

Proximal Aberrant Crypt Foci Associate with Synchronous Neoplasia and Are Primed for Neoplastic Progression

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Abstract

Aberrant crypt foci (ACF) are the earliest morphologically identifiable lesion found within the human colon. Despite their relatively high frequency in the distal colon, few studies have examined the molecular characteristics of ACF within the proximal colon. In the following study, clinical participants ($n = 184$) were screened for ACF using high-definition chromoendoscopy with contrast dye-spray. Following pathologic confirmation, ACF biopsies were subjected to laser capture microdissection (LCM), and epithelial cells were evaluated for somatic mutations with a customized colorectal cancer mutation panel using DNA-mass spectrometry. Samples were further characterized for microsatellite instability (MSI). Logistic models were used to associate proximal ACF with synchronous (detected during the same procedure) neoplasia. Thirty-nine percent of participants had at least one histologically confirmed proximal ACF. Individuals with a proximal ACF were significantly more likely to present with a synchronous neoplasm ($P = 0.001$), and specifically, a proximal, tubular, or tubulovillous adenoma

(multivariable OR = 2.69; 95% confidence interval, 1.12–6.47; $P = 0.027$). Proximal ACF were more likely to be dysplastic (52%) compared with distal ACF (13%; $P < 0.0001$). Somatic mutations to *APC*, *BRAF*, *KRAS*, *NRAS*, and *ERBB2* were detected in 37% of proximal ACF. Hyperplastic ACF were more often MSI-high, but there were no differences in MSI status observed by colonic location. In summary, ACF are identified in the proximal colons of approximately 40% of individuals undergoing chromoendoscopy and more often in patients with synchronous proximal adenomas.

Implications: This study provides the most complete set of data, to date, that ACF represent the earliest step in the adenoma–carcinoma sequence but remain below the detection limit of conventional endoscopy.

Visual Overview: <http://mcr.aacrjournals.org/content/molcanres/16/3/486/F1.large.jpg>. *Mol Cancer Res*; 16(3): 486–95. ©2017 AACR.

Introduction

The widespread application of screening colonoscopies, along with the identification and removal of precancerous lesions, has led to a significant overall reduction in colorectal cancer incidence in the United States (1–3). Despite the health benefits afforded by regular screening visits, several studies have raised

the possibility that colonoscopies may fail to prevent the occurrence of proximal colorectal cancers (4). Clinically, these limitations are underscored by patients who develop metachronous, or "interval" advanced adenomas or colorectal cancers between screening and subsequent surveillance visits. Overall, the majority of these cases have been attributed to undetected or incompletely resected lesions that were present during the index colonoscopy (5–8). Thus, there is a need to innovate beyond conventional endoscopic approaches to enable a more complete identification and removal of proximal colon lesions and to identify those individuals at increased risk for recurrent neoplasms at the time of index colonoscopy.

Aberrant crypt foci (ACF) are the earliest morphologically identifiable lesion found in the human colon. ACF are not routinely detected during conventional endoscopy due to their diminutive size (<5 mm in diameter; refs. 9–13). Despite clear evidence that ACF frequency is directly associated with the presence of recurrent adenomas, their role in the adenoma–carcinoma sequence is still debated (14–17). Most clinical studies that use ACF as a surrogate marker for colorectal cancer risk have limited their analyses to endoscopic identification within the distal colon, failing to provide a more complete histologic or molecular characterization to understand their neoplastic potential (18, 19). We have previously demonstrated the usefulness of high-definition chromoendoscopy to identify colonic ACF within the proximal colon and validated a high-throughput DNA-mass

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spectrometry (DNA-MS) platform to detect somatic mutations to proto-oncogenes and tumor suppressors within microdissected epithelial cells (20). However, to firmly establish the premalignant potential of proximal ACF and their potential application to risk prediction, it is necessary to establish their association with known colorectal cancer risk factors and to better understand their molecular defects.

Here, we describe the results of the largest clinical study to date to prospectively screen an endoscopy population for the presence of ACF. We hypothesize that proximal ACF may be associated with other aspects of colorectal cancer risk and share molecular features with more advanced neoplasia. First, we examined the association of proximal ACF with the presence of synchronous neoplasia while adjusting for multiple possible confounders. Next, we characterized the clinical and molecular features associated with proximal ACF by examining their histologic changes and microsatellite instability (MSI). Finally, using our previously established approaches (20), we designed a customized, multiplexed DNA-MS panel to examine the presence of 112 somatic mutations within 12 colorectal cancer-associated genes in microdissected proximal ACF. This study provides the most complete evidence to date that ACF represent an early step in the adenoma–carcinoma sequence.

Materials and Methods

Study population

Eligible healthy adults (18 years old or older) that were referred to the Division of Gastroenterology at John Dempsey Hospital/UConn Health (Farmington, CT) for screening or surveillance colonoscopy were approached and recruited to the study by study physicians during their initial office consultation. At this visit, interested participants provided written informed consent. Individuals who met the Amsterdam criteria for familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer or with a history of colorectal cancer were excluded. The study was approved by the Institutional Review Board of UConn Health (IRB#IE-10-068-OSJ) and conducted in accordance with the ethical principles of autonomy, beneficence, and justice as set forth in the Belmont Report and in compliance with internal policies, and Federal Regulations.

A total of 184 participants were enrolled on the clinical study to undergo high-definition chromoendoscopy and ACF screening between July 2010 and March 2014. From the population of 184 participants, 14 participants were excluded from the analysis for one of the following reasons: study withdrawal ($n = 5$), no dye-spray applied to the proximal colon ($n = 2$), failure to obtain biopsy specimens ($n = 5$), and missing medical history data ($n = 2$). The final study population was comprised of 170 participants.

Lifestyle and environmental factor collection

Prior to colonoscopy, all participants completed a study questionnaire. The questionnaire included information on smoking, current medication and supplement use, previous history of endoscopy, and family history of cancer. Body mass index (BMI, kg/m^2) was calculated from weight (kg) and height (m) measurements obtained during the initial office consultation.

Endoscopic procedure

One of three board-certified gastroenterologists performed the high-definition chromoendoscopy as described previously (20). Briefly, the proximal (cecum and ascending colon, including the

hepatic flexure) and distal 20 cm of the colorectum were sprayed with 1% indigo carmine solution administered through a spray catheter. Importantly, the number of ACF and polyps, location (cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure, descending colon, sigmoid colon, and rectum), and size of each lesion (diameter in mm or cm) were recorded by study staff during the procedure. Polyps were confirmed using the standardized endoscopy report (completed by the study endoscopists immediately following the procedure) and included within the electronic medical record.

ACF visualization, counts, and biopsy

ACF were identified from grossly normal-appearing colonic mucosa and counted by two independent observers during the endoscopy as described previously (20). Up to 10 ACF per subject were biopsied using standard Captura biopsy forceps with a central spike (Cook Medical). Colonic location, diameter of the lesion, and endoscopic appearance were recorded. When more than 10 ACF were identified in a single colon, all proximal ACF were biopsied, and the remaining biopsies (no greater than 10) were taken from randomly selected distal ACF. A normal biopsy specimen from both the proximal and distal colon was also collected. Biopsy specimens were immediately embedded in OCT media, flash frozen, and stored at -80°C .

ACF and polyp histopathology

A systematic pathologic analysis of ACF and normal biopsy specimens was performed by a gastrointestinal pathologist blinded to the endoscopic findings and a skilled technician, as described previously (20). A subset of ACF ($n = 96$) was analyzed by a second gastrointestinal pathologist with good interrater agreement across the four possible pathologies [77.1% agreement, $\kappa = 0.689$ (S.E. = 0.055; 95% confidence interval (CI), 0.58–0.80)]. Blinded biopsies were confirmed to be either dysplastic or hyperplastic ACF or normal mucosa. Hyperplastic ACF were subclassified into serrated and distended (nonserrated) pathologies. False positives were considered biopsy specimens with "pseudo" hyperplasia- or dysplasia-like pathologic appearance of colonic crypts not representative of a classic ACF due to the presence of a lymphoid nodule (21, 22). Approximately, 18% of all biopsy specimens were considered false positives by these criteria and were not considered histologically confirmed ACF.

Polyps detected during the procedure were confirmed via routine pathology reports generated by the Department of Pathology at John Dempsey Hospital (Farmington, CT). Data were extracted including location and diagnosis (tubular adenoma, tubulovillous adenoma, villous adenoma, carcinoma *in situ*, hyperplastic polyp, sessile serrated adenoma, traditional serrated adenoma). Polyp histopathologic diagnoses were largely based on current World Health Organization criteria (23). No villous adenomas, carcinoma *in situ*, or traditional serrated adenomas were detected in the study population. To account for the evolving pathologic guidelines with respect to sessile serrated adenomas that occurred during the study period (24), hyperplastic polyps and sessile serrated adenomas were grouped together and termed "serrated polyps," as we have described previously (25).

Laser capture microdissection, DNA purification, and DNA-MS profiling

Frozen serial sections of colon biopsies were prepared on Arcturus PEN membrane glass slides and prepared for laser

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capture microdissection and DNA-MS profiling (LCM; Applied Biosystems) as described previously (20). Briefly, approximately 3–5,000 cells (the equivalent of 1 mm² of collected tissue area) from morphologically distinct aberrant crypt populations were microdissected using a Veritas LCM instrument. DNA was extracted and purified from microdissected tissue as described previously (20). DNA-MS mutation profiling was performed at the Yale Center for Genome Analysis using the MASSArray platform (Sequenom, Inc.). The MASSArray platform uses matrix-assisted laser desorption ionization time-of-flight mass spectrometry to detect single-base mutations with increased sensitivity (26). A customized panel of 112 hotspot mutations present within 12 known colorectal cancer–related tumor suppressor and proto-oncogenes was created using data from our previous publication (20), pilot study data, and the COSMIC and TCGA databases for mutation frequencies for colorectal cancer. Genotype reports including mutant allele frequency were generated using the TYPER 4.0 software package (Sequenom Inc.). All mutation calls were assessed for the following technical quality control metrics. Any assay within a sample showing poor primer extension (high unextended primer peak), poor allele signal (background peaks at higher intensity than expected signal peaks), or failed assays (evidence of poor-quality wild-type signal in HapMap control) were excluded.

Assessment of MSI

MSI analysis was performed using five mononucleotide repeat microsatellite targets (BAT-25, BAT-26, NR-21, NR-24, and NR-27) in a pentaplex PCR assay (see Supplementary Methods). Primer sequences have been described previously (27, 28). Samples that demonstrated no instability in any loci were considered microsatellite stable (MSS), instability in one of five loci was considered to have an MSI-low phenotype (MSI-low), and instability in two or more loci was considered to have an MSI high phenotype (MSI-high). For group comparisons, MSS- and MSI-low were grouped together, as there are no established clinical, survival, or prognostic differences between MSI-low and MSS colorectal cancer (29–31).

Statistical analysis

Student *t* tests for continuous variables and χ^2 tests for categorical variables were used to detect significant differences ($P < 0.05$) between individuals with and without proximal ACF. For ACF histology by colonic location and MSI status by ACF histology analyses, Fisher exact test was used to assess significant differences ($P < 0.05$) in the 2×2 distributions. For DNA-MS-based mutation calling, following quality control filtering, samples were considered positive for the mutation if the mutant call had a Z-score higher than the significance threshold after correcting for multiple hypothesis testing. Following conservative Bonferroni correction based on the number of ACF samples ($n = 43$) and mutations assayed ($n = 112$), positive mutation calls were those with a Z-score greater than 4.4 [corresponding $P < 0.00001$; $(0.05)/(43 \times 112) = 0.00001$].

To test our primary hypothesis, sex and age-adjusted logistic regression modeling was used to identify the association of proximal ACF and risk of synchronous polyps and their subtypes. To further adjust for possible confounding, several covariates were identified *a priori* based on their prior association with colorectal neoplasia for use in multivariable logistic regression models: BMI (<25 vs. 25–30 vs. ≥ 30 kg/m²), smoking status (current vs. former

vs. ever), endoscopic screening history (yes/no), regular aspirin use (at least once/week), and family history of cancer in a first- or second-degree relative (yes/no). Pairwise comparisons between individuals with and without a histologically confirmed proximal ACF were performed to identify additional potential confounding variables. Distal ACF endoscopic count (continuous) and regular multivitamin use (at least once/week) significantly differed between groups and were included as covariates. In the secondary analyses of colonic location and lesion pathology, we also included a covariate for presence of the lesion in the opposite location or pathologic subtype to measure the association of proximal ACF with the specific lesion of interest in patients with multiple polyp subtypes. All logistic regression analyses were performed using SPSS 21 (IBM). All statistical tests were two sided.

Results

The characteristics of the study population are shown in Table 1. The mean (\pm SD) age of the 170 participants was 56.6 (± 7.9 years). The majority of the population was Caucasian (82%) and male (59%). A total of 3,137 ACF were counted during endoscopy throughout the distal and proximal colorectum (see Supplementary Fig. S1).

From these participants, we collected 757 ACF biopsies that were subjected to histologic confirmation (see Supplementary Table S1). Overall, 66.1% (95% CI, 62.5–69.4) of biopsied ACF were confirmed to have underlying histologic alterations. Subsequent pathologic confirmation did not differ according to colonic location. Although the endoscopic appearance and subsequent biopsy of an ACF was highly sensitive (95.4%), it was not highly specific (49.1%). Therefore, in subsequent analyses, we have only considered histologically confirmed ACF to specifically capture the risks associated with the detection of hyperplasia or dysplasia. Overall, those participants with a confirmed proximal ACF ($n = 67$) were similar to those without a proximal ACF ($n = 103$); however, individuals with a proximal ACF were less likely to use multivitamins and had higher distal ACF and synchronous polyp counts (Table 1; see Supplementary Figs. S1 and S2).

Proximal ACF are associated with synchronous neoplasia

To stringently test the observation that study participants with a proximal ACF had significantly higher mean polyp counts than those without a proximal ACF ($P < 0.001$; Table 1; see Supplementary Fig. S2), we performed logistic regression modeling adjusting first for sex and age, and second for multiple potential confounders. Following multivariable adjustment, those individuals with a proximal ACF were significantly more likely to also harbor a synchronous polyp (multivariable OR = 3.28; 95% CI, 1.62–6.64; $P = 0.001$; Table 2). We then performed polyp subtype [traditional dysplastic adenomas (tubular or tubulovillous adenoma) vs. serrated polyps (hyperplastic polyp or sessile serrated adenoma)] and colonic location (proximal vs. distal colon) secondary analyses. Polyp distributions according to location and pathology are presented in Supplementary Table S2. Significant associations were observed between the prevalence of proximal ACF and the prevalence of traditional adenomas (multivariable OR = 2.66; 95% CI, 1.32–5.36; $P = 0.006$) and proximal polyps (multivariable OR = 2.54; 95% CI, 1.25–5.19; $P = 0.010$). Finally, based on these findings, we examined the association of proximal ACF with proximal serrated or proximal traditional adenomas and found that the association for

Table 1. Demographic characteristics according to the presence of a histologically confirmed proximal ACF

	Overall (N = 170)	No proximal ACF (n = 103)	At least one proximal ACF (n = 67)
Age (y)	56.6 ± 7.9	56.2 ± 8.6	57.1 ± 6.7
Male	100 (58.8)	58 (56.3)	42 (62.7)
Caucasian	139 (81.8)	85 (82.5)	54 (80.6)
BMI (kg/m ²)			
Normal (<25)	39 (22.9)	25 (24.3)	14 (20.9)
Overweight (25-29.9)	59 (34.7)	40 (38.8)	19 (28.4)
Obese (≥30)	72 (42.4)	38 (36.9)	34 (50.7)
Smoking			
Current	21 (12.4)	12 (11.7)	9 (13.4)
Former	61 (35.9)	36 (35.0)	25 (37.3)
Never	88 (51.8)	55 (53.4)	33 (49.3)
History of screening colonoscopy (yes)	87 (51.2)	51 (49.5)	36 (53.7)
History of polyps	41 (24.8)	22 (22.0)	19 (29.2)
Regular any aspirin use	64 (37.6)	39 (37.9)	25 (37.3)
Daily baby aspirin (81 mg)	52 (30.6)	33 (32.0)	19 (28.4)
Daily regular strength (325 mg)	17 (10.0)	10 (9.7)	7 (10.4)
Regular other NSAIDs use	88 (52.1)	52 (51.0)	36 (53.7)
Regular multivitamin use	104 (61.2)	70 (68.0)	34 (50.7) ^a
Regular folic acid use	25 (14.7)	15 (14.6)	10 (14.9)
Regular calcium use	50 (29.4)	36 (35.0)	14 (20.9)
Regular vitamin D use	62 (36.0)	40 (38.8)	22 (32.8)
Family history of cancer	128 (80.5)	75 (79.8)	53 (80.3)
Number of synchronous polyps	1.1 ± 1.3	0.7 ± 1.1	1.6 ± 1.5 ^c
Endoscopic count of distal ACF	15.8 ± 15.1	13.1 ± 12.1	19.9 ± 18.1 ^b

NOTE: Values are means ± SD or n (%). Statistical comparisons are made using either Student *t* test for continuous variables or the χ^2 test for categorical variables. Synchronous polyps are defined as the number of polyps detected during the study procedure.

^a*P* < 0.05.

^b*P* < 0.01.

^c*P* < 0.0001.

synchronous neoplasia was specifically strongly associated with the presence of synchronous traditional adenomas (multivariable OR = 2.69; 95% CI, 1.12–6.47; *P* = 0.027).

Proximal ACF share multiple molecular and histopathologic characteristics with advanced neoplasia

We next characterized the molecular and histologic characteristics of the ACF biopsied during chromoendoscopy. A total of 500 ACF from the 757 biopsies collected were confirmed to have underlying morphologic changes representative of more advanced lesions. Overall, the majority of confirmed ACF biopsies were hyperplastic (79.4%). When stratified by colonic location, however, the frequency of dysplasia (52.2%) was significantly higher in proximally located ACF biopsies compared with distal ACF biopsies (13.5%; Fisher exact test, *P* < 0.0001; Fig. 1A). Within this population, dysplasia was seven times more likely to occur in proximally located ACF compared with those in the distal colorectum [OR (proximal, dysplastic) = 7.00; 95% CI, 4.25–11.5].

We also assessed MSI status among a subset of ACF with known histology from both the distal and proximal colon (*n* = 127). As shown in Fig. 1B, a significantly greater proportion of hyperplastic ACF (35%; Fisher exact test *P* = 0.038) was MSI-high compared with dysplastic ACF (13.5%). However, no significant differences were observed in the rates of MSI according to colonic location.

A total of 43 proximal ACF and 4 normal biopsy samples were evaluated for somatic mutations within 12 colorectal cancer-associated tumor suppressors and proto-oncogenes using our custom DNA-MS panel (Table 3). Overall, 17 somatic mutations were identified in a total of five different genes (Fig. 2). A greater proportion of dysplastic ACF (8/19; 42.1%) were positive for a

single mutation compared with hyperplastic ACF (8/24; 33.3%). No mutations were observed in normal biopsy specimens, nor in the HapMap genomic DNA control sample, suggesting specificity to the lesion. Furthermore, there were no mutations detected in *CDKN2A*, *EGFR*, *FLT3*, *HRAS*, *MET*, *PIK3CA*, and *TP53*.

Five dysplastic ACF were positive for nonsense point mutations to *APC*; these point mutations were limited to either *APC*^{R876*} (*n* = 3) or *APC*^{R1450*} (*n* = 2). One ACF was positive for a frameshift deletion of *APC*, resulting in a premature stop codon downstream of serine at position 1465. In addition, one dysplastic ACF was positive for a *BRAF*^{V600E} mutation and a second was positive for a *KRAS*^{G12D} mutation. Notably, no hyperplastic ACF harbored an *APC* mutation. However, eight of 24 hyperplastic ACF (33.1%) were positive for any mutation, and each of these mutations occurred in genes encoding proteins associated with the MAPK signaling pathway. Four ACF were positive for mutations within codon 12 of *KRAS*, including a G12D (*n* = 1) and G12V (*n* = 3) mutation. Two hyperplastic ACF were each positive for *BRAF*^{V600E} mutation. Finally, two additional hyperplastic ACF were positive for *NRAS* mutations, one with a missense mutation in codon 13 (G13D) and the other harboring a missense mutation in codon 12 (G12D). The latter ACF also carried an insertion of four amino acids (YVMA) within the *ERBB2* gene at position 776. In fact, this was the only ACF examined that had more than one mutation detected by the panel.

To gain further insight into the possible clinical significance of a proximal ACF harboring an oncogenic mutation, we performed an exploratory, stratified analysis within the subset of patients with available mutation status data. Although the sample size is somewhat limited (*n* = 35), 10 of 14 patients with a confirmed mutation had at least one synchronous polyp (71.4%), whereas only 13 of 21 patients without a confirmed ACF mutation

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Table 2. Association of proximal ACF with synchronous neoplasia in participants

Synchronous neoplasia	No proximal ACF (n = 103)	At least one proximal ACF (n = 67)	P
Any			
Number of cases (%)	44 (42.7)	49 (73.1)	
Sex and age-adjusted OR (95% CI)	1 (referent)	3.58 (1.84–7.00)	<0.001
Multivariable OR (95% CI) ^a	1 (referent)	3.28 (1.62–6.64)	0.001
Traditional adenoma			
Number of cases (%)	25 (24.3)	31 (46.3)	
Sex and age-adjusted OR (95% CI)	1 (referent)	2.67 (1.38–5.18)	0.004
Multivariable OR (95% CI) ^b	1 (referent)	2.66 (1.32–5.36)	0.006
Serrated polyp			
Number of cases (%)	22 (21.4)	24 (35.8)	
Sex and age adjusted OR (95% CI)	1 (referent)	1.26 (0.66–2.39)	0.480
Multivariable OR (95% CI) ^b	1 (referent)	1.62 (0.76–3.49)	0.214
Distal colon polyp			
Number of cases (%)	22 (21.4)	23 (34.3)	
Sex and age-adjusted OR (95% CI)	1 (referent)	1.93 (0.96–3.88)	0.063
Multivariable OR (95% CI) ^b	1 (referent)	1.50 (0.69–3.24)	0.305
Proximal colon polyp			
Number of cases (%)	29 (28.2)	35 (52.2)	
Sex and age-adjusted OR (95% CI)	1 (referent)	2.76 (1.44–5.29)	0.002
Multivariable OR (95% CI) ^b	1 (referent)	2.54 (1.25–5.19)	0.010
Proximal traditional adenoma			
Number of cases (%)	13 (12.6)	20 (29.9)	
Sex and age-adjusted OR (95% CI)	1 (referent)	3.02 (1.34–6.82)	0.008
Multivariable OR (95% CI) ^b	1 (referent)	2.69 (1.12–6.47)	0.027
Proximal serrated polyp			
Number of cases (%)	14 (13.6)	13 (19.4)	
Sex and age-adjusted OR (95% CI)	1 (referent)	1.53 (0.67–3.52)	0.314
Multivariable OR (95% CI) ^b	1 (referent)	1.69 (0.67–4.27)	0.265

^aLogistic regression models adjusted for age, sex, smoking status, BMI (<25 vs. 25–30 vs. ≥30 kg/m²), screening history, regular aspirin use (at least once/week), multivitamin use (at least once/week), and endoscopic counts of distal ACF.

^bLogistic models adjusted for the covariates denoted by (^a) and the presence of the other synchronous lesion subtype, e.g., when traditional adenoma is modeled as the outcome, presence of a serrated adenoma is included as a covariate and vice versa.

(61.9%) had a synchronous polyp. Among the former group, the median number of polyps was marginally higher than in the latter group (2 vs. 1 polyps/colon, respectively; $P = 0.19$), suggesting that proximal ACF with a somatic mutation may have some clinical relevance. No clear trend beyond what has already been described was observed between polyp location or subtype among those with proximal ACF harboring a cancer-related mutation (Supplementary Tables S3 and S4).

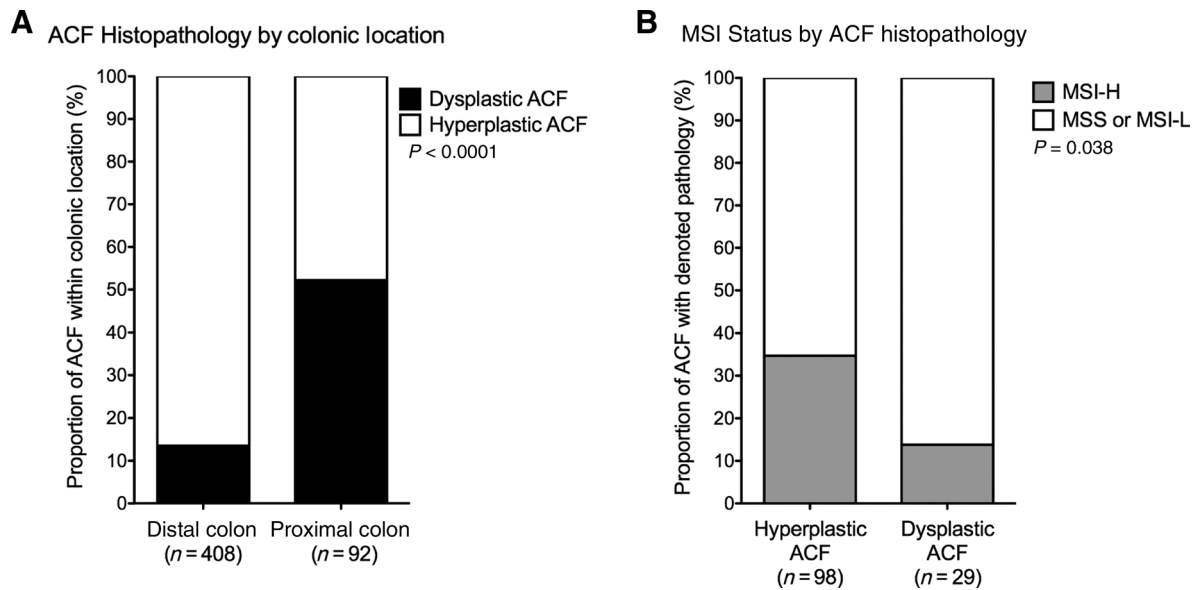
Synchronous polyp tissue was unavailable for sequencing analysis. However, 8 participants contributed multiple proximal ACF to the analysis. From this subset of patients, when a mutation was detected in one ACF, the same mutation was not observed in other proximal ACF, even in the cases where the synchronous ACF shared the same histology (Fig. 2; see patients C, E, I, K, N, P, T, and Y). Although limited in sample size, the mixture of ACF histologies across synchronous ACF and lack of shared mutations across multiple lesions suggests that these mutation events may be independent and lesion specific, but further investigation is required.

Discussion

In this clinical study designed to systematically evaluate human ACF within the proximal colon, we have identified several neoplastic characteristics of proximal ACF that support their role in colorectal carcinogenesis and their value as a surrogate marker of cancer risk. First, we have shown that individuals with a proximal ACF are more than three times as likely to harbor a synchronous

neoplasm regardless of the neoplasm's underlying pathology (traditional or serrated adenomas) or location (proximal or distal). Upon further analysis, proximal ACF are specifically associated with the presence of traditional adenomas in the right colon. Second, despite their diminutive size, the endoscopic appearance of an ACF is representative of abnormal mucosal pathology. Moreover, cytologic dysplasia, a hallmark of adenomas and more advanced neoplasia, is much more frequent in proximal ACF, whereas hyperplasia is more common in distal ACF. Third, although no differences were observed by colonic location, pathology-specific differences were established for MSI status, specifically, hyperplastic ACF were significantly more likely to be MSI-high compared with dysplastic ACF. Finally, using a customized somatic mutation screen on microdissected epithelial cells from proximal ACF, we were able to detect mutations within colorectal cancer-associated proto-oncogenes (*BRAF*, *KRAS*, *NRAS*, *ERBB2*) and tumor suppressors (*APC*) in 37% of the samples. Interestingly, mutations to *APC* seemed to be correlated with early dysplasia, while hyperplastic ACF typically harbored mutations in genes that are critical in MAPK signaling. Taken together, these findings support the preneoplastic nature of these diminutive lesions, placing them definitively within the context of neoplastic progression.

Although several previous endoscopic studies have described the presence of ACF in the proximal colon (20, 32, 33), the molecular and histopathologic features of proximal ACF have not yet been comprehensively defined. Consistent with our data, Shpitz and colleagues (1998) noted a higher prevalence of ACF in

**Figure 1.**

Histopathologic features and MSI of ACF. **A**, A total of 500 ACF were histologically evaluated (H&E stain) by a board-certified pathologist. In the distal colorectum, where ACF are more prevalent, a small proportion (14%) of ACF are dysplastic. In contrast, in the proximal colon, a significantly greater proportion of ACF are dysplastic (52%; Fisher exact test $P < 0.0001$). **B**, A subset of ACF were assessed for MSI status and stratified by histology. A significantly higher proportion of hyperplastic ACF (35%) are MSI-H compared with dysplastic ACF (13.8%; Fisher exact test $P = 0.038$).

the distal compared with proximal colon in a limited sample set obtained from surgical resections of colorectal cancer patients. We have recently reported a similar trend for endoscopic counts of ACF within our cancer-free population (20), findings that are consistent within the current larger study population.

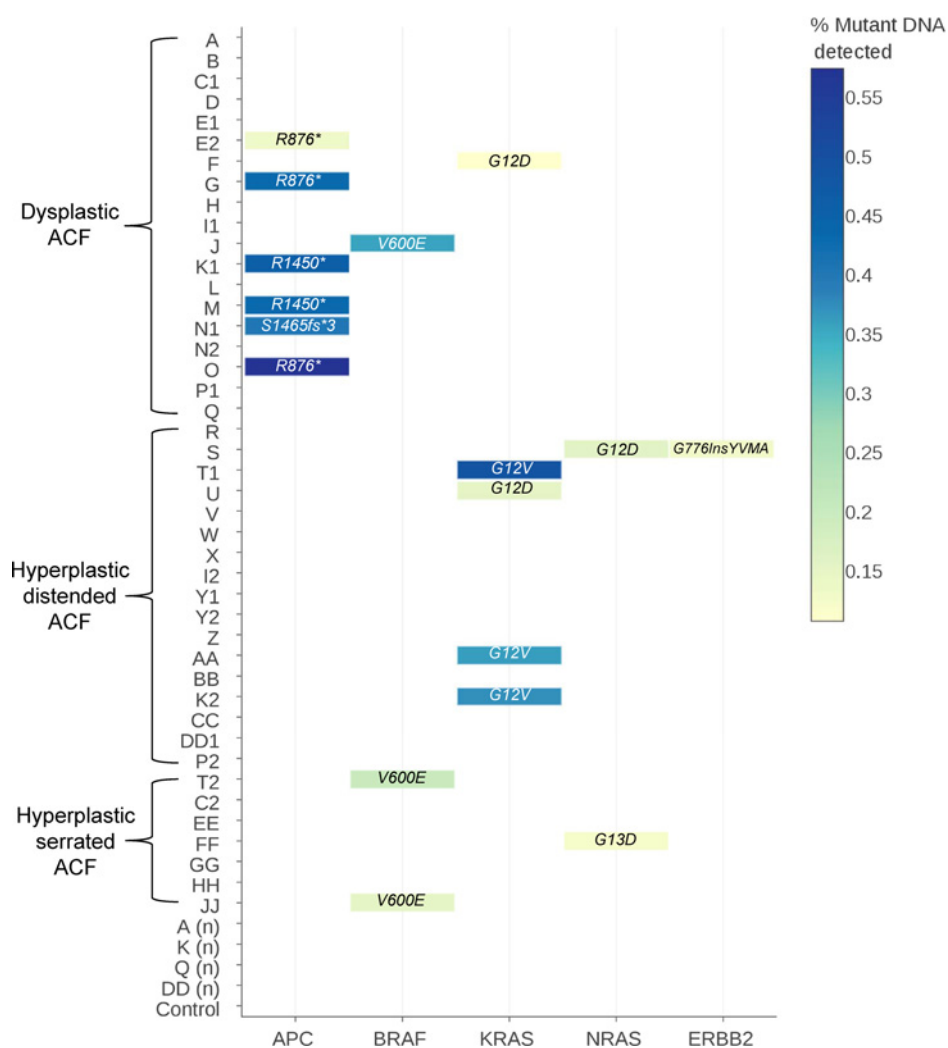
Some (14, 17, 34–38), but not all (14, 38), earlier studies found positive correlations between endoscopic counts of ACF and colorectal adenomas. These inconsistencies, especially in studies that have focused on distal ACF, may be explained in part by the limited histopathologic confirmation of ACF, the highly variable frequency of distal colon ACF in normal subjects, and interobservational variability between gastroenterologists (39). To our knowledge, our study is the largest to date that focuses specifically on the detection and characterization of proximal ACF and their potential association with synchronous adenomas.

More recently, a study by Inoue and colleagues (32) describes an association between proximal ACF and the prevalence of proximal serrated adenomas. Our results do not confirm these recent findings with respect to serrated adenomas, although the estimate appears suggestive of a possible association. Rather, we have identified a strong association between proximal ACF and traditional adenomas in the right colon. Moreover, we believe that our study design has several strengths that support the generalizability of our observations: (i) we recruited a large number of asymptomatic individuals undergoing routine screening or surveillance colonoscopy with unknown current polyp status and specifically excluded individuals with a known diagnosis of familial colorectal cancer syndromes. Our study was not specifically weighted toward individuals with a history of serrated adenomas, as in Inoue and colleagues (32), or those whose symptoms caused referral to the endoscopic practice; (ii) all

Table 3. Colorectal cancer-associated mutations included in the custom DNA-MS screen for microdissected ACF epithelium

Gene (number of mutations)	Mutation screened
<i>APC</i> (11)	E1306*, E1379*, N1661*, Q1338*, Q1367*, Q1378*, Q1429*, R1114*, R1450*, R876*, S1465*
<i>BRAF</i> (5)	D594G, V600E/G/L/M
<i>CDKN2A</i> (7)	D84Y, E61*, E69*, E88*, H83Y, R58*, R80*
<i>EGFR</i> (15)	G719SC, L858R, L861Q, E746delGGA, E746delAAT, E746delATT, L747delTTA, L747delTAA, S752IF, A750P, T751I, P753Q, T751PAS, R108K, T790M
<i>ERBB2</i> (8)	A775_G776insYVMA, D769H, G776SLC, G776VC, L755P, P780_Y781insGSP, S779_P780insVGS, V777L
<i>FLT3</i> (2)	D835del, D835HY
<i>HRAS</i> (5)	G12VD, G13CRA, Q61H/K/LRP
<i>KRAS</i> (11)	A59T, G12A/C/D/R/S/V, G13V/D, Q61H/LRP
<i>MET</i> (5)	M1268T, R988C, T1010I, Y1248C, Y1253D
<i>NRAS</i> (19)	A18T, G12V/A/D/C/R/S, G13V/A/D/C/R/S, Q61E/K/L/R/P/H
<i>PIK3CA</i> (14)	C420R, C901F, E542K, E545K, H1047R/L/Y, H701P, M1043I, N345K, P539R, Q546K, R38H, R88Q
<i>TP53</i> (9)	D281G/Y/H, G245SRC, R248GW, R273C/H/L, V143A

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**Figure 2.**

DNA-MS screen detects colorectal cancer (CRC)-associated mutations in microdissected epithelium from proximal ACF. Results appear by gene (*x*-axis; specific mutation appears in respective cells) and are grouped by histology (hyperplastic or dysplastic). Hyperplastic ACF are further separated into distended and serrated subtypes. Letters refer to the participant (A-JJ, $n = 35$). Some participants (e.g., participants C, E, I, N) contributed multiple ACF to the analysis. Numbers denote separate samples from the same participant [e.g., two ACF, a dysplastic (I1) and hyperplastic (I2), were included from participant "I"]. Heatmap depicts the mutant allele frequency (presented as the percentage of total area under the peaks measured for both wild-type and mutant allele spectra). Four normal biopsies, "(n)," from 4 participants and one HapMap control, "control," were also included. Although mutations for *CDKN2A*, *EGFR*, *FLT3*, *HRAS*, *MET*, *PIK3CA*, and *TP53* were included in the panel, no samples were positive for mutations in these genes, and they were excluded from the heatmap.

participants considered to have a proximal ACF in our analysis were independently confirmed by a pathologist blinded to endoscopic findings limiting false positives from relying solely on endoscopic detection; (iii) our prospective study design and data collection through administered questionnaires supplemented by the electronic medical record enabled us to adjust for several important potential confounders that were not included in the Inoue and colleagues' (32) study and that may have led to residual confounding.

Our results further suggest that at an early stage, ACF may acquire molecular and histologic changes representative of, and perhaps predicting their likelihood to develop into more advanced neoplasia. *APC* mutations were only observed in ACF with cytologic dysplasia, while mutations to MAPK signaling components, specifically, *BRAF*, *KRAS*, *NRAS*, and *ERBB2*, were observed within hyperplastic ACF. Similarly, our results suggest that MSI is specifically associated with hyperplastic ACF, but this association is not location dependent within the colon. These findings indicate significant overlap with two important signaling pathways that contribute to colon carcinogenesis, namely the traditional adenoma–carcinoma sequence as proposed by Vogelstein and characterized by *APC* mutations, aberrant

Wnt/ β -catenin signaling, and chromosomal instability (40–42) and the alternate serrated pathway characterized by aberrant MAPK signaling (*BRAF* mutations) and epigenomic deregulation (CIMP/MSI; refs. 43–46). Given their presumed early stage, the separation of molecular and histologic subtypes observed in ACF suggests that these pathways may diverge at the earliest stages of tumor initiation, but this requires further confirmation.

We acknowledge that our study has several limitations. First, although we have adjusted for multiple potential confounders, we cannot completely rule out the possibility of residual confounding. Second, we have used synchronous adenomas as a measure of individual risk, providing us with only a cross-sectional snapshot of individual risk. Eventually, long-term follow-up of our ACF study participants will provide valuable insight into the utility of proximal ACF as surrogate markers of colorectal cancer risk or adenoma recurrence, but these data are currently unavailable. Moreover, tissue from synchronous polyps was not routinely collected as a part of this study, which would provide further insight into the molecular relationship between ACF and developing neoplasms. Third, although we have designed our mutation screen to capture the most common somatic variants within colorectal cancer–associated oncogenes and tumor suppressor

genes, the entire mutational landscape of these lesions has not been examined. Thus, as only 37% of assayed ACF were found to harbor a mutation using our screen, we cannot rule out that other genes may play an important role in proximal ACF formation. Similarly, the potential role of epigenetic dysregulation as a key mechanism contributing to early stages of neoplastic transformation has recently been published by our group (47), but remains unmeasured in our current approach. However, we believe that our sensitive, multiplexed DNA-MS approach has allowed us to expand beyond routine Sanger sequencing of single mutational targets to provide the largest genetic study (in terms of number of mutations screened) of microdissected, histologically confirmed, human ACF to date.

Nonetheless, we believe that our results have significant clinical impact and can be extended to the general screening population. The associations reported here imply that individuals found to have a polyp and, specifically, a proximal tubular or tubulovillous adenoma, are more likely to have a proximal ACF. The detection of ACF within patients at increased risk for colorectal cancer (those found to have an adenoma or other advanced neoplasm; ref. 1), combined with the growing evidence that interval colorectal cancers are largely due to missed lesions during screening endoscopy (5–8), underscores the limitations of conventional endoscopy as a strategy for prevention of proximal colorectal cancer (4). The synchronous presence of neoplasia with ACF that have pre-neoplastic pathologic features and colorectal cancer-associated somatic mutations within the proximal colon further support the premalignant potential of a subset of ACF. Long-term follow-up of these patients will clarify the prognostic utility of proximal ACF.

Several possible interpretations of our findings exist. The association may be explained by the presence of a "field effect." This theory proposes that a "field" (e.g., the colonic mucosa) may be influenced by overlapping carcinogenic mechanisms, perhaps as a result of dietary, lifestyle, or environmental exposures that ultimately prime a region for the development of multiple neoplasms (48). Alternatively, synchronous polyps may "seed" an ACF, meaning that ACF are directly descended from nearby developing adenomas, although our results do not seem to appear to support this despite the absence of more detailed molecular data from the synchronous polyps. First, we observed a mixture of ACF and polyp pathologies within the same patient, even within the same segment of the colon. Although our study is the first to expand upon endoscopic identification and to confirm the presence of proximal ACF histologically, no clear correlation between specific ACF histologic subtypes and the presence of synchronous polyps has emerged. Our results support that histologic assessment is an important consideration for any ACF study, and future studies should be designed to simultaneously collect polyp tissue that will enable a direct molecular comparison. Second, we observed no evidence of common mutations that are shared across proximal ACF, even when similar histologically. In our previous study (20), we examined the mutation status of synchronous polyps present in a single patient. This patient, who was also included in the current analysis as patient "O," had a proximal dysplastic ACF that was found to be positive for an *APC*^{R876*} mutation in both studies. None of the three synchronous proximal tubular adenomas harbored the same somatic mutation. Although speculative, our results suggest that ACF may arise in a field that is prone to carcinogenic insult, perhaps as a result of some individual risk

factor, resulting in multiple independent or lesion-specific initiation events, rather than being the product of nearby adenomas or polyps. Future studies designed to examine these factors and how they influence the formation of proximal ACF and synchronous polyps may provide key insight into how certain exposures promote the earliest stages of neoplastic initiation.

In summary, we report that proximal ACF are present within approximately 40% of the general screening population, typically have acquired dysplastic features, and harbor significant molecular aberrations to *APC* and several MAPK effectors. Moreover, they are associated with synchronous neoplasia, most often proximal tubular and tubulovillous adenomas. We postulate that proximal ACF may contribute to the prevalence of metachronous neoplasia in those individuals with a history of adenomas, but prospective studies will be required to specifically assess this question. These studies further highlight the importance of a rigorous surveillance of the proximal colon in endoscopy-based prevention strategies and the need for implementation of additional strategies (e.g., chemoprevention, advanced endoscopic techniques, modified screening intervals) to more completely prevent neoplastic development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: T.J. Devers, D.W. Rosenberg

Other (statistical analysis): J.J. Grady

Other (contribution to pathologic analysis): F.A. Farouhar

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Proximal Aberrant Crypt Foci Associate with Synchronous Neoplasia and Are Primed for Neoplastic Progression

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