Perspective

Sporadic Aberrant Crypt Foci Are Not a Surrogate Endpoint for Colorectal Adenoma Prevention

Peter Lance[1] and Stanley R. Hamilton[2]

Although colorectal cancer (CRC) remains the second most frequent cause of cancer-related death in industrialized countries, reductions in the numbers of deaths from this cancer are responsible for reductions in the total numbers of U.S. cancer deaths for the past 2 years (1). To understand the earliest identifiable precursors to this malignancy has been a decades-long goal of research to prevent CRC. The venerable concept of the adenoma-carcinoma sequence (2, 3) proposes that almost all CRCs develop from adenomatous epithelium or polyps (adenomas), i.e., visible lesions that protrude above the surrounding mucosal surface and are characterized histopathologically by the presence of intraepithelial neoplasia (dysplasia). Proposed more than four decades ago, this concept has evolved to include flat (nonpolypoid) adenomas and likely will require additional modification to include some types of serrated polyps (called "traditional serrated adenoma" and "sessile serrated adenoma") that lack the usual adenomatous epithelium but are uncommon precursors of CRC (4). The adenoma-carcinoma concept is otherwise essentially unscathed after many years of intensive study, and removing adenomas is an important CRC prevention strategy. Aberrant crypt foci (ACF) were identified as a result of research to describe the earliest morphologic precursor lesions. Since the late 1980s (5, 6), much effort has gone into aligning knowledge of the molecular pathogenesis of colorectal carcinogenesis with the sequential morphologic changes that take normal-appearing epithelial cells through stages of premalignant neoplastic change to become invasive CRCs. The seminal report "Aberrant Crypt Foci in the Adenoma Prevention with Celecoxib (APC) Trial" of Cho et al. (7) in this issue of the journal provides an important opportunity for putting ACF research into a new perspective.

ACF were first described in 1987 as the earliest putative premalignant lesion in the murine colon (8). Rodents do not spontaneously develop CRC, but progressive neoplastic changes take place in their colonic mucosa following repeated administration of azoxymethane and other carcinogens. ACF were identified at a magnification of ×40 in the resected, unsectioned, formalin-fixed, methylene blue–stained colons of azoxymethane-treated mice (8, 9). The majority of ACF were in the distal colon and easily recognizable by their increased size, pericryptal area, and thicker epithelial lining.

Some ACF showed unequivocal intraepithelial neoplasia/dysplasia (9).

Over the next 10 years, investigators looked for ACF in humans, first in formalin-fixed specimens, next in *ex vivo* fresh tissue specimens, and then *in vivo* in patients undergoing colonoscopy (Table 1). The first-identified ACF (in formalin-fixed specimens from carcinogen-treated mice) differ substantially from the ACF identified later *in vivo* in humans. ACF were first reported by investigators conducting macroscopic studies of the human colorectum in 1991 (10), although adenomatous change that would now be called dysplastic ACF was described in the 1970s by *en face* histopathologic examination of nonpolypoid colorectal mucosa of familial adenomatous polyposis (FAP) patients (11, 12). Grossly normal-appearing colonic mucosa was obtained from various sources: a consecutive series of surgically resected CRCs; patients who underwent colonic resection for nonneoplastic conditions; and autopsies of patients without CRC. Fresh mucosal strips were peeled from the submucosa, fixed in paraformaldehyde, stained with methylene blue, and examined at a magnification of ×40 or ×60. ACF in this series (10) varied from single altered glands to plaques of >30 crypts. The number of ACF per square centimeter was higher in patients with CRC than in patients without CRC or its predisposing conditions, but at least one ACF was found in each left colon of the 13 autopsy patients without CRC. ACF histology was not examined in this study.

ACF frequency and histology were investigated in a series of 27 patients with autosomal dominantly inherited FAP (5 cases), sporadic CRC (12 cases), and benign colonic disease (10 cases) undergoing colonic resection (13). ACF were identified in all FAP and sporadic CRC patients and in 6 of the 10 patients with benign colonic disease. After scoring for ACF frequency, 4-mm^2 areas of colon (55 specimens) were excised and embedded in paraffin for preparation of 5-μm sections. ACF frequency per square centimeter was significantly higher in colons from FAP patients (20 ± 19) compared with that in patients with sporadic CRC (0.37 ± 0.41) or benign disease (0.18 ± 0.35). Twenty-six of the ACF found in the 55 histologically examined specimens displayed low-grade or high-grade dysplasia and were termed "microadenomas." Whether ACF could be identified in fresh tissue was not known at that time.

To answer this question, investigators scored both fresh (unfixed) and fixed flat normal-appearing colonic mucosae for ACF under a dissecting microscope after methylene blue staining in a series of 10 colonic resections for CRC (14). The number of ACF per square centimeter and the average number of crypts per foci correlated highly in unfixed (fresh) and fixed mucosa. The topographical feature of a slit-like lumen in the crypts of ACF on the mucosal surface correlated with the presence of histopathologic dysplasia.

By the early 1990s, increasing numbers of investigators from various backgrounds were focusing their attention on ACF.

Table 1. ACF: chronology of when first identified according to source and methodology

Year	Source	Methodology
1987, Bird (8)	Azoxymethane-treated C57BL/6J mice *ex vivo*	Formalin-fixed whole colon Methylene blue stained, ×40
1991, Pretlow et al. (10)	Human colons resected for colorectal cancer Normal-appearing segments *ex vivo*	Paraformaldehyde-fixed whole-mount preparations Methylene blue stained, ×40
1991, Roncucci et al. (13)	Human colons resected for colorectal cancer Normal-appearing segments *ex vivo*	Unfixed, fresh colon Methylene blue stained, ×20
1998, Takayama et al. (16)	Human subjects undergoing colonoscopy *in vivo*	Magnifying chromoendoscopy Methylene blue spray, ×40

Basic scientists saw ACF as an attractive source of tissue in which to explore the early pathobiology of colorectal carcinogenesis. Experts in cancer chemoprevention saw ACF as a potentially attractive surrogate intermediate endpoint for assessing the efficacy of novel chemopreventive agents and strategies. Experts in risk assessment saw ACF as a potential tool for identifying subjects at increased risk for CRC. It was clear that the ability to identify, biopsy, and follow ACF over time *in vivo* would further energize this diverse field. In 1998, a group of Japanese investigators did just this. They used magnification chromoendoscopy (which is endoscopy supplemented with the application of methylene blue to the mucosal surface; ref. 15) to study ACF in 171 normal subjects, 131 patients with colorectal adenomas, and 48 patients with CRC (16). A total of 3,155 ACF were identified, of which 161 (5%) were dysplastic. There were significant correlations between the number of ACF, the presence of dysplastic ACF, ACF size, and the number of adenomas, as well as the provocative findings of a reduction in ACF in a substudy of the effects of a nonsteroidal anti-inflammatory drug that are discussed later. Stimulated by this Japanese study, investigations of ACF and their potential use as a surrogate endpoint of clinical prevention trials proceeded rapidly (17–20).

There is space here to mention only a selected few of the numerous ACF-related publications of the last decade. These published studies can be arranged in three groups: mechanistic studies in ACF tissue of the molecular pathogenesis of colorectal neoplasia; investigations relating ACF findings to CRC risk; and preclinical and clinical intervention studies of the effects of dietary and chemopreventive agents on ACF.

Mechanistic ACF research has shown that activation of the APC/β-catenin pathway plays an important role in colorectal tumorigenesis occurring through chromosomal instability with losses and gains of chromosomal regions. Cytoplasmic and nuclear expression of β-catenin is absent in normal colonic cells but is common in adenomas and CRCs, providing evidence of chromosomal instability. Cytoplasmic expression of β-catenin was reported in 54% of dysplastic, paraffin-embedded ACF from surgical specimens and in 80% of CRCs in the same series (21). Loss of heterozygosity of tumor suppressor gene loci is seen in most chromosomal instability–associated CRCs. In a series of 32 paraffin-embedded ACF from surgical specimens, loss of heterozygosity was shown in 5 (22), but loss of heterozygosity did not correlate with the histopathology of the ACF.

Just as evidence of chromosomal instability–mediated mechanisms common in adenomas has been shown in some ACF (discussed above), evidence of mechanisms mediated by microsatellite instability has also been shown in ACF. Microsatellite instability lacks the structural chromosomal changes of chromosomal instability but induces extensive nucleotide mutations. Precursor lesions for colonic adenocarcinomas characterized by high levels of microsatellite instability include sessile serrated adenomas (4, 23). This finding contributes to the earlier comment that the adenoma-carcinoma sequence will likely need to be modified by the addition of some types of serrated polyps as precursors of CRC (4). Microsatellite instability in ACF was first detected in concurrent ACF of CRC patients (24) and later in 24% of the ACF obtained by biopsy during chromoendoscopy in a series of 45 patients without CRC (25).

An inverse relationship exists at the molecular level between sessile serrated adenomas and conventional adenomatous polyps with respect to *BRAF* and *KRAS* mutations (26–28): *BRAF* mutations are strongly associated with serrated histology and are rare in nonserrated adenomas, whereas *KRAS* mutations are common in nonserrated adenomas (22) and are rare in serrated polyps. ACF also have a serrated variant, which is the predominant form of nondysplastic ACF. Serrated and nonserrated ACF have an inverse relationship with respect to BRAF and KRAS mutations that is similar to that between serrated and nonserrated adenomas (26, 29, 30). The definition of hyperplastic ACF in the study by Cho et al. (7) included serrated architecture, and >90% of the nondysplastic ACF in this study were serrated (shown in their Fig. 1).

Another inverse relationship at the molecular level, this time with respect to *KRAS* and *APC* mutations, has been observed between ACF from FAP patients and ACF from sporadic CRC or adenoma patients (31, 32). *KRAS* mutations are common in sporadic ACF but are rare in FAP-associated ACF, whereas *APC* mutations occur in 100% of FAP-associated ACF but are rare in sporadic ACF. These genes are often mutated in both sporadic and FAP-associated adenomas. It therefore has been suggested that the acquisition of an *APC* mutation in sporadic ACF and of a *KRAS* mutation in FAP-associated ACF may be the gateway for progression from ACF to adenoma in the respective phenotypes (32). Distinct gene methylation patterns also distinguish sporadic and FAP-associated ACF (33, 34).

Magnification chromoendoscopy allows the collection of sufficient ACF tissue for performing multiple analyses (e.g., example, histology, genotyping, real-time PCR, immunohistochemistry, and Western blotting; ref. 35). Studies using

this approach have shown that histologically dysplastic ACF were hyperproliferative and possessed significant increases in four epidermal growth factor receptor signaling components.

The extensive literature on the genomic and gene-product profiles of ACF indicates that many features of genomic instability associated with the early stages of colorectal carcinogenesis can be found in ACF, suggesting the hypothesis that selected ACF can be the precursors of colorectal adenomas and CRC. The literature on the clinical significance of ACF and their potential risk of progression is modest in comparison. A recent systematic review yielded only 13 studies that could be defined as "epidemiologic" in that some estimate of association between the presence of ACF and subject characteristics was provided (36). Results of studies in which ACF within a defined area of the rectum were counted lead to the mundane conclusions that persons with adenomas (37) or CRC (38) have more rectal ACF than persons without, and that older age is a risk factor for ACF growth (37). One study reported a higher mean number of ACF in subjects with a family history of CRC than in those without (39).

The literature on preclinical ACF interventions is extensive (40, 41). Many studies have investigated the ability of agents to reduce ACF number in carcinogen-induced rodent carcinogenesis models. These ACF are preponderantly dysplastic and may be useful for screening preventive agents. The relevance of preclinical ACF research to the clinical setting is undefined and under investigation. Highlighting the ongoing importance of understanding ACF, a recent U.S. Food and Drug Administration decision to approve an obesity drug despite data showing that it caused ACF in rodents sparked a lively debate on the appropriateness of the Food and Drug Administration action (42–44). If ACF were a precursor of colorectal adenomas or cancer, the rodent ACF model would be a good toxicity screen for new drugs; if ACF are not a precursor, causing ACF in rodents may not be clinically relevant.

The literature on clinical ACF interventions is scant and includes no reports prior to this issue of *Cancer Prevention Research* of prospective randomized controlled trials using ACF number or characteristics as an endpoint. A small unblinded observational substudy (16) found that ACF decreased in number after therapy with the nonsteroidal anti-inflammatory drug sulindac, disappearing in 7 of 11 patients. The data from this study do not differentiate between dysplastic and nondysplastic ACF. Another study determined ACF prevalence in normal-appearing mucosal samples from colorectal resection specimens obtained from patients undergoing surgery for sporadic CRC, some of whom reported long-term aspirin use (45). ACF number and density were significantly lower in the mucosae from the aspirin group compared with the mucosae from the non-aspirin group. Aspirin was associated with a statistically nonsignificant reduction in dysplastic ACF. However, because all patients in both groups had CRC, the lower ACF prevalence in the aspirin group had no clinical significance and raised the possibility that this might be a misleading endpoint for chemoprevention trials. A major drawback to the use of changes in ACF prevalence as an endpoint in clinical trials has been the wide variability in reported ACF prevalence (46). This prevalence issue probably results largely from variability in the criteria used for endoscopic identification and classification of ACF. Other drawbacks to the use of

ACF in clinical trials are technical challenges of applying this technology, the lack of magnifying endoscopes in most clinical endoscopy facilities, and the inability of most endoscopists to spend the additional time required for rigorous ACF assessment.

Can ACF be used as a reliable surrogate endpoint for CRC in chemoprevention trials? The article by Cho et al. in this issue of the journal (7) provides the first evidence from a prospective randomized controlled trial to help answer this important question. They report the results of a substudy to their previously reported Adenoma Prevention with Celecoxib (APC) trial, which was conducted in a total of 2,035 patients at 91 clinical sites (47). Adenoma recurrence was significantly reduced by the celecoxib intervention compared with placebo in the APC trial. In the substudy, a subset comprising 45 patients at five clinical sites randomized to placebo ($n = 17$), celecoxib (200 mg twice daily; $n = 15$), or celecoxib (400 mg twice daily; $n = 13$) underwent magnification chromoendoscopy to identify, count, and biopsy ACF within the rectum at baseline and after 8 to 12 months of treatment. A total of 665 ACF were identified in the 45 patients. Seventy of these ACF were examined histologically, none of which were dysplastic. The presence or number of the nondysplastic ACF did not correlate with a higher risk of synchronous advanced adenomas (diameter ≥ 1 cm, or adenomas of any size with villous histology or high-grade dysplasia). The overall results indicated that nondysplastic ACF were not an accurate surrogate endpoint biomarker of recurrent colorectal adenomas in the APC trial. How does this report of Cho et al. affect the status of ACF?

The unequivocal answer is that the use of ACF cannot be justified as a surrogate endpoint in CRC chemoprevention trials. We arrive at this conclusion from our diverse perspectives of gastroenterology and clinical chemoprevention and of gastrointestinal and molecular pathology. The finding of a complete absence of dysplastic ACF in the 70 histologically examined rectal ACF and its implications for the overall APC clinical trial population are of striking importance, presaged by a molecular pathology study reported more than a decade ago (48). Also finding no evidence of dysplasia in human sporadic ACF, this study led to the conclusions that dysplastic ACF are extremely rare and that hyperplastic ACF were different from hyperplastic polyps and fundamentally different in biology from FAP-associated ACF and, therefore, were rarely an intermediate step within sporadic colorectal carcinogenesis. Cho et al. also found no relationship between nondysplastic ACF and hyperplastic polyps, and the relationship of this category of ACF to the various histopathologic types of serrated polyps remains uncertain. A previous clinical study in Japanese patients apparently similar to APC trial patients found only a 5% minority of dysplastic ACF among a total of >3,000 ACF (16). In contrast to FAP-associated ACF, sporadic dysplastic ACF seem to be extremely rare in U.S. patients, illustrated by a study in which only 1 of 104 biopsied ACF had dysplasia, which was low grade (37). Therefore, it is not anomalous that Cho et al. found no dysplastic ACF in their sample of 70 ACF, which seems to be a fair reflection of a very small prevalence of dysplasia among the sporadic ACF of the APC trial.

The higher prevalence of dysplastic ACF among Japanese patients could be due to a larger sample size or differences

in other factors such as ethnicity, diet, environmental exposures, imaging methods, or histopathologic interpretation. Even if dysplastic ACF occur in as many as 5% of patients at risk of sporadic CRC, this prevalence is likely too low to be useful for assessing outcome in a sporadic CRC prevention clinical trial, regardless of the possibility that certain drugs may modulate dysplastic ACF correlatively with clinical outcome. Such modulation has not been shown to occur, however, even in FAP. The scarcity of dysplastic sporadic ACF *in vivo* in humans may surprise many people because (we believe) of the complementary indications of the Japanese study (5% dysplasia) and the high frequency of dysplastic ACF reported in rodent carcinogen-induced tumorigenesis models. Although it is conceivable that future research might identify a patient population, excluding FAP patients, with dysplastic ACF or ACF with other characteristics denoting increased risk for CRC, this scenario seems unlikely. In addition, the discordance between the relatively high prevalence of sporadic colorectal adenomas and the low frequency of dysplastic ACF in patient populations is unexplained. It seems that adenomatous polyps are the colorectal equivalent of pigeons: the babies are very rarely seen (49).

Cho et al. (7) found no differences between nondysplastic ACF and adjacent normal mucosa with respect to cyclooxygenase-2 expression, microvessel density, nuclear localization of β-catenin, or expressions of the tumor suppressors SMAD4, estrogen receptor α, or O^6-methylguanine-DNA methyltransferase. There was, however, a highly significant increase in Ki67 proliferative index in the nondysplastic ACF versus normal mucosa. Although most of the data from Cho et al. and others suggest that nondysplastic ACF are not an adenoma precursor, these lesions could be a marker of global epigenetic changes that promote tumor formation (50). Cho et al. found in ACF that reduced baseline nuclear SMAD4 expression was associated with reduced risk of metachronous colorectal adenoma recurrence. Transforming growth factor-β signaling requires nuclear expression of SMAD4, which is a tumor suppressor gene. SMAD4 expression occurs in zones of terminal differentiation in colorectal crypt epithelium (51); therefore, reduced nuclear SMAD4 expression in ACF, which also have increased proliferation by immunohistochemistry for Ki67, is not entirely unexpected although the ACF are not dysplastic. On the other hand, loss of SMAD4 expression is a common feature of colorectal cancer epithelium (52, 53) and a relatively late event in tumorigenesis (54). A higher frequency of subsequent adenomas would *a priori* have been more expected rather than the lower frequency that Cho et al. found. Additional molecular genetic and epigenetic studies of the relationship between the molecular changes that characterize colorectal mucosal field defects and the early lesions including ACF that develop within the field changes are warranted.

The substudy by Cho and colleagues is a complex, rigorously conducted translational research within the major, large-scale randomized controlled APC trial. The clinical, molecular, and methodologic effort documented in this report rivals or surpasses any other correlative analyses (we know of) in labor intensiveness, expertise, and seminal importance. The seemingly inescapable conclusion that we must discard rectal ACF as a surrogate endpoint for CRC chemoprevention trials need not preclude studies using ACF tissue to address mechanistic and other questions of relevance to colorectal carcinogenesis and its therapy and prevention. Although questions remain about what sporadic ACF are, this study has told us a great deal about what they are not.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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