2). However, because only a fraction of MDF (25%) harbors mutations in the rat gene coding for β-catenin (*Ctnnb1* gene), other components of the Wnt pathway might be altered in these lesions.

The *Apc* gene is a component of the Wnt-signaling pathway as a member of the macromolecular complex which degrades β-catenin (7). Mutations in either the *Apc* or *Ctnnb1* gene make β-catenin resistant to degradation, thus causing its accumulation in the nuclei and activation of the Wnt-signaling pathway. Germ line *APC* mutations are responsible for the inherited predisposition for familial adenomatous polyposis (FAP) and for intestinal tumors in the *Min* mouse carcinogenesis model (8, 9). Somatic *APC* mutations are also common in sporadic colorectal cancers (8) and are present with variable frequencies in chemically induced colon cancer of rodents (10, 11).

On the basis of these considerations, we studied *Apc* mutations in rat MDF as a possible cause of Wnt pathway activation observed in these lesions. Moreover, the presence of a mutation so relevant for colon carcinogenesis such as the *Apc* would strengthen the hypothesis that MDF are preneoplastic. To compare MDF with more advanced stages of transformation or with other purported preneoplastic lesions, we also studied *Apc* mutations in colon tumors and aberrant crypt foci (ACF), well characterized in rodents and humans (12, 13).

Materials and Methods

Carcinogenesis induction. Male F344 rats, 4 to 5 weeks old, were obtained from Nossan (Correzzana, Milan, Italy). They were housed according to the European Union Regulations on the Care and Use of Laboratory Animals (14) and fed a high-fat diet (23% corn oil w/w) based on the American Institute of Nutrition-76 diet (2). The experimental protocol was approved by the Commission for Animal Experimentation of the Italian Ministry of Health. At 6 to 7 weeks of age, rats were treated twice, 1 week apart, with s.c. injections of 1,2-dimethylhydrazine (DMH; 150 mg/kg × 2). Rats were divided into two groups. One group was sacrificed by CO_2 asphyxiation 15 weeks after the first injection, and the other was sacrificed 28 weeks afterward. The colons were excised and opened longitudinally. Those lesions which were visible by the naked eye at sacrifice were defined as tumors following subsequent histopathologic examination as described (2). To collect MDF, ACF, or tumors, the colons were then fixed flat, as reported (1, 2), using 70% ethanol for no more than 3 h.

Identification of ACF and MDF. MDF are foci of crypts depleted of mucins in a background of normal crypts using the high-iron diamine Alcian blue (HID-AB) technique, which allows differentiation between sialomucin and sulfomucin production (1). MDF have also been identified by other authors using Alcian blue (AB), which stains in blue all acid mucins (6). However, we found that both staining methods (HID-AB or AB) degrade DNA, making it unsuitable for relatively long amplification by PCR (data not shown). Therefore, we used a milder staining procedure (Alcian blue-neutral red [AB-NR]) consisting of a shorter AB incubation (10 min versus 30 min

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for standard AB staining), followed by a brief counterstaining with neutral red (0.5% aqueous solution for 10 s) to identify MDF in this study. In fact, with the AB-NR staining, the epithelial layer of the normal mucosa is shown as a reddish background dotted with blue spots representing the opening of normal crypts full of mucus stained with AB. In this blue-dotted background, MDF is shown as a reddish spot in which the crypts, often distorted and smaller than normal, do not produce mucins (therefore devoid of blue; Fig. 1*A*). The correspondence between the original identification method (HID-AB) and the AB-NR method was studied in seven rat colons in which all MDF observed with the AB-NR staining were confirmed as MDF with a subsequent HID-AB staining (62 out of 62 total MDF scored in AB-NR). An example of this correspondence is shown in Fig. 1 (*A* and *B*). ACF were also clearly visible using AB-NR as foci elevated from the plane of the mucosa, formed by crypts larger than normal, with increased pericryptal space and a thicker layer of epithelial cells (Fig. 1*C*). Each lesion identified as MDF or ACF in AB-NR–stained colons was marked with permanent ink and processed as previously described (2).

***Apc* analysis.** ACF, MDF, and tumors were laser microdissected (PALM MicroBeam system) from longitudinal paraffin 5-μm-thick sections (two to four sections per lesion), and the DNA was extracted using the Qiamp DNA Micro Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. We analyzed a fragment of the rat *Apc* gene encompassing the homologous region of the human MCR (ref. 8; nucleotides [nt] 3,900–4,589 of the rat sequence, GenBank accession no. D38629) and a region upstream of the MCR (nt 3,078–3,899). Importantly, this segment comprises the region from nucleotides 3,078 to 3,997, where the majority of *Apc* mutations have been described in experimentally induced rodent tumors (10, 11). The *Apc* gene region was divided in four overlapping fragments (fragments 1–4) for mutational analysis. Due to the very low amount of DNA that could be extracted from microdissected MDF and ACF, a nested PCR approach was planned. For each lesion, in the first round of amplification, the entire region of the *Apc* gene was obtained by two duplex PCR in which two nonoverlapping fragments were coamplified in the same reaction. In the second round of PCR amplification, each fragment was amplified separately by nested primers using a dilution of the first PCR amplification as template. The primer sequences were designed using the published rat *Apc* cDNA sequence (GenBank accession no. D38629). The primers used for the first round of amplification were fragment 1 (nt 2,900–3,507): 1Fw: 5′-TGCCAAGGTGGAATATAAGAGA; and 2Rv: 5′-CATGCTGTTCTTCCTCAGAATA; fragment 2 (nt 3,260–3,859): 3Fw: 5′-GAACACCAATTTTCCCGTCT; and 4Rv: 5′-GAGAAACATATTGGGGTGTCTT; fragment 3 (nt 3,696–4,226): 5Fw: 5′-GCTGCATCTGCACCTTCAT; and 6Rv: 5′-TGGAGCGACTCTCAAAACTG; and fragment 4 (nt 4,033–4,662): 7Fw: 5′-CTAGAACCAAGCCCAGCAGA; and 8Rv: 5′-CAGTTTCATTCCCATTGTCG.

The primers used in the nested amplifications were fragment 1Nested (nt 2,985–3,455): 1NFw: 5′-AGCCCTCGGTTGAGTCCTAC; and 2NRv: 5′-TTCATAGTCGTCTTCCTGACACA; fragment 2Nested (nt 3,322–3,811): 3NFw: 5′-ATTTTGGGCAACAAGAGTGC; and 4NRv: 5′-TGGTTGATGGAGGGGACTT; fragment 3Nested (nt 3,768–4,197): 5NFw: 5′-CAGACTCCAAAAGGGACTGC; and 6NRv: 5′-TGAATACAAGGGGCGTCTCC; and fragment 4N (nt 4,091–4,640): 7NFw: 5′-TAAAGCTGTTGAGTTTTCTTCAG; and 8NRv: 5′-TTCCTGAACTGGAGGCATTA.

PCR reactions were carried out in a 25-μL volume containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 0.3 mmol/L deoxynucleotide triphosphates, 0.4 μmol/L of each primer and 1.25 units of Taq polymerase (Advanced Biotechnologies, Epsom, United Kingdom). Cycling conditions were initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension at 72°C for 1 min with a final

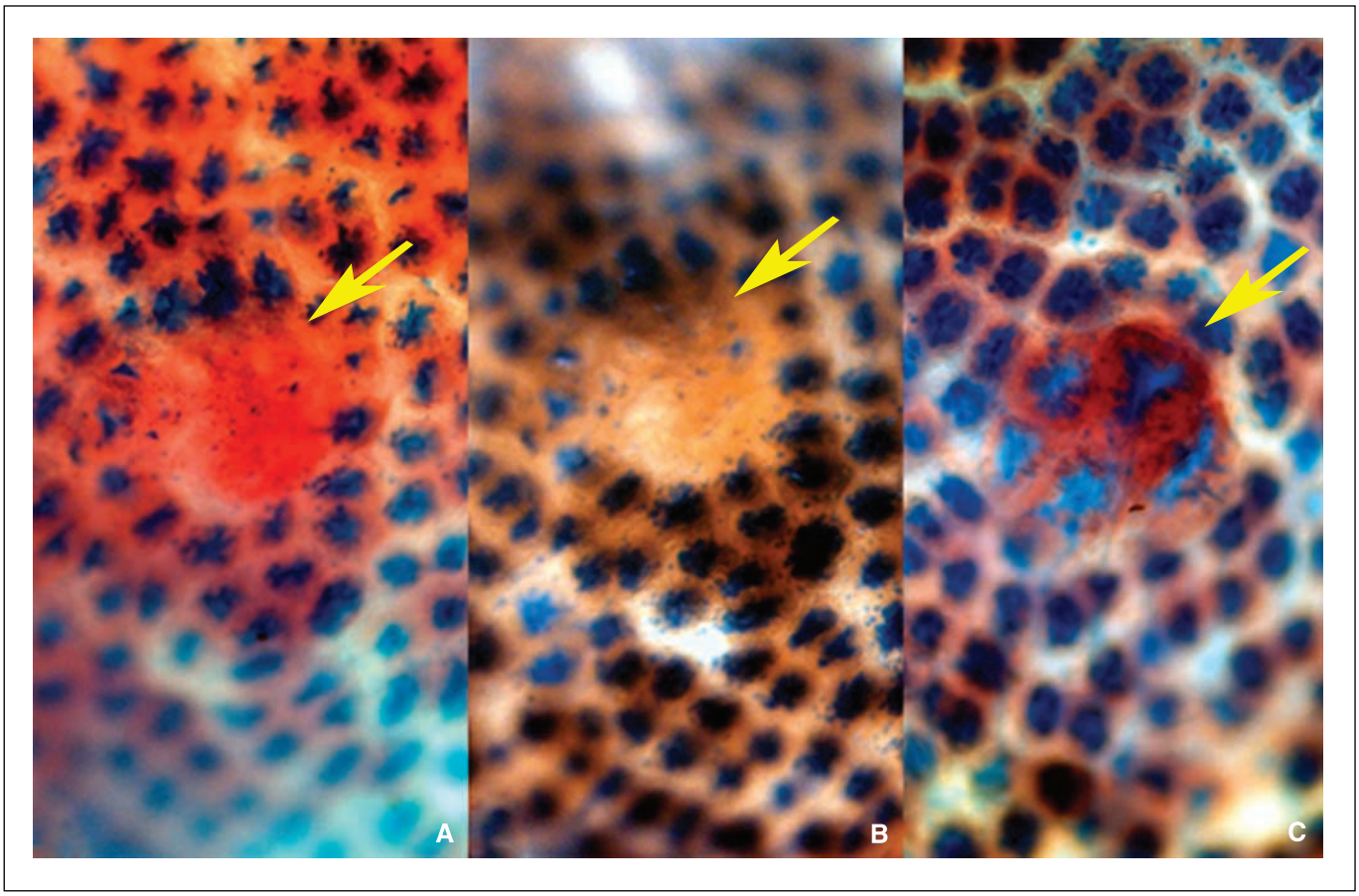

Figure 1. Microscopic appearance of MDF or ACF in Alcian blue-neutral red (AB-NR)– or HID-AB–stained unsectioned colons. *A,* identification of an MDF (*yellow arrow*) in Alcian blue-neutral red–stained colon. *B,* the same MDF (*yellow arrow*) observed in *A* after restaining of the colon with the HID-AB technique. *C,* typical appearance of an ACF (*yellow arrow*) in AB-NR–stained colon. Original magnification, ×100.

Table 1. *Apc* mutations in MDF and tumors induced by DMH in rat

Lesion	Histology	Crypts/focus	Nucleotide position	Amino acid position	Nucleotide change	Amino acid change
Tumors	Adenocarcinoma		3,186	1,045	GAA → TAA	Glu → Stop
	Adenoma		3,194	1,047	TGG → TGA	Trp → Stop
	Adenoma		3,194	1,047	TGG → TGA	Trp → Stop
	Adenocarcinoma		3,194	1,047	TGG → TGA	Trp → Stop
	Adenoma		3,246	1,065	CAA → TAA	Gln → Stop
	Adenocarcinoma		3,387	1,112	CGA → TGA	Arg → Stop
	Adenoma		3,810	1,253	CAA → TAA	Gln → Stop
MDF		80	3,084	1,011	CAT → TAT	His → Tyr
		15	3,194	1,047	TGG → TGA	Trp → Stop
		9	3,194	1,047	TGG → TGA	Trp → Stop
		11	3,231	1,060	CAA → TAA	Gln → Stop
		6	3,822	1,257	CAG → TAG	Gln → Stop
		7	4,444	1,464	GGT → GAT	Gly → Asp

extension step for 10 min at 72°C. The final products were separated on a 2.5% agarose gel and visualized by ethidium bromide staining. Final PCR products from nested amplifications were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and directly sequenced in both directions using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA); sequences were analyzed using the DNA sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems) following the manufacturer's protocol.

Statistical evaluation of the data. *Apc* mutation frequencies in ACF, MDF, and tumors were evaluated with Fisher's exact test, followed by the determination of odds ratios (OR) to evaluate the statistical significance of the difference between lesions. A trend analysis (Cochran-Armitage trend test) was also used to evaluate the frequencies of mutations in the three different lesions (ACF, MDF, and tumors). All the statistical calculations were done using Exact Methods Statxact6 statistical package (Cytel Software Corporation, Cambridge, MA).

Results

***Apc* mutational analysis in tumors.** Twenty-three tumors (adenomas $n = 12$; and adenocarcinomas $n = 11$) were analyzed for *Apc* mutations. Seven of these tumors were mutated (frequency of mutated lesions, 30.4%); four out of the seven *Apc* mutated tumors were adenomas, and three were adenocarcinomas (Table 1). All mutations were single-base changes resulting in a stop codon. Six mutations were transitions (three G:C → A:T and three C:G → T:A), one was a G:C → T:A transversion (Table 1). All *Apc* mutations were found in a region between nt 3,186 and 3,810, upstream of the homologous region of the human MCR (nt 3,900–4,589); (Table 1; Fig. 2).

***Apc* mutational analysis in MDF and ACF.** Six out of the 24 MDF studied harbored *Apc* mutations (frequency of mutated lesions, 25%). All mutations were single-base substitutions resulting in transition mutations (three G:C → A:T and three C:G → T:A) (Table 1). Four of these mutations resulted in a stop codon, whereas two mutations were missense (Table 1). All but one mutation were localized upstream of the homologous region of the human MCR (Table 1; Fig. 2).

Most (four out of six) of the mutated MDF were formed by <15 crypts (11, 7, 6, and 9 crypts/focus); only one had a multiplicity of about 80 crypts/focus [the mean number of crypts forming each MDF: 15.50 ± 4.8 (SE); range, 3–100].

The same mutational analysis of the *Apc* gene was also done in 24 ACF (mean multiplicity, 8.0 ± 0.5 SE; range, 4–13). None of the ACF analyzed had *Apc* mutations.

The mutation frequencies of the different lesions (0:24, 6:24, and 7:23 in ACF, MDF, and tumors, respectively) were significantly different ($P = 0.006$ by Fisher's exact test); differences between ACF and MDF, ACF and tumors were statistically significant (exact P values for OR were 0.02 and 0.008, respectively), whereas the frequency of mutation in MDF and tumor was similar (exact P value for OR, 0.93). Moreover, a trend analysis calculated on the frequencies of mutation in the three different lesions (ACF, MDF, and tumors) showed that the trend was statistically significant (exact $P = 0.011$ for Cochran-Armitage trend test).

Discussion

The aim of this study was to test whether MDF were mutated in *Apc*, a key gene in colorectal carcinogenesis. We analyzed a region homologous of the human MCR and a segment upstream, where the majority of mutations in carcinogen-induced rats have been reported (10, 11). About 30% of all the tumors we analyzed (adenomas and adenocarcinomas) harbor mutations in the *Apc* gene. The mutations were nonsense and were located upstream (nt 3,186–3,810) of the homologous region of the human MCR (nt 3,900–4,589), a finding in agreement with previous experimental studies (10, 11). Interestingly, all but one mutation were found in the region (nt 3,150–3,452) between the first two human homologous 15-amino acid repeats for β-catenin binding (Fig. 2), thus suggesting that mutations in this location may induce a growth advantage in our experimental model.

Our results also clearly show that 25% of the MDF analyzed harbor *Apc* mutations, a frequency similar to that found in tumors. As observed in tumors, the mutations in MDF were mainly located upstream of the MCR (Table 1; Fig. 2) and had the same nucleotide change profile as tumors. In fact, 42% of tumor mutations were G:C → A:T and 42% C:G → T:A. Similarly, MDF mutations were 50% G:C → A:T and 50% C:G → T:A. Interestingly, two MDF harbored the same mutation (TGG → TGA, codon 1,047) found in three tumors (two adenomas and one adenocarcinoma), thus suggesting that at least some MDF may be true tumor precursors.

We also found that not all MDF mutations resulted in a truncated protein because two out of the six mutations found were missense (Table 1). One of the two missense mutations was found in a rather large MDF formed by about 80 crypts, whereas the

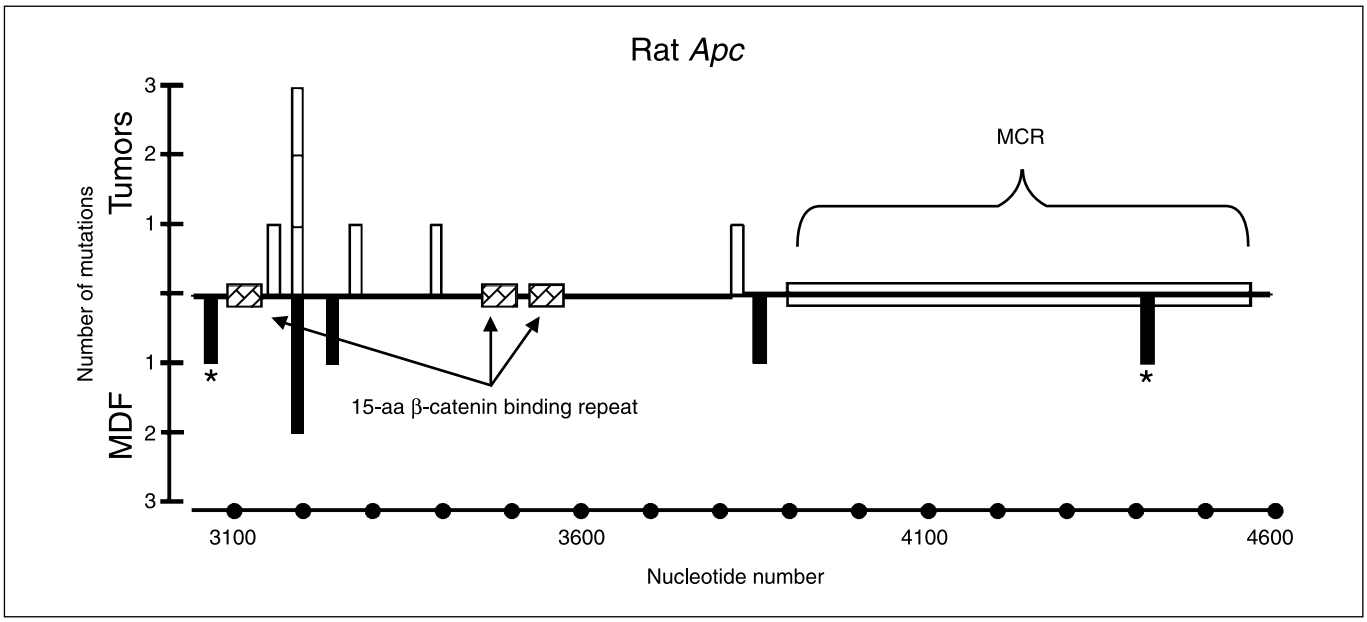

Figure 2. Schematic representation of the *Apc* region analyzed and localization of the mutations found in MDF and tumors. The nucleotide numbers are assigned according to the published rat sequence (GenBank accession no. D38629). *, missense mutations.

other one was carried by an MDF formed by 7 crypts. Although it has been reported that most mutations in the *Apc* gene result in a truncated protein (15), it is worth noting that a previous experimental study reported a high frequency of missense mutations in DMH-induced colon tumors (10), whereas a recent study of human colorectal tumors (16) reported the same.

Regarding ACF, none of the lesions tested had *Apc* mutations, a result in agreement with a previous study by our group in which these lesions were analyzed using the protein truncation test (11). ACF are putative preneoplastic lesions that have been extensively characterized in rodents and humans (12, 13). Although ACF show preneoplastic characteristics, they are heterogeneous lesions that often regress to a more normal phenotype, whereas only a few progress to more advanced stages. We previously reported that ACF show less dysplasia than MDF (1), a phenomenon that could be related to the absence of *Apc* mutation. In fact, *Apc* mutations in ACF have been reported in human studies in very large dysplastic ACF (about 200 crypts; ref. 17) or in one dysplastic ACF formed by about 30 crypts in another study (18). However, other authors have reported lack of *Apc* mutations in ACF irrespective of their dysplasia (19).

Our data do not permit to evaluate how tumors develop from preneoplastic lesions; however, it is a fact that only a fraction of all the lesions formed after carcinogen administration evolves into tumors. Previous results from our group (2) showed that in DMH-treated rats, the number of MDF increases after carcinogen treatment, but decrease later, when macroscopic tumors appear; this suggests that at least some MDF may transform into tumors (2). The results obtained in the present study suggest that the MDF tumor transformation may be primed by *Apc* mutations, occurring quite early because many mutated MDF were formed by <15 crypts. As for the relation between ACF and MDF, we found a significantly different *Apc* mutation frequency between these two lesions. Although the analysis of the data showed a significant trend of *Apc* mutation in the sequence "ACF-MDF tumors," we cannot say at the moment whether MDF evolve from ACF acquiring mutations in *Apc* or in other cancer-related genes, or if they develop independently from ACF.

In conclusion, the fact that *Apc* mutations occur in MDF and tumors with a similar frequency indicates that these lesions are closely related. Moreover, these results, together with our previous findings (2), indicate that *Ctnnb1* (β-catenin) and *Apc* mutations in MDF are the main determinants for Wnt pathway activation, a phenomenon that can also induce a down-regulation of mucin production (20). Thus, the loss of a physical and chemical barrier of mucus could lead MDF in strict contact with inflammatory stimuli or food mutagens and increase the probability of acquiring further mutations and a more advanced transformed phenotype.

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