Review

Aberrant Crypt Foci

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Abstract. *Colon cancer evolves through epithelial cell deregulation and inappropriate proliferation. These histopathological characteristics are exemplified in the biochemical, immunohistochemical, genetic and epigenetic elements detected within colonic mucosa. Early detection is paramount for the prevention of colon cancer deaths. Aberrant crypt foci (ACF) are thought to be the earliest identifiable neoplastic lesions in the colon carcinogenetic model. The progression of ACF to polyp and, subsequently, to cancer parallels the accumulation of several biochemical alterations and mutations whereby a small fraction of ACF evolve to colon cancer. Recent data indicate that, not uncommonly, some ACF bypass the polyp stage in their carcinogenesis thus reinforcing the importance of their early detection and our understanding of their pathogenesis. Since ACF were first detected in carcinogen-treated mice, research efforts have focused on these microscopically visible lesions both in animal and human models. ACF show variable histological features, characterized by Kudo (20) and, therefore, can be grouped into differing categories by in vivo examination with high-magnification-chromoscopic-colonoscopy (HMCC). As expected, ACF are more frequently detected in distal animal and human colons coinciding with the geographic distribution of colorectal cancer (CRC). Various proteomic (Prot) markers may be altered within ACF suggesting possible prospective pathological changes. These markers include Calreticulin, Transgelin, Serotransferrin, Triphosphate isomerase and Carbonic anhydrase II. Other markers of importance include carcinoembryonic antigen (CEA), B-catenin, placental cadherin (P-cadherin), epithelial cadherin (E-cadherin), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and P16INK4a. Genetic mutations of K-ras, B-Raf, APC and p53 have been demonstrated in ACF as well as the epigenetic alterations of CpG island methylation. Genomic instabilities (GI), illustrated by a higher GI Index (GII), microsatellite instability (MSI), loss of heterozygosity (LOH) and defects in mismatch repair (MMR) systems, are also expressed. These transformations may lead to the identification of the earliest pathological features initiating colon tumorigenesis. In this review, the advances in ACF research as precursors of CRCs are highlighted.*

Abbreviations: ACF, aberrant crypt foci; aCGH, array comparative genomic hybridization; AOM, azoxy-methane; GI, genomic instability; GII, genomic instability index as determined by ISSR-PCR; GM, gene mutations; HMCC, high-magnification-chromoscopic-colonoscopy; ISSR-PCR, inter-(simple sequence repeat)-PCR; LOH, loss of heterozygosity; MSI, microsatellite instability; Prot, proteomics.

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Colorectal cancer (CRC) is the second most common fatal malignancy in the United States (1). In 2004, approximately 149,000 newly diagnosed CRC cases resulted in 57,000 deaths (2). Screening of risk populations currently centers on colonoscopy and the possible discovery of neoplastic polyps. If in fact aberrant crypt foci (ACF) represent pre-polyp abnormalities, a better understanding, prevention and treatment of this disease might result. Accordingly, the

importance of a powerful new screening tool such as high-magnification-chromoscopic-colonoscopy (HMCC) cannot be overemphasized.

ACF lesions may be identified by HMCC in otherwise normal appearing colonic mucosa. They are comprised of crypts that are microscopically elevated above the normal mucosa, are composed of excessively thickened epithelia and have altered luminal openings clearly circumscribed from the normal adjacent crypts.

These lesions were first observed at higher frequency in the colons of rodents treated with colon-specific carcinogens and, later, similar observations were noted in the colons of higher risk patients with sporadic and inherited colon cancer. Thus, it was hypothesized that ACF might be precursors of CRC. Subsequent biochemical, genetic and morphological studies have shown similar alterations within colon tumors and ACF further supporting the proposed hypothesis (3-12).

Since the first detection of ACF in carcinogen-treated mice by Bird in 1987 (4) and the hypothesis of ACF as the earliest precursors of CRC, numerous studies have centered on their evolution. The current studies are highlighted in Table I.

The Clinicopathological Profile of ACF

ACF and Colorectal Carcinogenesis. There appear to be two differing pathological pathways to CRC: *The Exophytic Adenoma-Carcinoma Sequence* and *the De Novo-Flat Adenoma-Carcinoma Sequence.*

Although the actual specifics require clarification, ACF appear to play a central role in both carcinogenic pathways. Several lines of evidence support ACF as preneoplastic: primarily their presence in carcinogen-treated susceptible rodents and high risk human CRC groups, genetic and cell dynamic alterations and histochemical abnormalities.

A. Exophytic Adenoma-Carcinoma Sequence. Reported initially by Morson and Muto *et al.* (13, 14), adenomatous polyps are generally recognized as precursors of CRC. Presumably, over a number of years to decades, some benign adenomas enlarge, accumulate genetic alterations and develop malignant characteristics. This sequence is believed to account for roughly two-thirds of CRCs and constitutes the current clinical rationale for early screening and therapeutic intervention. In fact, polypectomy has conclusively demonstrated a decrease in the incidence of CRC by interrupting this pathological progression (15, 16).

B. The De Novo or Flat Adenoma-Carcinoma Sequence. The remaining third of CRCs are thought to develop by "*de novo*" transformation. Although initially thought of as an exclusively Japanese phenomenon, Rembacken (22) reported similar findings in the Western hemisphere. These

Table I. *Updated highlights of human ACF in recent years (from NCBI).*

Major fields	Magazines and authors	Year
1-Histopathology	Takayama T *et al.* NEJM	2004
	Douglas G *et al.* Gastrointest Endosc	2002
	Kristt D *et al.* Hum Pathol	1999
	Nascimbeni R *et al.* Am J Surg Pathol	1999
	Shipitz B *et al.* Hum Pathol	1998
	Siu IM *et al.* Am J Pathol	1997
	Roncucci L *et al.* Cancer Epidemiol	1997
	Roncucci L *et al.* Hum Pathol	1991
2-Magnifying/ chromoscopic colonoscopy	Hurlstone DP *et al.* Br J Surg	2002
	Matsumoto T *et al.* Am J Gastroenterol	1999
	Takayama T *et al.* N Engl J Med	1998
	Yokota T *et al.* Gastrointest Endosc	1997
	Dolara P *et al.* Cancer Detect Prev	1997
3-Dietary agents	Moxon D *et al.* Clin Gastoentero Hepoatol	2005
	Nascimbeni R *et al.* Cancer Epidemiol Biomarkers Prev	2002
	Johnson IT *et al.* Food Chem Toxicol	2002
	Alabaster O *et al.* Mutat Res	1996
4-Chemoprevention	Murillo G *et al.* Int J Cancer	2003
	Osawa E *et al.* Gastroenterology	2003
	Kassie F *et al.* Carcinogenesis	2003
	Osawa E *et al.* Life Sci	2002
	Mori H *et al.* Biofactors	2000
	Chung FL *et al.* Carcinogenesis	2000
5-Genetic, epigenetic/ phenotype alterations of genes CpG island methylation	Alrawi SJ *et al.* In press	2005
	Luo L *et al.* Int J Cancer	2005
	Luo L *et al.* Cancer Res	2003
	Chan AO *et al.* Am J Pathol	2002
	Sakurazawa N *et al.* Cancer Res	2000
6- Gene mutations	Takahashi M *et al.* Cancer Sci	2004
	Kazuya H *et al.* Cancer Sci	2004
	Yasuhiro Y *et al.* Carcinogensis	2003
	Yuan P *et al.* World J Gastroenterol	2001
	Takayama T *et al.* Gastroenterology	2001
	Otori K *et al.* Cancer	1998
	Bjerknes M *et al.* Am J Pathol	1997
	Losi L *et al.* J Pathol	1996
	Zaidi NH *et al.* Carcinogenesis	1995
	Smith AJ *et al.* Cancer Res	1994
7- Loss of heterozygosity	Yuan P *et al.* Article in Chinese	2002
8- Microsatellite instability	Pedroni M *et al.* Cancer Res	2001
	Heinen CD *et al.* Cancer Res	1996
	Augenlicht LH *et al.* Oncogene	1996
9- Proteomics/ cell dynamics and proliferation	Drew JE *et al.* Bioch & Bioph Res Com	2005
	Roncucci L *et al.* Cell Prolif	2000
	Kristt D *et al.* Pathol Oncol Res	1999
	Otori K *et al.* Cancer Res	1995
	Roncucci L *et al.* Cancer Res	1993
10- Oncoproteins	Dong M *et al.* Carcinogenesis	2003
	Hao XP *et al.* Cancer Res	2001
	Shpitz B *et al.* Anticancer Res	1999
	Pretlow TP *et al.* Gastroenterology	1994
11- Signal transduction pathways	Boon EM *et al.* Cancer Res	2002

cancers were initially thought to develop in the absence of any associated adenomatous tissue. Muto, however, in 1985, suggested that *de novo* CRCs developed from flat or depressed adenomas, as previously described by Kariya and further studied and classified by Kudo. These flat adenomas were described as appearing "reddish", less than 1 cm in diameter and often containing central cavitations. Minamoto suggested that 30% of colorectal lesions are flat adenomas with a high proportion of over 40% demonstrating more aggressive malignant potential with high-grade dysplasia. Other studies support the concept that flat adenomas are more aggressive neoplastic lesions potentially taking an alternate route in CRC evolution (17-25).

Submucosal invasion was reported in the majority of flat carcinomas by Tada, while Shimoda compared early polypoid *vs.* non-polypoid CRC and found double the rate of invasion into the submucosa, venous and lymphatic vessels and muscularis propria in non-polypoid lesions when compared to polypoid CRC, despite the latter's smaller size. Other studies support these findings, Minamoto *et al.* reporting nodal metastasis in flat CRCs of less than 10mm in diameter. The "*de novo*" pathway seems to be more likely to become malignant earlier, when smaller in size, and spread more aggressively. These flat CRCs and adenomas, because of their flat shape and size, can often be difficult to detect endoscopically but with the aid of HMCC, these and their putative precursors, the ACF, are more readily identified (26-31).

The transformation in both the *de novo* and exophytic sequences is heralded by the accumulation of numerous genetic abnormalities in oncogenes, tumor suppressor genes and mismatch repair genes (MMR), resulting in genomic instability (GI). These early changes are thought to take place throughout ACF evolution (10-12, 32-35).

Early carcinogenesis. Normal colonic crypts contain regenerative stem cells located at their base. Through a series of dynamic mechanisms, stem cells extrude epithelial cells replacing the crypt approximately every five days. The normal proliferative zone is located within the lower two-thirds of the crypt. All crypt cells, both normal and aberrant, arise from a single stem cell, as demonstrated by crypt monoclonality in both humans and mice. Replication of both normal and aberrant crypts occurs through a process called "*fission*" involving basal budding, branching and finally separation. The mechanisms controlling crypt multiplicity are thought to be important early aberrations in the formulation of ACF (36-41).

Studies in the 1970s and early 80s examined CRC carcinogenesis in human and rodent models. Further analysis showed remote histological abnormalities occurring in normal appearing mucosa distant from the malignant lesions. It is unknown whether this is a field or lesion-effect phenomenon, although alterations in crypt zone proliferation, apoptotic malfunction and abnormal cell dynamics have been demonstrated in early CRC and in ACF (4, 42-45).

ACF description. ACF were first described and induced in a dose- and time-dependent manner by Bird in 1987 in mice after treatment with azoxy-methane (AOM) (4, 46). Using stereomicroscopy and methylene blue, Bird visualized the colonic mucosa and noticed clusters of heavily stained irregular crypts with larger, thicker epithelial linings and frequently altered lumens. He identified these aberrant crypts and hypothesized that they were possible precursors of colon cancer (4, 47).

Then, in 1991 Pretlow and Roncucci were the first to establish the presence of ACF in human surgical specimens, noting their marked similarities to rodents' (48, 49). Their first description was of the normal, finely organized circular or oval crypt lumens surrounding more heavily stained, irregular ones consisting of from a single crypt to more numerous complexes numbering in the tens to hundreds of crypts (50). The ACF are often slightly elevated above the normal mucosa and have visible dilated, circular, oval or slit-like irregular openings (51-54).

In addition to mucosal elevation, McLellan *et al.* suggested individual ACF occupy at least double the surface area of the normal crypt. Thus, multiple aberrant crypts would form a much larger focus than a similar number of normal crypts and their heavier staining allows for ready visualization (55). Subsequent work involving HMCC, using methylene blue or indigo carmine staining techniques, illustrated ACF with the same appearance endoscopically (55-59).

High-magnification-chromoscopic-colonoscopy. New magnification endoscopes are now available from Pentax, Olympus and Fujinon allowing for 13-150x magnification displayed on a 20-inch monitor. Amplification is adjusted by a thumb-controlled lever or foot pedal. With HMCC, their heavy staining and pattern of crypt lumens or "pit pattern" readily identifies ACF. Likewise the "pit pattern" of other lesions, such as polyps and CRCs, can also be visualized.

Several successful colonoscopy lavage and staining techniques have been developed. Takayama *et al.* used a water wash, often with 0.5% glycerine to remove mucous and then stained with the vital dye 0.1%-0.5% methylene blue followed by another wash a minute or two later. Hurlstone *et al.* recommended Indigo carmine (0.2%-2%), a blue stain not absorbed systemically. They occasionally employed Crystal Violet 0.05%, which has been associated with *in vitro* toxicity but can be used to further clarify the pit pattern in certain circumstances. An initial wash with the mucolytic acetylcysteine (2 mg/ml) was recommended. These dyes accentuate the landscape of the mucosa and allow for examination at the microscopic level (59-63).

Table II. *Kudo's classification of ACF. (20,64,66-69)*

Type	Description of pit pattern	Size	Comments
Type I	Normal round crypt openings	0.07±0.02mm	Seen in normal, metaplastic, hyperplastic
Type II	Stellate or papillary crypt openings	0.09±0.02mm	colorectal mucosa
Type III L	Tubular, long , large crypt openings	0.22±0.09mm	Seen in tubulovillous adenoma
Type IV	Gyrus, cribriform	0.93±0.32mm	and tubular adenoma
Type III S	Tubular or rounded smaller than type I	0.03±0.01mm	Seen in invasive neoplastic lesions (early)
Type Va	Irregular pattern and sizes (IIIL,IIIS,IV)		deeper but probably still mucosal
Type Vn	No discernable pit pattern		deeper than mucosal

Pit pattern and the Kudo classification. Kudo *et al.* developed the current classification system for pit pattern description by correlating findings made through HMCC followed by stereomicroscopy and histological evaluation of normal, hyperplastic, adenomatous and malignant specimens (Table II; (20, 62-64)). Accurate correlations and reproducibility with *in vivo* HMCC and *ex vivo* stereomicroscopy have been reported (20, 47, 60-67).

Histological Classification of ACF. As reported by various authors, ACF descriptions include non-dysplastic, dysplastic and mixed types (6, 50, 52, 55, 59, 68-72).

A. ACF with Normal Mucosa (Non-Dysplastic)

1. Non-Hyperplastic ACF. This subtype of ACF possess enlarged crypts at least 1.5 times larger than normal, but lack significant abnormalities in the epithelial cells lining the crypts. Minimally enlarged and elongated nuclei are occasionally seen, but no crowding, stratification, or mucin depletion is present. In this subtype, cells staining positive for proliferating cell nuclear antigen (*PCNA*) and *Ki-67* remain localized to the lower parts of the crypts, as seen in normal tissue.

2. Hyperplastic ACF. This class embodies the overall pathology of the hyperplastic polyp. They maintain larger crypts with an elongated appearance, having both side and apical branching. The crypt profile is serrated and may exhibit cellular tufting slightly above the surrounding mucosa. The epithelium reveals no dysplasia, but goblet cells are interwoven with absorbing cells and demonstrate partial mucin depletion. Although these nuclei are enlarged and sometimes appear crowded, they lack stratification. In contrast to the aforementioned class, the cells staining positive for *PCNA* and *Ki-67* extend from the bottom into the middle parts of the crypts.

B. Dysplastic ACF

These ACF demonstrate significant variability from the normal crypt pit pattern. Goblet cells are decreased and mucin production is depleted. The epithelial cells are enlarged, stretched and show stratification with depolarized nuclei. Dysplastic tissues manifest one or more of the following cytological features: stratification, nuclear enlargement, pleomorphism, hyperchromasia, or increased nuclear-to-cytoplasmic ratios, with or without architectural distortion (73). Their major sites for positive staining cells by *PCNA* and *Ki-67* extend into the upper part of the crypts.

Sporadic (non-familial) dysplastic ACF are similar to dysplastic ACF in familial adenomatosis polyposis (FAP) patients but show uncommon *APC* mutations with more frequent methylation abnormalities (50, 74, 75). Dysplastic ACF are found in the majority of FAP patients but are infrequent in sporadic ACF patients (52, 76, 77). In addition, serrated adenomatous ACF have been described as similar to serrated adenomas with respect to their associated histopathology (78, 79).

C. Mixed ACF

The WHO classification only draws distinction between hyperplastic and dysplastic ACF. Additionally, the Otori classification adds a stage I class, which may represent a precursor of the dysplastic or adenomatous ACF with its similarly high proliferation indices and zonal expression using PCNA and Ki-67 (80-86).

However, ACF with mixed histology contain various proportions of pure adenomatous dysplasia and pure hyperplastic features without dysplasia (78). Takayama *et al.* report ACF as being small (1-9 crypts), medium (10-19), or large (20+), and further subdivide these into foci containing dysplasia, hyperplasia, or neither (74). They also studied the validity of endoscopic diagnosis by the degree of endoscopic and histological correlation. He found the corresponding sensitivity and specificity for non-dysplastic, non-hyperplastic foci to be 96.4% and 97.6%, respectively, thus highly in agreement. Non-dysplastic, hyperplastic foci were 86.4% and 97.3% and dysplastic foci were 100% and 97.4%, respectively. Hurlstone reinforced the accuracy of HMCC in ACF detection and typing by this *in vivo* optical biopsy methodology (47). In most series, with proper technical

implementation, HMCC identification of ACF is comparable to microscopic evaluation.

ACF Features and Characteristics

1. ACF Histological Variability. The reported incidence of dysplastic ACF in non-FAP (sporadic ACF) patients ranges from 5%-54% (5, 50, 68-72, 77, 87). The histological variability of ACF may be due in part to the definition of "dysplasia", but also a transition phase from hyperplastic to dysplastic may occur (50, 70, 80, 81). A single ACF may focally express differing pathological types including carcinoma *in situ* (6, 70, 78) and these remote from lesion ACF have been identified in patients with colonic carcinoma as well as within invasive carcinoma in the rat model, supporting their role as precancerous lesions (6, 50, 51, 72, 82, 88-90).

2. Anatomic Location of ACF. Nucci *et al.* reported that only 35% of ACF are located in the proximal colon (74). The ACF density is higher in the left colon, but proximal colon ACF tend to have more crypts per ACF *i.e.* larger "*crypt multiplicity*" (6, 49, 68, 88). Although ambiguous, current data indicate larger ACF are more frequently dysplastic. Therefore, rectal ACF are more commonly hyperplastic while right-sided ACF are frequently dysplastic (12-37). Furthermore, there is no evidence to suggest gender differences in ACF location or anatomy. Increased crypt multiplicity has been associated with a higher incidence of carcinoma or adenoma when compared with benign disease such as diverticulosis, but no correlation with the distance from a tumor has currently been established (48, 49, 59, 69, 71).

3. Prevalence of ACF. Roncucci *et al.* reported that regions with high colon cancer rates also have a higher prevalence of ACF (71). Although Yokota *et al.* and a number of other studies have demonstrated an increased density of ACF in the presence of CRC, there are other reports showing their increased frequency in benign disease such as diverticulitis (48, 54, 64, 71, 91). Takayama found that 10% of normal subjects under the age of 40 had ACF, but that the prevalence rose precipitously to 53.6% in the 40-49 age group and 65.7% in the 60-69 age cohort. An additional association with ACF and the presence of an adenoma became apparent with 75% of patients under 40 having an ACF followed by 90.2% in patients studied between 60-69 years of age. When a CRC was actually present, the prevalence of ACF was 100% in all age groups (59).

Even more remarkable were the relative risk ratios for these groups when dysplastic foci were examined. Compared to the normal, the presence of an adenoma and CRC increased the relative risk ratio for dysplastic ACF by 4.26 and 18.14, respectively, and 1.14 and 1.29 for non-dysplastic foci. Takayama *et al.* further described a clear connection between the number of ACF and the number of adenomas and an even stronger correlation between the number of adenomas and the presence of dysplastic ACF. The size of the ACF depends on crypt multiplicity, which also correlates with the total number of adenomas (59).

4. Sporadic ACF and ACF associated with Familial Adenomatosis Polyposis (FAP). Patients with FAP and Gardner's syndrome demonstrate increased ACF size and density compared with non-familial CRC, indicating that hereditary disease predisposes patients to higher numbers and multiplicity of ACF compared with sporadic CRC-associated ACF (48, 49). This difference suggests at least two differing pathways in this carcinogenic pathway (62). Histologically the majority of *FAP*-associated ACF are dysplastic or microadenomas, while sporadic ACF are mostly hyperplastic and less frequently form microadenomas (72).

Most *FAP* ACF form without *K-ras* mutations. In contrast, the majority of sporadic ACF cases has *ras* mutations and is hyperplastic (45). *K-ras* mutations were detected in 82% of non-dysplastic, sporadic ACF and in 63% of dysplastic, sporadic ACF. However, only 13% of dysplastic *FAP* ACF showed *K-ras* mutations and 100% of *FAP* ACF and *FAP* adenomas demonstrated somatic *APC* mutations (80). Similarly, in experimental mice (Min/+) with germline mutations homologous to human *APC*, dysplastic ACF are noted to progress to adenoma and carcinoma by rapid crypt division and abnormal *β-catenin* expression (6, 77, 92). Meanwhile, sporadic ACF in AOM-treated rodents showed normal growth and *β-catenin* expression and infrequently developed dysplastic ACF and neoplasia (62, 92, 93). Similarly, CpG island methylation is more common in non-dysplastic, sporadic ACF while FAP ACF usually lack methylation (92-94). It is postulated that only dysplastic ACF or microadenomas progress to an adenoma or carcinoma and since FAP ACF quickly become dysplastic most will progress while sporadic ACF are usually hyperplastic and relatively infrequently become neoplastic (77).

Thus, a clear genetic divergence exists between familial and sporadic CRC with respect to ACF. *β-catenin* abnormalities exist in familial APC while sporadic CRC occurring from carcinogenic exposure evolve independently from this mechanism and are most probably the result of inappropriate methylation.

Histochemistry of ACF

Strong evidence supports the neoplastic potential of ACF progression to CRC as expressed in cell and crypt dynamics. Various authors demonstrated similar increases in cellular proliferation in rat ACF and colon tumors. Extensive evidence in both rodent and human data supports the contention that abnormalities and errors in epithelial crypt dynamics are central to the neoplastic transformation (43, 56-58).

Genetic and Proteomic Profiles of ACF

The morphological characterization of ACF pathways are distinct in that dysplastic foci are more likely to progress to adenoma, while those showing hyperplastic characteristics typically remain stationary or develop into polyps with little malignant potential, further implying differences within the genetic profile (70). There is a variety of genetic profile expression including:

1. Inter simple sequence repeat PCR and comparative genomic hypridization. Some degree of genomic instability (GI) is expressed by ACF with the majority of them exhibiting neither band to band variability detected by inter- (simple sequence repeat) PCR, nor major fluttering differences (amplifications or deletions) expressed by array comparative genomic hybridization (aCGH) (95-99). The GI index of these ACF, calculated by the Alrawi *et al.* technique (100), is a surrogate of genetic aberrations and shows that a more stable set of ACF (75%) would be static and thereby unlikely to evolve further. In contrast, less than a quarter of the ACF showed higher indices at levels in the vein of that seen in most adenomas and carcinomas, indicative of substantial genomic reparation occurring in their infancy. Since a small percentage of these lesions progress to overt carcinoma, detection of these unstable crypts would serve as a superb screening tool.

Additionally, small events of GI expressed by ISSR-PCR apparently precede the larger ones in the CRC evolutionary pathway concealed by aCGH. These events evidently happen before blatant mutations occur, with other reports showing ACF rarely containing aberrant genetic mutations. This critical process, generating small event instability, may initiate the first steps in CRC progression, inflicted by various carcinogens including smoking. However, smoking itself might be a significant benefactor to the genomic damage detected in early ACF, since most ACF with elevated GI develop in patients who smoke (57, 68, 100-104).

These findings may have significant implications for CRC surveillance and cancer prevention. Patients with ACFs harboring high indices may benefit from frequent observation in follow-up programs as these lesions might be proven as useful indicators for CRC pathogenesis. Consequently, ACF characterizations themselves could become a valuable screening tool. Needless to say, preventive measures, reducing the overall number or size of ACF without impacting the total showing GI, will probably achieve little in terms of cancer prevention (76, 105).

Still, GI is a valuable parameter in evaluating ACF since some might progress to CRC without ever passing through the polyp phase. Accordingly, further attempts should focus on comparing those indices with the histopathological parameters for effective risk stratification.

2. Proteomics analysis in protein profiles of ACFS. Proteomic evaluation of colonic tissue after carcinogenic exposure provides useful biomarkers in assessing genetic and epigenetic profiles associated with altered homeostasis in early CRC carcinogenesis. Various indicators include acetylation, phosphorylation, glycosylation, ubiquitination and altered expression of different protein isomers. Altered or defective normal cellular functions are considered a signature for the potential metabolic changes observed in both human and rodent CRC pathogenesis (106-115).

The first biomarker of scientific concern is *calreticulin,* which has been implicated in CRC pathogenesis. Calreticulin is one of the cytoplasmic calcium-binding proteins associated with nuclei and extracellular compartments. In addition, it has an assumed role in many functions involved in cell homeostasis, including hormone receptors binding to modify gene (*MUC2*) expression (116-118).

The second biomarker is *transgelin*, an anti-inflammatory calponin-related protein found in various muscular tissues. Transgelin is up-regulated in soluble fractions from the colon of rats treated with AOM. Thus, malignancy may be prevented by counteracting the initial inflammatory events in tumor pathogenesis. Subsequent tumor progression leads to a loss of transgelin gene expression down-regulating tumor progression in CRC by *ras*-dependent and *ras*-independent mechanisms. This broadens supports for counteracting the inflammatory pathways activated by AOM treatment as this increases prostaglandin production (119).

The third biomarker is the *carbonyl reductases* (CBRs). These enzymes are monomeric, cytosolic enzymes known to catalyze the reduction of endogenous prostaglandins, steroids, aliphatic aldehydes and ketones, and a wide variety of polycyclic aromatic hydrocarbon-derived xenobiotic quinones. CBRs appear to be involved in a variety of cellular and molecular reactions associated with drug metabolism, detoxification, drug resistance, mutagenesis and carcinogenesis. CBRs might play a role in the metabolic processes associated with altered levels of prostaglandin expression, as measured in AOM-treated rats in CRC (120, 121).

The fourth marker is the *serotransferrin* precursors, which have a potential role in stimulating cell proliferation and might be considered growth factors in the CRC pathway through the activation of transferrin receptors at target level. However, serum transferrin and C-reactive protein are considered biomarkers of inflammatory bowel disease and are independently linked to CRC pathogenesis (122).

The fifth one of importance is *triosephosphate isomerase I*. This enzyme catalyzes the conversion of D-glyceraldehyde 3-phosphate to glycerone phosphate, which plays a role in multiple metabolic pathways including the reversible transfer of phosphate by creatine kinase. These isoenzymes play a pivotal part in energy transduction in muscular tissue including cardiac and skeletal muscles which have large,

capricious energy demands. Various metabolic and pathological changes could be encountered in colonic tissue after fluctuations in the levels of triosephosphate isomerase I and creatine kinase post carcinogen exposure.

Other proteomics of less clinical importance are *carbonic anhydrase II (CA-II)* and *tropomycin*. CA-II is an enzyme responsible for regulating the pH in different body fluids, thus optimizing fluid absorption, digestion and waste excretion. Its expression and function is altered in pre-cancerous colon pathology in rodent models. Finally, tropomycin isoforms are associated with CRC and ulcerative colitis (114, 115).

Altered biochemical homeostasis was observed in both pre-cancerous states and CRC as evident by elevations in prostaglandin levels and altered indices of oxidative stress. These findings are linked to the dynamic processes of proliferation, differentiation and apoptosis, regulating crypt characterization and subsequent induction of tumorigenesis. In conclusion, proteomics linked to CRC pathologies may prove effective investigative approaches to colon cancer prevention and surveillance.

3. Methylation in aberrant crypt foci and cancers. Epigenetic alterations are common in CRC tumorigenesis, occurring at very early stages of ACF and polyp formation. DNA methylation was found in 53% of sporadic ACF at 5 major loci. Methylation was reported in various promoter regions including *CRBP1* (acts as a chaperone for retinol signaling involved in cell growth and differentiation) and *CDH13* (a member of the cadherin family of glycoproteins involved in cell adhesion and recognition) genes in human ACF. Li *et al.* found *SLC5A8* hypermethylated in 47% of ACF, and Suzuki *et al.* found *SFRP1* and *SFRP2* hypermethylated in 93% and 87% of ACF, respectively. These loci are known to control functions that could promote CRC tumorigenesis (123-129).

The smaller numbers of epigenetic alterations detected in ACF compared to CRC from the same patients support the hypothesis of ACF as early precursors of some cancers going through a polyp stage and others moving directly to cancer formation. The different genetic make up of the ACF and cancer samples contributed to the various frequencies of loci methylation in several series. Although *MINT31* is characterized as being specifically methylated in cancer and not in normal tissue, the low frequency of methylation of *MINT31* in normal crypts supports the hypothesis that ACF are precursors to CRC (94, 130, 131).

Concordant methylation and lack of methylation at the same loci in both benign and malignant lesions from the same patient, or methylation of cancers with no precursor lesions, are also reported. Numerous data supports the ACF, like adenomas, are monoclonal lesions arising independently during colon tumorigenesis. The high frequency of ACF with methylated loci suggests that this particular epigenetic alteration occurs early and may play an important role in tumorigenesis. The lack of concordance among lesions from the same patient suggests some epigenetic changes are more likely independent events, similar to genetic alterations (38, 92, 132, 133).

Table III. *Gene alterations in colon cancer, adenoma and ACF.*

Lesion	Mutation frequency %				
	APC	*B-Catenin*	*K-ras*	*DCC*	*P53*
Adenocarcinoma	40-80%	15%	40-60%	40-70%	50-80%
Adenoma	40-65%	0%	0-40%	0%	0%
ACF	<5%	0%	10-95%	0%	0%

4. Gene mutation and altered gene expression in colon cancer carcinogenesis. Table III illustrates the gene alterations in colon cancer, adenoma and ACF.

Gene mutations in colon carcinogenesis. Colon carcinogenesis is a multi-step process with frequent genetic mutations including *K-ras*, *APC, DCC* and *p53* in various developmental stages of ACF, polyps and cancer (Table III). Although the *DCC* and *p53* genes are involved in the late stages of carcinogenesis, *K-ras* and *APC* gene mutations are involved in the relatively early stages. These *APC* defects are typically seen in adenomas while *K-ras* mutations are frequently found in the ACF stage. However, with respect to *K-ras,* a wide spectrum exists from trace to 95% mutation rates among various population bases, with an increase noted in Eastern populations. Most ACF are hyperplastic and positive for *K-ras* mutations, but about 5% of ACF are dysplastic and harbor *APC* mutations. (5, 83, 100, 134-139)

K-ras is an oncogene encoding an intracellular signaling molecule. Oncogenic mutations in *ras* (codons 12 and 13) result in the constitutive activation of *ras* and its downstream signaling pathway such as *Raf*/*MEK*/*MAPK* and *PI3K*/*Akt*/*PKB*. The relationships between ACF and the corresponding colon tumors were studied by various authors and were highly prevalent in colorectal carcinogenesis (140-142). Interestingly, it was found that the *K-ras* base composition at codon 12 and 13 was relatively concordant in both ACF and in the consequent colonic tumors in most cancer patients studied. The same was applicable for wild type *K-ras* in ACF and colon tumors.

These observations suggest that ACF arise as a result of clonal genetic alterations and that *K-ras* mutations may contribute to their development (143). However, certain

mutations confer a greater chance of progression to macroscopic tumor than others located in the vicinity of the tumor. ACF positioned closer to the corresponding colonic tumors may be considered more likely to progress to malignant lesions. At the same time there is heterogeneity among different ACF from the same patients with regards to the K-ras mutation and ACF may even arise from independent clones (105, 137, 144).

The other three mutations noted involve tumor suppressor genes. DCC encodes a protein exhibiting homology to cell adhesion molecules, while p53 protein is a transcription factor which regulates the cell cycle and apoptosis. The identification of the *APC* gene is implicated in the inherited colon cancer syndrome adenomatous polyposis coli. Here, the APC protein forms a complex with *β-catenin* thus stimulating its degradation (145,146). Mutations in the GSK-3b phosphorylation consensus motif of the *β-catenin* gene, as well as *APC* mutations, cause stabilization of *β-catenin* in the cytoplasm and induce constitutive transcriptional activation with Tcf-4, a specific type of DNA binding protein. In fact, Sparks *et al.* found mutations of the *β-catenin* gene in half of human colon tumors possessing an intact *APC* gene. In *APC*-deficient tumors, *β-catenin* mutations are generally not detected; hence, most colon tumors feature changes within the *APC*/*β-catenin*/Tcf pathway (147-149).

Analysis of *p53* (exon 4-9) mutation showed the presence of *p53* mutations in colonic carcinomas from 50-80% of cancer patients. However, it was expected that *p53* mutation would rarely be detected in ACF. Thus, *p53* mutations may appear in CRC independent of ACF. Of note, *p53* is found in less than 30% of adenomas, demonstrating that ACF have genetic features further maintaining their distinctive role in the formation of precancerous lesions. However, presence of the same *p53* mutation in ACF and corresponding carcinoma (while absence of *p53* mutation in the other ACF and corresponding adenoma) suggests that the combined existence has a higher malignant potential (102).

5. Microsatelitte instability (MSI) and mismatch-repair protein expression in hereditary (HNPCC) and sporadic colorectal carcinogenesis. The presence of microsatellite instability (MSI) in ACF suggests that the MSI-positive phenotype may occur early in colorectal carcinogenesis. ACF and adenomas from patients with HNPCC and sporadic unstable CRCs expressed MSI 100% and 91%, respectively. Thus, MSI represents an early event in HNPCC colorectal carcinogenesis. Analysis of *hMLH1* and *hMSH2* protein expression in HNPCC patient DNA authenticates that mismatch-repair genes appear altered in the early phases of the carcinogenic pathway. Supportive evidence confirms 61% of premalignant lesions examined (including ACFs and adenomas) show inactivation of *hMLH1* or *hMSH2* protein with few of them retaining protein expression resembling the genetic imprint of their carcinomas. This implies homozygous or inactivating mutations in mismatch-repair genes occur at an early stage of HNPCC colorectal carcinogenesis.

Such data provide strong evidence for the premalignant nature of ACF in HNPCC. This might explain the low density of ACF and the rapid growth of premalignant lesions in these patients since the early loss of mismatch-repair genes allows ACF and adenomas to progress faster than sporadic colorectal carcinogenesis. On the other hand, 22% of ACF and no adenomas from patients with sporadic carcinoma showed instability (151, 152). Further research indicates right-sided colon carcinomas show higher rates of MSI than left-sided ones, although this does not seem the case for adenomas (6-153, 154). This data lends support to the understanding of the pathogenesis of CRC; however, loss of *hMLH1* protein expression in sporadic carcinomas could be caused by epigenetic mechanisms leading to gene inactivation (*i.e.* hypermethylation of the promoter) (150-154).

MSI is not associated with the histological features of ACF and is found in both dysplastic and in hyperplastic ACF from HNPCC patients. Although MSI does not possess these characteristics, it is associated with a defective mismatch repair system and underlies the initial steps in HNPCC colorectal carcinogenesis present in small ACF. In addition, MSI in sporadic ACF also seems independent of the size of the ACF (4, 6, 71, 80, 151-157).

6. Loss of heterozygosity (LOH) in aberrant crypt foci of human colon. LOH of microsatellite loci at 18q12, 18q21, 5q12, 5q21, 3p21, 2p16, 17q21, 17q11 and 11p13 was detected in various studies. ACF had lower rates of LOH than the counterpart carcinoma (41.18% *vs.* 68.57%). The profile of LOH rates at loci 18q12, 5q12, 3p21, 17q21, 17q11, 11p13 and 2p16 in ACF was similar to that in carcinoma. The LOH frequencies on 18q12, 18q21, 5q12, 5q21 and 3p21 were higher than that on 17q11 and 11p13. However, the rate at 18q21 and 5q21 in ACF was much lower than that in the carcinoma. The co-existing carcinomas displayed more polypoid growth patterns and were typically located at the sigmoid colon and rectum. LOH in carcinomas did not correlate with the location, size, or type of the carcinoma and did not show a relationship with the Duke's stage, indicating that ACF are putative preneoplastic lesions presumably representing the earliest morphological lesion with the alteration occurring at the molecular level (158).

While analysis of the ACF samples reported positive for *p53* mutation for LOH at the D18847 locus (a marker on chromosome 18q adjacent to DCC and DPC4), LOH could not be identified at that locus. This can be explained by the fact that LOH has often been associated with later stages of carcinogenesis (5, 83, 140-144). Furthermore, the

adenocarcinoma and adenoma may evolve from two different groups of ACF with respective genetic alterations.

Interestingly, fewer LOH in ACF were detected at D14s1006 and D14s1010 compared to carcinoma, but higher LOH existed at the same loci in adenoma when compared to ACF. This further supports the previous studies in that LOH is part of the genetic alteration evidenced in the pathogenesis of CRCs (Alrawi *et al.,* unpublished).

In conclusion, this data suggests and further supports the evidence previously reported by various authors for the existence of an ACF subset with higher multiplicity and a prominent genetic profile which are more likely to progress to advanced lesions. Efforts to localize these particularly aggressive foci should contribute to the colon cancer prevention strategy.

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