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ABERRANT CRYPT FOCI - ENDPOINT IN COLORECTAL CANCER

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Abstract

Aberrant crypt foci (ACF) can be used as the endpoint in colorectal cancer development because aberrant crypts are postulated to be the earliest identifiable potential precursors of colorectal cancer. ACF were first described by Bird and defined as crypt that: (1) have altered luminal openings; (2) exhibit thickened epithelia; (3) are larger than adjacent normal crypts. ACF may eventually evolve into polyps and, subsequently, colorectal cancer in the case of adenoma-carcinoma sequence; it provides a simple and economical tool for preliminary screening of potential chemopreventive agents, and it allows a quantitative assessment of the mechanisms of colon carcinogenesis.

ACF are morphological lesions that represent an early stage in the stepwise progression of colon cancer. These surface abnormalities often appear in the distal colon within 2 weeks of carcinogen treatment. Sequential analyses suggest that these early lesions increase in size and multiplicity and often exhibit nuclear atypia and dysplasia. ACF also shows increased proliferative activity, growth factor signaling and K-Ras mutations, suggesting that at least a subset of ACF are putative precursors of colon cancer. Human ACF shares a similar morphology and is present in grossly normal-appearing colon tissue from patients with colorectal cancer (CRC). It has been suggested that the nature and order of acquired genetic changes can profoundly impact ACF morphology and the likelihood of tumor progression. Although limited in numbers, flat dysplastic ACF appeared to be more likely to become tumors than the elevated ACF. Clearly, the expanding diversity of ACF identified in rodent carcinogen models provides researchers with exciting new tools for studying the earliest stages of CRC. Lesions characterized in these models may ultimately become useful for predicting cancer risk in humans.

Keywords: *Aberrant crypt foci (ACF), colorectal cancer (CRC), chemopreventive agents.*

Rezumat

Focarele de cripte aberante (ACF) sunt identificate ca punct final în dezvoltarea cancerului colorectal, deoarece criptele aberante sunt postulate a fi precursori potențiali identificabili ai cancerului colorectal. ACF au fost descrise pentru prima dată de Bird și definite ca o criptă care:



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(1) are deschideri luminale modificate; (2) prezintă epiteliu îngroșat; (3) sunt mai mari decât criptele normale adiacente. ACF pot evolua în cele din urmă în polipi și, ulterior, în cancer colorectal conform secvenței adenom-carcinom; acestea oferă un instrument simplu și economic pentru screeningul preliminar al potențialilor agenți chimiopreventivi și permite o evaluare cantitativă a mecanismelor carcinogenezei colonului. ACF sunt leziuni morfologice care reprezintă un stadiu incipient în progresia treptată a cancerului de colon. Aceste anomalii de suprafață apar adesea în colonul distal în primele 2 săptămâni de carcinogeneză indusă experimental. ACFprezintă activitate proliferativă crescută, semnalizarea factorului de creștere și mutații K-Ras, sugerând că ACF sunt precursori presupuși ai cancerului de colon. ACF identificate la subiecții umani prezintă modificări morfopatologice similare celor experimentale , putând fi identificate și în colonul cu aspect normal de la pacienții cu cancer colorectal (CRC).

S-a sugerat că natura și ordinea modificărilor genetice dobândite pot afecta profund morfologia ACF și probabilitatea progresiei tumorii. Deși limitat ca număr, ACF displazic plat părea a fi mai probabil să sufere transformare tumorală decât ACF supradenivelat. În mod clar, diversitatea de creștere a ACF identificată în modelele carcinogenetice experimentale oferă cercetătorilor noi instrumente interesante pentru studierea etapelor incipiente ale CRC. Leziunile caracterizate în aceste modele experimentale ar putea deveni utile pentru predicția riscului carcinogenetic uman.

Cuvinte cheie: *Focare de cripte aberante (ACF), cancer colorectal (CRC), agenți chemopreventivi.*

Background

It is widely believed that colorectal carcinogenesis is a representative multi-step tumor genesis with events of genetic alterations. Cancer develops through four definable stages: initiation, promotion, progression and malignant conversion. The process of malignant transformation from normal tissue to invasive carcinoma comprises several stages (Figure 1):

1. **Genetic alterations**: It is generally believed that the first step towards malignant transformation is the accumulation of genetic alterations in proliferative cells (stem or transit cells).
2. **Abnormal crypt dynamics**: Loss of coordination between the processes of

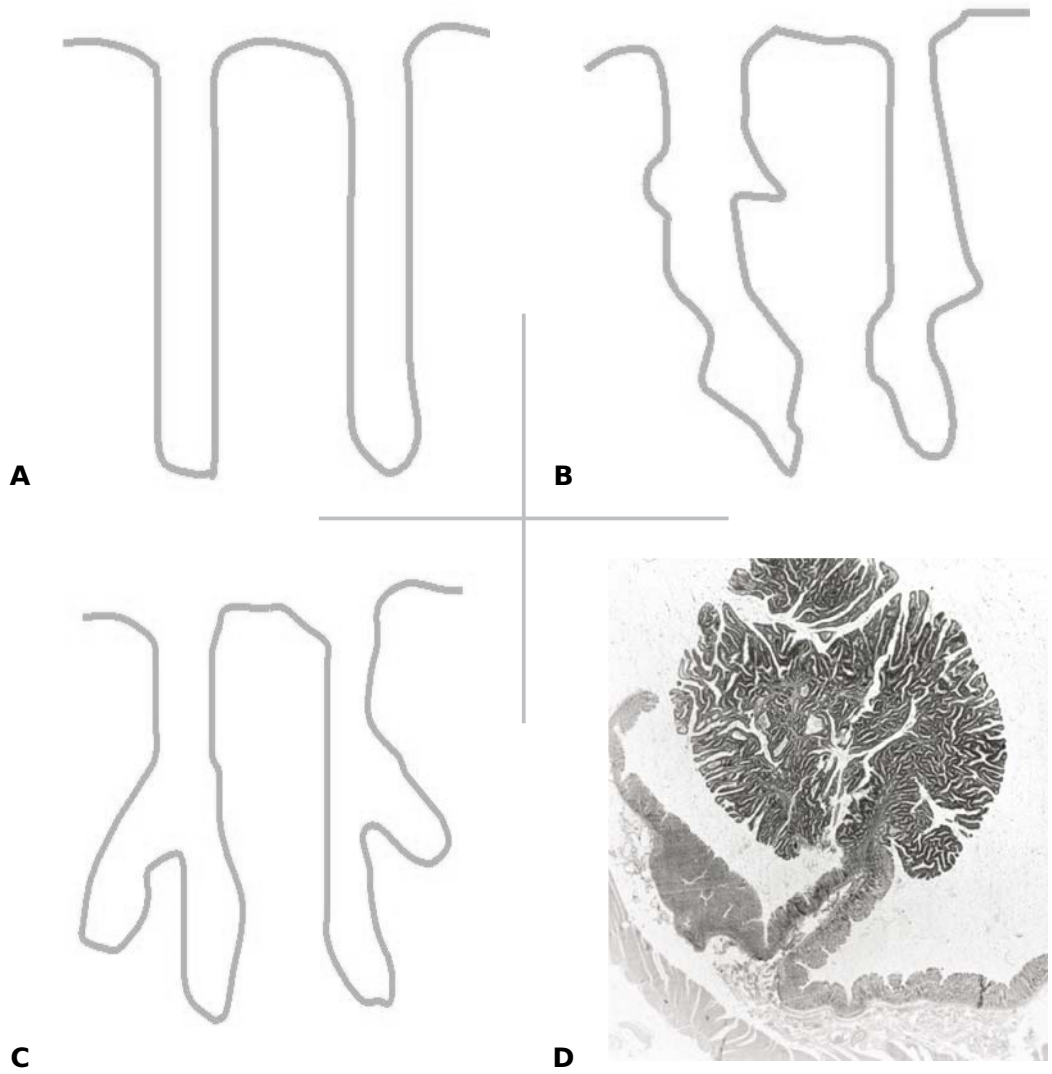


Figure 1. *Progression towards malignancy*

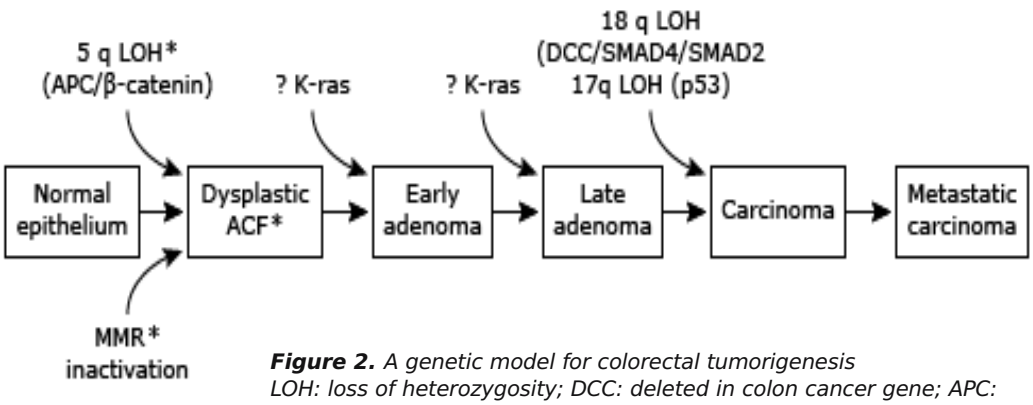


Figure 2. *A genetic model for colorectal tumorigenesis*
LOH: loss of heterozygosity; DCC: deleted in colon cancer gene; APC: adenomatous polyposis coli gene; ACF: aberrant crypt foci; MMR: DNA mismatch repair enzyme. Modified from: Lynch, JP, Hoops, TC. The genetic pathogenesis of colorectal cancer. Hematol Oncol Clin North Am 2002; 16:775[7]



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cell proliferation at the bottom of the crypt and differentiation and death at the top of the crypt leads to a net increase in the number of cells in the crypt.

3. **Crypt deformation and fission** (figures I B and 3C): The excessive cell number inflicts biomechanical stress on the wall of the crypt, which might cause it to fold and eventually induce crypt fission.
4. **Polyp formation** (fig. I D): Successive series of crypt fission usually led to the formation of a benign polyp.
5. **Tumor progression:** Further genetic alterations are required for progression to malignancy and invasiveness.
6. **Metastasis*:*** Some colorectal cancers acquire the ability to spread to other parts of the body, often the liver.

A. Normal mucosa: In the normal mucosa, a tight coordination of the processes of proliferation, migration, differentiation, and cell removal ensures the maintenance of the integrity of the crypt structure.

B. Deformation: Deregulation of the processes involved in crypt dynamics and the consequentincrease in cell numbers can cause the surface epithelium to deform and fold

C. Crypt fission: Further increase in the cell numbers leads to crypt budding and fission

D. Polyp: Subsequent series of cell proliferation and crypt fission cause the mass of aberrant tissue to protrude from the mucosa, giving rise to a polyp.

In humans, adenomatous polyposis coli (APC), beta-catenin (CTNMB1), Ki-ras (KRAS1) oncogene and p53(TP53) genes are thought to play important roles at different stages of colo-rectal carcinogenesis[1-3]. In no heritable colon cancer, at least seven independent genetic events are needed over decades and in the correct order to develop colorectal cancers[4]. This process begins with a normal colonic epithelial cell developing an adenomatous polyposis coli (*APC*) mutation, migrating to the top of the colonic crypt, expanding, and then forming an early adenoma[5,6]. Accumulation of a K-*ras* mutation then promotes intermediate adenoma formationfollowed by the transition to a late adenoma after mutations on chromosome *18q21* (candidate genes DCC, DPC4, JV18) occur. Mutations in the *p53* gene then transform the premalignant lesion to invasive carcinoma, and other additional genetic hits leadto metastasis (Figure 2).

There are two heritable forms of colon cancer: HNPCC and FAP. In HNPCC, germ line mutations in two genes are commonly found, *h*MSH2 and *h*MLH1.These genes encode for mismatch repair proteins, which when

abnormal will lead to genomic microsatellite instability and a two- to three-times higher mutation rate[8-11]. FAP is defined by an autosomal dominant germline mutation inthe *APC* gene. Patients with FAP develop hundreds to thousands of adenomatous polyps in the colorectum by their teenage years and colorectal carcinoma by the fourth decade of life.

Aberrant Crypt Foci-potential precursors of colorectal cancer

In present colorectal carcinogenesis research are directed to the use of surrogate end-point biomarkers that are altered early in carcinogenesis, before polyp (adenoma) formation, to predict the clinical effectiveness of chemopreventive agents or drugs because it takes 10-20 years for a normal cryptal cell to undergo molecular changes and to be clinically detected as a neoplasm. Aberrant crypt foci (ACF) can be used as the endpoint in colorectal cancer development because aberrant crypts are postulated to be the earliest identifiable potential precursors of colorectal cancer.ACF were first described by Bird and defined as crypt that: (1) have altered luminal openings;(2) exhibit thickened epithelia;(3) are larger than adjacent normal crypts. The analysis of ACF may facilitate the study of the early pathological and molecular changes that precede the development of an adenoma to colorectal cancer[12-13].

ACF may eventually evolve into polyps and, subsequently, colorectal cancer in the case of adenoma-carcinoma sequence; it provides a simple and economical tool for preliminary screening of potential chemopreventive agents, and it allows a quantitative assessment of the mechanisms of colon carcinogenesis. Beta-catenin-accumulated crypts and mucin-depleted foci are also early lesions that develop into colorectal cancer, but the detection of these lesions from human specimens is not easy by routine laboratory techniques.

ACF is microscopically visible in the unsanctioned colon stained with methylene blue and high-iron diamine Alcian blue, respectively[14-16] (Figure 3).

ACF is recognized early on the colonic lumen surface in rats treated with colon-specific carcinogens, azoxymethane (AOM), 1,2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazol pyramid (PhIP). These ACF are characterized by crypts with altered luminal openings, thickened epithelia and being larger than the adjacent normal crypts. The numbers of ACF increase with time after exposure to the carcinogen, but there is only weak evidence correlating tumor development with the expression of ACF.

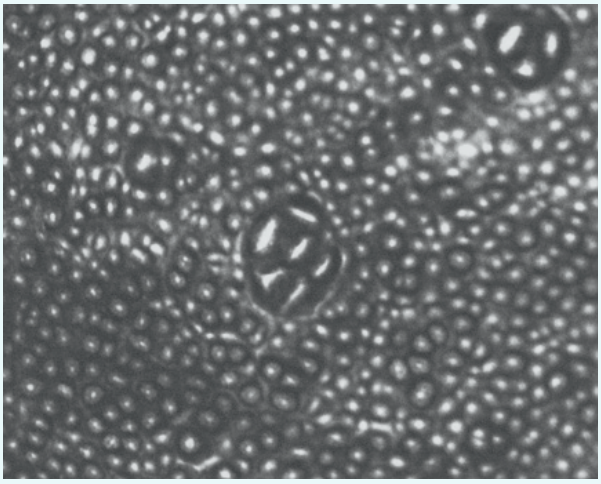


Figure 3. *Aberrant crypt foci (H&E, methylene blue)*

In the human colon, orifices of the colonic crypts have been referred to as “pits”, and the specific arrangement of the openings of the colonic glands is called the “pit pattern”. Kudo et al.[17,18] classified the pit patterns into five types: type I, round pit detected in the



normal mucosa; type II, asteroid; type III L, tubular or round pit, which is larger than a normal pit (type I); type III S, tubular or round pit, which is smaller than a normal pit (type I); type IV, dendritic or gyrus-like pit; and type V, irregular or amorphous pit. Applying their classification, typical ACF seems to correspond to the types III L and II pit pattern. In the previous studies, types III S and V pit patterns, which seemed to be different lesions from ACF, had stronger dysplasia than type II and type III L pit patterns (typical ACF pattern)[17.19].

Some lesions with type III S and type V pit patterns are smaller than ACF[19]. The evidence strongly suggests that, as in the rodent cases, there will be small dysplastic lesions without typical ACF appearance, and such lesions could be direct precursors for colon cancers in humans. Reliable intermediate biomarkers for colon carcinogenesis need to be identified to use them to evaluate several agents for their carcinogenic risk or their potential chemopreventive efficacy against colon tumors.

Aberrant crypt foci (ACF) have been recognized as early preneoplastic lesions. The specificity study showed that several other colon-specific carcinogens such as 1, 2-dimethylhydrazine, 4-aminobiphenyl, N-nitroso-N-methyurea and 3- methylcholan-trene also induce ACF, indicating that development of these lesions in the colon is clearly related to the genotoxic events. Genetic studies of ACF revealed that k-ras mutations are frequently identified in these lesions. Because colonic tumors induced by chemical carcinogens are also reported to have frequent mutations in the k-ras gene, there is some similarity in the genotype of colonic ACF and tumors, suggesting that this genetic alteration may play a key role in the development of colonic ACF as well as tumors.

Aberrant Crypt Foci- a short-term assay to identify modulators of colon carcinogenesis

Based on the proposition that these lesions appear preneoplastic, ACF is being used as a short-term assay to identify modulators of colon carcinogenesis prior to a long-term assay using tumor occurrence as end prior. However, some lines of evidence suggest that the results of the ACF assay and long-term analysis whose end point is colon tumor occurrence contradict each other. For example, there is a large body of evidence from clinical and metabolic epidemiological studies showing a positive association between the levels of secondary bile acids in intestinal lumen and colon cancer risk.

The long-term model assays using rodents have provided convincing evidence showing that administration of cholic acid (CHA), one of the primary bile acids, in the diet or

intrarectally is converted by bacterial 7-alpha-dehydroxylase to deoxycholic acid, which has been shown to induce cell proliferation and act as a strong tumor promoter in the colon. In contrast, a previous study indicated that dietary CHA significantly decreases the formation of AOM-induced colonic ACF in the short-term assay.

This disparity between the results of ACF formation and later tumor development by an agent was also documented previously on these parameters because of genistein administration.

Genistein was thought to be a promising chemopreventive agent against colon carcinogenesis based on epidemiological observations that consumption of soybeans and soybean-based products containing a large amount of genistein might reduce the risk of colon cancer.

While a short - term animal bioassay showed that genistein inhibits the development of azoxymethane-induced colonic ACF using tumors as end point demonstrated that dietary administration of this compound significantly enhances colon carcinogenesis. Comparative model studies using several strains of mice also ACF formation and susceptibility to azoxymethane-induced colon tumor genesis, demonstrated that azoxymethane treatment induces ACF in the colonic mucosa of resistant mice with no tumor occurrence at later time points, whereas the same treatment with the carcinogen produces a similar number of ACF as well as several colon tumors in highly susceptible mice; these observations leave little doubt that colonic ACF are not reliable biomarkers for colon tumor genesis.

During the last decade, beta-catenin signaling pathway is closely associated with the development of colon cancer. The beta-catenin protein and its Drosophila homolog armadillo have been established as critical downstream factors in the Wnt signaling pathway in vertebrates and the conserved Drosophila Wingless pathway[20].

Glycogen synthetase kinase 3 phosphorylates specific serine and/or threonine residues in the N-terminus of beta-catenin. Phosphorylation of these N-terminal sequences of beta-catenin promotes its interaction with F-box proteins and subsequent ubiquitarian and rapid degradation by the proteasome[21-23].

When the beta-catenin protein escapes from degradation and accumulates in the nucleus, it binds to T-cell factor (TCF) or lymphoid-enhancer factor (Lef) proteins and stimulates transcription of TCF/Lef-target genes. Recently the target genes of the beta-catenin signaling pathway were determined as growth-promotion genes, such as c-myc and cyclin D1, suggesting that the pathway is potentially oncogenic[23,24].

As might be predicted from such models of beta-catenin function in the Wnt pathway, bi-allelic inactivation of the APC gene or mutations in the beta-catenin gene in colon cancers give rise to stabilization of beta-catenin, its translocation to the nucleus and constitutive activation of TCF/Lef transcription. These alterations finally lead to activation of the oncogenic beta-catenin signaling pathway. It is now apparent that most colon cancers in both humans and rodents have alterations in the Apc gene or beta-catenin gene, which lead to beta-catenin accumulation[25,26].

Furthermore, it has recently been indicated that expression of nuclear beta-catenin is correlated with the size of colon neoplasms.

In rats or mice with a predisposition to develop CRC, histological sections reveal dysplastic crypts with excessive beta-catenin accumulation, called BCAC. These have disrupted cell morphology and increase with time after carcinogen treatment, BCAC does



not have the appearance of ACF and is usually not recognizable on the mucosal surface. BCAC has greater dysplasia than ACF and is associated with Paneth cells, as are CRCs, but not normal colonic epithelium[26]. Interestingly, the whole mounts colons, BCAC do not have a typical ACF-like appearance, with was originally characterized by Bird. The majority of BCAC is not recognized in the mucosal of end face preparations. In the end face sections, typical ACF does not represent excessive beta-catenin protein, which is associated with most colon cancers and suggested to play a critical role in colon carcinogenesis[28].

These results suggest that BCAC are independent early-appearing lesions of ACF. It is noteworthy that BCAC has a higher grade of histological dysplasia and proliferative activity than ACF[26]. Thus, it is reasonable to conclude that BCAC are more likely to progress into malignant transformation than ACF. It is also interesting to note that Paneth cells are frequently associated with BCAC, cells known to provide several cell growth-related factors, such as tumor necrosis factor-alpha, guanilin, epidermal growth factor and matrilysin. Oh them, matrilysin has been shown to play a role in the early stage of intestinal carcinogenesis[29], suggesting that Paneth cell metaplasia could promote intestinal carcinogenesis. BCAC has an increased expression of matrilysin at both mRNA and protein levels. These findings indicate a differentiating potential of BCAC towards the Paneth cell lineage and the results suggest that BCAC have the similarity to colon cancers in terms of their biological characteristics. It may be possible that Paneth cell differentiation could be a molecular target for early detection of colon cancers.

It is not clear whether BCAC represents a subgroup of ACF or can be early delineated as a separate entity. These foci are disseminated over several crypts, possibly by crypt fission or by cell migration. Clearly, not all cells are malignant because the number of colorectal cancers is less than the number of foci. There are many cells in each microadenoma or ACF/BCAC. If another mutation conferring carcinogenic potential occurs presumably it would be in a single cell and arise in the field of abnormal cells (in particular, the i3-catenin-accumulating cells). The potential size of that field is not known.

The most common mutation of the beta-catenin gene seen in BCAC is the same and representative type detected in colon tumors[28,30]; this genetic evidence seems to support the notion that BCAC is the pre-malignant lesion and implies that these mutations play gatekeeper roles in the development of colon tumors. The results that BCAC have beta-catenin gene mutations more frequently than typical ACF may

therefore indicate the potential of BCAC to progress into malignant lesions.

A portion of ACF harbors mutation in beta-catenin gene and lacks accumulation of beta-catenin. Such data suggest that although the beta-catenin mutation itself is insufficient for the detectable accumulation of beta-catenin, a part of ACF with beta-catenin mutations may possess neoplastic potential leading to colon cancers.

Thus, it is conceivable that other genetic and/or epigenic alterations relating to the translocation of beta-catenin into the cytoplasm and/or nuclei are present in BCAC, but not in most ACF. K-ras mutations are recognized in more than 70% of total ACF in rats treated with azoxymethane[30]. The majority of human non-dysplastic ACF has K-ras mutations, indicating that K-ras mutations are closely associated with the formation of typical ACF. That mouse who strains carrying oncogenic alleles of K-ras develops ACF in the colon[31], suggesting again that K-ras activation may be implicated in the formation of ACF.

Aberrant Crypt Foci and Chemical Carcinogens

The observation that chemical carcinogens may induce macroscopic alterations within the colonic epithelium has a long and interesting history. Initial descriptions of preneoplastic alterations within the colonic mucosa were made by Delapierre et al.[32] who monitored the descending colon of rats by transmission electron microscopy and observed preneoplastic lesions at 3 weeks after treatment with DMH. Wargovich et al.[33] reported several histological alterations, including mucin changes and alterations in crypt proliferation rates after four injections of DMH to C57BL/6J and CF1 mice.

While these and other studies have identified colon crypts with altered histology, it was not until Bird[34] developed a methodology that could readily identify and quantify macroscopic alterations on the surface of the colon that they referred to as ACF. Using methylene blue dye staining, it was found that carcinogen-induced cryptal lesions could be distinguished by their increased size, thicker epithelial lining, and increased peri cryptal zone. ACF are morphological lesions that represent an early stage in the stepwise progression of colon cancer.

These surface abnormalities often appear in the distal colon within 2 weeks of carcinogen treatment. Sequential analyses suggest that these early lesions increase in size and multiplicity and often exhibit nuclear atypia and dysplasia. ACF also shows increased proliferative activity, growth factor signaling and K-Ras mutations, suggesting that at least a subset of ACF are putative precursors of colon cancer. Human ACF shares a similar morphology and is present in grossly normal-appearing colon tissue from patients with CRC. It has been suggested that the nature and order of acquired genetic changes can profoundly impact ACF morphology and the likelihood of tumor progression.

Since these early observations, several other investigators have described subsets of ACF with various histological criteria, including hexosaminidase-altered foci, BCAC, flat dysplasia and mucin depleted foci. The Pretlow laboratory[35] demonstrated a marked reduction in hexosaminidase activity in methacrylate-embedded aberrant crypts taken from the distal colons of F344 rats treated with AOM. Tsukamoto et al.[36-37] then examined the transcription of the hexosaminidase a and b subunits in altered foci and reported that both markers were down-regulated in aberrant crypts of DMH-treated rats.



The same group then examined by scanning electron microscopy the three-dimensional structure of DMH-exposed rat colons and found that hexosaminidase-altered foci had abnormal budding within the middle of the crypt body, distinct from the symmetrical budding that occurred in hexosaminidase normal crypts. BCAC are reportedly a premalignant lesion in the colon that lack some of the characteristic histological features of traditional ACF, but may have a greater likelihood for malignant transformation than ACF[38].

When the frequency of BCAC was studied in AOM-exposed resistant and sensitive mouse strains, BCAC was found at a greater frequency in the distal colon of the sensitive strain at a site associated with subsequent tumor formation .On the other hand, ACF were more common in the proximal colon and occurred at a similar frequency in the sensitive and resistant strains, suggesting that BCAC may be a more reliable prognostic lesion. Mucin-depleted foci, originally characterized by their defective mucin production by the Caderni et al.[39] are dysplastic lesions that are found in the colons of carcinogen-exposed rats.

These lesions are induced by DMH, have a higher degree of dysplasia than ACF, increase in response to tumor promoters such as cholic acid or hem-rich diets and carry alterations in the Wnt-signaling pathway, including mutations in b-catenin .In addition, it was shown that the cancer-preventing agent polyethylene glycol suppressed their growth, whereas a high-fat diet resulted in a modest increase in mucin-depleted foci/colon with no change in ACF density. Paulsen et al.[40] identified flat dysplastic ACF in the colons of ApcMin mice that have different macroscopic characteristics than carcinogen induced ACF, as well as histological features consistent with microadenomas.

More recently, these investigators have identified flat dysplastic ACF in the colons of AOM-treated A/J mice, as well as F344 rats). In both species, these flat ACF grew more rapidly than classical elevated ACF, possibly because of Wnt activation and accelerated crypt multiplication.

Although limited in numbers, flat dysplastic ACF appeared to be more likely to become tumors than the elevated ACF Clearly, the expanding diversity of ACF identified in rodent carcinogen models provides researchers with exciting new tools for studying the earliest stages of CRC. Lesions characterized in these models may ultimately become useful for predicting cancer risk in humans.

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