Epidermal Growth Factor Receptor Signaling Is Up-regulated in Human Colonic Aberrant Crypt Foci

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Abstract

Aberrant crypt foci (ACF) are collections of abnormal colonic crypts with heterogeneous molecular and pathologic characteristics. Large and dysplastic ACF are putative precursors of colon cancer with neoplastic risk related to increased proliferation. In this study, we examined the role of epidermal growth factor receptor (EGFR) signaling in regulating ACF proliferation. Using magnification chromoendoscopy, we collected large ACF with endoscopic features of dysplasia and separately biopsied adjacent mucosa. Transcript levels were measured by real-time PCR, proteins were assessed by Western blotting, and Ras was activated in 46% of ACF (3.2 ±fold; P < 0.05). At the RNA level, 38% of ACF were hyperproliferative, with proliferating cell nuclear antigen (PCNA) mRNA ≥2-fold of adjacent mucosa. Hyperproliferative ACF had significantly increased mRNA levels of EGFR (6.0 ±1.7-fold), transforming growth factor-α (14.4 ±5.0-fold), heparin-binding EGF-like growth factor (4.5 ±1.4-fold), cyclin D1 (4.6 ±0.7-fold), and cyclooxygenase-2 (COX-2; 9.3 ±4.2-fold; P < 0.05). At the protein level, 46% of ACF were hyperproliferative (PCNA, 3.2 ±1.2-fold). In hyperproliferative ACF, 44% possessed significant increases in four EGFR signaling components: EGFR (9.5 ±1.3-fold), phosphoactive ErbB2 (2.6 ±0.4-fold), phosphoactive extracellular signal-regulated kinase (3.7 ±1.1-fold), and cyclin D1 (3.4 ±0.8-fold; P < 0.05). Ras was activated in 46% of ACF (3.2 ±0.4-fold; P < 0.05), but K-ras mutations were present in only 7% of ACF. In contrast to COX-2 mRNA, the protein was not increased in hyperproliferative ACF. In summary, we have shown that ACF with up-regulated PCNA possess increased EGFR signaling components that likely contribute to the enhanced proliferative state of dysplastic-appearing ACF. (Cancer Res 2006; 66(11): 5656-64)

Introduction

Understanding events that drive growth in early colonic carcinogenesis is important, as premalignant lesions are more likely arrested or even reversed compared with invasive disease. Aberrant crypt foci (ACF) were first described as collections of colonic crypts with expanded pericryptal zones and increased methylene blue staining in colons of mice treated with a carcinogen (1). These lesions are monoclonal and are believed to be the earliest identifiable precursors of colon cancer in animal models and perhaps in human colon cancer (1–3). In the azoxymethane model of experimental colon cancer, most tumor-promoting and tumor-inhibiting agents cause parallel changes in the incidences of ACF and tumors (4). As in animal models, in humans there are significant correlations between the incidence and size of ACF and the risk of synchronous adenomas or carcinomas (5–7). The prevalence, number, and size of human ACF also increase with age. Furthermore, many of the molecular derangements described in colon cancers and ACF are similar, including K-ras, adenomatous polyposis coli, and β-catenin mutations and growth-promoting alterations in cell cycle–controlling genes (8–10). Thus, studies in humans and findings in experimental animal models strongly support the premalignant potential of ACF.

Otori et al. reported previously that 34% of large human ACF were dysplastic, with histologic features of microadenomas (11). The proliferative rates were increased in dysplastic ACF, supporting the significance of crypt cell hyperproliferation as a biomarker of ACF with greater neoplastic potential (11). Proliferating cell nuclear antigen (PCNA), an important enzyme in DNA replication and a surrogate marker of proliferation, was also increased in dysplastic ACF (12). Thus, increases in PCNA mark ACF thought to be at higher risk for malignant progression. Little is known, however, regarding the mechanisms driving hyperproliferation in human ACF.

The epidermal growth factor receptor (EGFR) plays important roles in regulating normal growth and contributes to malignant growth of several tumor types, including colon cancer (13). EGFR is a transmembrane receptor tyrosine kinase belonging to the ErbB family of proteins. EGFR (ErbB1) has an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity. EGFR signaling is activated by ligands that induce EGFR homodimerization or heterodimerization with other ErbB members (13). ErbB2, for example, is an important heterodimeric partner of EGFR that is up-regulated in some human and experimental colon cancers (14, 15). Ligand-activated EGFRs phosphorylate tyrosine residues in trans within the cytoplasmic domain of the receptor to activate Ras and other downstream effectors. Ras effectors include the extracellular...
signal-regulated kinases (ERK). ERKs, in turn, induce EGFR targets cyclin D1 and cyclooxygenase-2 (COX-2; refs. 16, 17). Cyclin D1 is a key cell cycle regulator and COX-2 is the rate-limiting enzyme in prostanoid biosynthesis (18, 19). Signals from these pathways control cellular proliferation and survival, and when deranged, can contribute to transformed features, including hyperproliferation and apoptotic resistance.

EGFR as well as receptor ligands, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin, are frequently overexpressed in colonic adenocarcinomas (20–23). In human colon cancer, overexpression of EGFR has been associated with increased cell proliferation and metastasis and possibly with decreased survival (24). ErbB2 is the preferred dimeric partner for EGFR and can be up-regulated and activated in human and experimental colon cancers (15, 25). Blockade of EGFR signaling by anti-EGFR antibodies or receptor tyrosine kinase antagonists have been shown to effectively inhibit growth of colon cancer cells in vitro and tumor xenografts in vivo (26). Recently, combination therapy with cetuximab (C225), a monoclonal anti-EGFR antibody, improved the clinical course of patients with advanced colorectal cancer (27).

Studies in animals also suggest that EGFR signaling is important in tumorigenesis. For example, inactivating EGFR mutations nearly abolished tumor growth in Min mice, a model of intestinal tumorigenesis (28). In preliminary studies, we have found that Iressa (gefitinib) inhibited EGFR activation, hyperproliferation, and ACF formation in azoxymethane-treated mice and blocked tumor development in azoxymethane-treated rats (29, 30). Taken together, elevations in EGF Rs and ligands in human colon cancers and inhibition of experimental tumorigenesis by EGFR blockade support our hypothesis that EGFR signaling plays an important role in colonic carcinogenesis. The goal of this study was to determine whether dysregulated EGFR signaling contributes to the hyperproliferative state of human ACF. We are unaware of any study that has quantified EGFR signaling events in individual human ACF. Insights into the signaling events contributing to ACF hyperproliferation would advance our understanding of the mechanisms promoting their growth.

Materials and Methods

Materials. For magnification chromoendoscopy, glycercin was purchased from Fisher Scientific (Pittsburgh, PA). Sterile methylene blue (1%) was supplied by Faulding Pharmaceuticals (Puerto Rico). For detection of K-ras codon 12 and 13 mutations, primers and probes were purchased from TIB Molbiol (Berlin, Germany). Genomic DNA was isolated with the DNA Clean and Concentrator kit (Zymo Research, Orange, CA). For RNA isolation and PCR, RNA later RNA storage solution and DNA-free DNase I kit were purchased from Ambion (Austin, TX). TRIZol RNA/DNA/protein isolation reagent was obtained from Life Technologies (Gaithersburg, MD). Ribogreen RNA quantitation reagent and kit were purchased from Molecular Probes (Eugene, OR). Custom PCR primers were ordered from Invitrogen Life Technologies (Carlsbad, CA). Taqman probes were supplied by Synthegen (Houston, TX). Other PCR reagents, including Moloney murine leukemia virus reverse transcriptase, random hexamers, and SYBR Green, were supplied by Applied Biosystems (Foster City, CA). Primer and probe sets were designed to amplify the relevant regions of the ras genes. Primer and probe sequences are available upon request.

PCR. PCR was carried out in a reaction containing 1× Taqman universal PCR master mix (Applied Biosystems, Foster City, CA), 0.2 μM of each primer, and 0.4 μM of each probe. The PCR cycling conditions were 1 cycle of 50°C for 2 minutes followed by 40 cycles of 95°C for 10 seconds, 60°C for 1 second, and 72°C for 1 second. The PCR products were run on a 2% agarose gel and visualized with ethidium bromide.

Western Blotting. Western blotting was performed on 30 µg of protein per lane using standard protocols. Primary antibodies were from DAKO Cytomation (Carpinteria, CA). For Western blotting, precast polyacrylamide gels and prestained molecular weight markers were purchased from Bio-Rad (Hercules, CA). Polyvinylidene difluoride membranes (Immobilon-P) were supplied by Millipore, Inc. (Bedford, MA). PCNA monoclonal antibodies (K110) were provided by Biomedra (Foster City, CA). Polyclonal anti-pan-EGFR antibodies were supplied by Upstate Biotechnology (Charlottesville, VA). Polyclonal antibodies to phospho-EGFR and phosphoactive ErbB2, cyclin D1, COX-1, and HuR and monoclonal antibodies to phosphotyrosine and COX-2 were provided by Cayman Chemical (Ann Arbor, MI). Polyclonal anti-CUGBP2 antibodies were from Alpha Diagnostic International (San Antonio, TX). Monoclonal anti-β-actin antibody was purchased from Sigma (St. Louis, MO). Enhanced chemiluminescence (ECL) Western blotting detection reagents and anti-mouse IgG and anti-rabbit IgG antibodies conjugated to HRP were obtained from Amersham Biosciences (Chicago, IL).

Patient selection. The study was approved by the University of Chicago Hospitals Institutional Review Board for Human Investigations. Formalin-fixed colons were obtained from surgical pathology under institutional review board–approved protocols. Patients included in the chromoendoscopy study had one of the following American Society of Gastrointestinal Endoscopy–approved indications for colonoscopy: (a) history of colonic polyps with advanced neoplasia (size >1 cm, villous features, high-grade dysplasia), (b) history of colon cancer, or (c) surveillance in high-risk patients with a family history of colorectal carcinoma or adenoma. Individuals excluded from the study included patients with a bleeding diathesis or patients unable to provide informed consent. Two patients were excluded for technical reasons.

Magnification chromoendoscopy. ACF were identified in situ using image magnification chromoendoscopy of the rectosigmoid colon as described previously (5). The distal 30 cm of the left colon was washed with 0.5% glycercin, stained with 0.2% methylene blue solution, and examined under magnification for ACF using an Olympus CF-Q160LZ. Magnifying video colonoscope (Olympus America, Melville, NY) at half-maximal magnification. Large ACF (>50 crypts per focus) that appear dysplastic (5, 31) were selected for biopsy. These surface features included ACF with elongated, compressed, or closed lumens. Colonic biopsies were immediately placed in RNAlater storage solution or flash frozen in liquid nitrogen. For Western blotting studies, biopsies were placed in 2× Laemmli buffer or flash frozen. Biopsy sites were reexamined to confirm accurate and complete removal of ACF. From each patient, we obtained several additional control biopsies of adjacent normal-appearing mucosa that were at least 5 cm from ACF. Biopsies were stored at –70°C until analyses. Differences in extraction procedures precluded simultaneous measurements of RNA and protein or microscopic examination of the same samples (see below).

Ras activation. Samples for Ras activation and K-ras and B-Raf mutations were extracted as described (15). The Ras activation state was assessed by the ratio Ras-GTP / (Ras-GTP + Ras-GDP). Briefly, Ras was immunoprecipitated from colon lysates under conditions to inhibit Ras GTPase activity. GDP and GTP bound to Ras were separated and converted to ATP using coupled reactions. ATP was quantified by a sensitive luciferin/luciferase system (15). Samples with Ras activation levels ≥2-fold above control were defined as activated.

K-ras mutations. Genomic DNA was extracted from colonic biopsies. PCR (40 cycles) was done using a pair of primers designed to amplify K-ras exon 1 containing codons 12 and 13. Amplification was done with 50 to 100 ng DNA in a 20 μL volume containing 2 μL of 10× LightCycler DNA Master hybridization probes, 1.6 μL of 25 mmol/L MgCl2, 40 pmol of each primer, and 2 pmol of each probe. The PCR cycling variables were 40 cycles of denaturation at 94°C for 1 second, annealing at 50°C to 54°C for 20 seconds, and extension at 72°C for 10 seconds. For detection of K-ras codon 12 and 13 mutations, a LightCycler PCR assay was done with fluorescence melting curve analysis (FMCA) of the PCR products as described previously (32). Two fluorescently labeled oligonucleotide probes were used that hybridized adjacent to one another, with one probe overlapping the mutation site. Postamplification FMCA was done by the ratio Ras-GTP / (Ras-GTP + Ras-GDP). Briefly, Ras was immunoprecipitated from colon lysates under conditions to inhibit Ras GTPase activity. GDP and GTP bound to Ras were separated and converted to ATP using coupled reactions. ATP was quantified by a sensitive luciferin/luciferase system (15). Samples with Ras activation levels ≥2-fold above control were defined as activated.
used as a negative control. DNA from cell lines with a codon 12 K-ras mutation (SW480 cells) or codon 13 K-ras mutation (HCT-116 cells) served as positive controls.

**B-Raf mutations.** Genotyping was done by real-time Taqman PCR using primers and probes designed, synthesized, and validated by the Applied Biosystems custom Genotyping Assay Service. Both wild-type and mutant B-Raf (V599E) were assayed in a single reaction. The master mix included a probe specific for the wild-type sequence and a probe specific for the mutant sequence as well as all reaction components, except AmpliTaq Gold polymerase, which was added at the end of assay. Each probe carried a nonfluorescent quencher and a minor-groove binder. The latter increased the specificity of probe binding. Reactions were done on a Stratagene (La Jolla, CA) Mx4000 real-time thermocycler with suitable optical filters. RKO cells that carry a B-Raf mutation at codon 599 and Caco-2 cells that are wild type for B-Raf were included as positive and negative controls, respectively.

**Real-time PCR.** Colonic biopsies were homogenized in 1 mL TRIzol reagent and total RNA was extracted according to the manufacturer’s protocol. RNA was dissolved in DEPC-treated water and genomic DNA was removed by DNase I using the DNA-free kit. RNA (1 µg) quantified using Ribogreen was reverse transcribed in a 20 µL reaction containing 5 mmol/L MgCl₂, 1 × PCR Buffer II, 4 mmol/L deoxynucleotide triphosphates (dTTP), 20 units RNase inhibitor, 2.5 µmol/L random hexamers, and 50 units Moloney murine leukemia virus reverse transcriptase. Samples were reverse transcribed in a PTC-100 thermal cycler at 42°C for 60 minutes and the temperature was increased to 95°C for 5 minutes to denature the reverse transcription. For real-time PCR, reactions contained 5 µL (1:5 dilution) cDNA, 1 × PCR Buffer II, 5.5 mmol/L MgCl₂, 200 µmol/L dNTPs, 200 µmol/L forward and reverse primers, 100 µmol/L Taqman probe (or 0.5 µL of 1:2,000 dilution of SYBR Green), 0.75 units HotStarTaq DNA polymerase, and water to adjust the final volume to 25 µL. The reactions were incubated at 95°C for 15 minutes to activate the DNA polymerase followed by 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. COX-1 levels were used to normalize for total RNA (33). In preliminary experiments, PCR products of the expected sizes were visualized on an agarose gel and their predicted levels in ACF and control biopsies were synthesized in parallel, along with negative controls, omitting reverse transcriptase. PCR reactions were run in triplicate, and mRNA levels were normalized to COX-1 mRNA abundance and expressed as fold change of control. (See Supplementary Table S1 for sequences of primers and Taqman probes).

**Immunohistochemical staining.** Surgically resected colon specimens were fixed flat in 10% buffered formalin. The mucosa was stained with 0.2% methylene blue and ACF were identified using a ×20 dissecting microscope and isolated by punch biopsy. Biopsies were paraffin embedded and sectioned along the vertical axis of the crypt. Briefly, for Ki-67 immunohistochemical staining, paraffin sections were deparaffinized in xylenes, rehydrated through graded ethanol solutions to distilled water, and washed in TBS. Antigen retrieval was carried out by heating sections in 3% H₂O₂ in methanol for 20 minutes. Sections were then incubated for 1 hour at room temperature with 5 minutes. Nonspecific binding sites were blocked using Protein Block for 5 minutes. Antigen retrieval was carried out by heating sections in xylenes, rehydrated through graded ethanol solutions to distilled water, and electroblotted onto Immobilon-P transfer membranes. Blots were probed with highly specific antibodies with overnight incubations at 4°C. The sequence of primary antibodies was as follows: monoclonal PCNA antibodies (1:1,000) followed by anti-pan-EGFR antibodies (1:1,000), anti-phosphoactive ErbB2 antibodies (1:2,500), anti-cyclin D1 antibodies (1:2,500), and anti-phosphoactive ErbB2 antibodies (1:2,500). Similarly, samples were probed on a separate blot with anti-COX-2 (1:1,000) followed by anti-COX-1 (1:1,000), anti-CUGBP2 (1:250), and anti-HuR (1:250) antibodies. The combination of antibody specificities, differences in electrophoretic mobility, and adjustment of timed exposures of blots for film chemiluminescence detection allowed these proteins to be assessed on the same blot while circumventing the need to strip the blot between primary antibody incubations. Separately, control biopsies from several individuals were run on the same gel to assess mean ± SD of these proteins in normal-appearing crypts. To confirm comparable protein loading among the samples, 5 µg protein/sample were run on a separate gel and probed for β-actin. After incubation with primary antibodies, blots were incubated with peroxidase-coupled secondary antibodies. Antigen-antibody complexes were detected with ECL. Blots were digitized with an Epson (Long Beach, CA) Expression 1680 flat bed scanner in transparency mode and captured with SilverFast scan software (Lasersoft Imaging, Kiel, Germany). Band intensity was quantified using the pixel value of 1 pixel Gel software (Scanalytics, Rockville, MD) running on a G4 Apple Macintosh computer (Cupertino, CA). Protein expression levels in ACF were expressed as fold change of control.

**Statistical methods.** Protein and mRNA expression levels in adjacent control biopsies (two to four biopsies per patient) were averaged (mean ± SD). Protein and mRNA expression levels in ACF were expressed as fold change of matched adjacent control. ACF were defined as hyperproliferative if PCNA mRNA or protein levels were ≥2-fold of control. Similarly, EGFR, pErbB2, pERK, or cyclin D1 was defined as elevated if expression levels in ACF were ≥2-fold of matched control. In general, ACF proteins with expression levels ≥2-fold of control exceeded the mean ± 3 SDs of matched controls. Statistical comparisons of relative RNA or protein expression levels in ACF and control biopsies were assessed by unpaired Student’s t test. Ps < 0.05 were considered statistically significant.

**Results**

**Clinical characteristics and ACF features of chromoendo-scopy patients.** The clinical characteristics of patients for our study are summarized in Table 1. Seventy-three patients underwent magnification chromoendoscopy. Patients ranged from 56 to 78 years old. Half the patients had a history of colon neoplasia and nonsteroidal anti-inflammatory drug (NSAID) use, and nearly a third had a family history of colon cancer. Many patients also had a history of tobacco use. Interestingly, no ACF were identified in two patients with hereditary nonpolyposis colon cancer syndrome. Large ACF (>50 aberrant crypts) were present in 77% of patients. Previous studies have indicated that ACF are heterogeneous with respect to proliferation (11). The surface features of ACF selected in this study, with elongated or closed lumens, suggested many were dysplastic (Fig. 1A). We identified ACF with normal proliferation and hyperproliferation and those with dysplastic features (Fig. 1B-D). We showed that some hyperproliferative ACF overexpressed EGFR (Fig. 1H). We also confirmed that some ACF with increased proliferation (Fig. 1D) and up-regulated EGFR expression (Fig. 1H) had histologic features of dysplasia (Fig. 1E). Although formalin-fixed specimens were satisfactory for Ki-67 proliferation studies, the RNA was often degraded following prolonged operative ischemia and phosphoactive proteins could not be quantitatively extracted for Western blotting studies. For these reasons, we used
image magnification chromoendoscopy to isolate ACF in situ. This allowed us to rapidly preserve the samples.

**Increased PCNA mRNA levels define a hyperproliferative group of ACF.** For the chromoendoscopy study, 48 ACF were analyzed by real-time PCR (see Supplementary Table S2). PCNA is induced by EGFR and widely used as a marker of cell proliferation (34, 35). Because expression levels of PCNA mRNA and protein levels correlate closely (36), we used PCNA mRNA abundance to assess the proliferative status of ACF analyzed at the RNA level. In samples for RNA analysis, hyperproliferative ACF were defined as ACF with increases in PCNA mRNA abundance ≥ 2-fold of adjacent mucosa. By this criteria, 38% of ACF (18 of 48 ACF) were hyperproliferative (PCNA mRNA, 7.7 ± 1.2-fold of control; \( P < 0.05; \) Table 2).

Patients with hyperproliferative ACF were significantly older than patients without hyperproliferative ACF (69 versus 60 years old; \( P < 0.05; \) Table 1). Although not significant, there was a more frequent history of colonic adenomas or adenocarcinomas in the group with hyperproliferative ACF compared with those without hyperproliferative ACF (64% versus 43%, \( P = 0.11 \)). Because dysplastic ACF are also more frequent in patients with a history of colonic neoplasia (7), this is consistent with our observations that many of these large ACF had dysplastic-appearing surface features. Use of NSAIDs, smoking history, family history of colon cancer or colonic polyps, or gender, however, did not seem to be related to the proliferative state of ACF.

**Transcripts for EGFR, EGFR ligands, and CCND1 are up-regulated in hyperproliferative ACF.** Because EGFR ligands are regulated transcriptionally, their protein expression levels reflect mRNA abundance (20). We, therefore, measured TGF-\( \alpha \), HB-EGF, and amphiregulin mRNA in ACF using real-time PCR. In hyperproliferative ACF, mRNA levels of TGF-\( \alpha \) (14.4 ± 5.0-fold) and HB-EGF (4.5 ± 1.4-fold), but not amphiregulin, were significantly increased compared with normal-appearing mucosa (\( P < 0.05; \) Table 2). EGFR mRNA was also significantly increased 6.0 ± 1.7-fold in hyperproliferative ACF compared with normal-appearing mucosa (\( P < 0.05; \) Table 2). In addition, the EGFR-inducible gene encoding cyclin D1 (CCND1) was up-regulated 4.6 ± 0.7-fold in hyperproliferative ACF. In contrast, in nonhyperproliferative ACF, mRNA levels of EGFR, TGF-\( \alpha \), and CCND1 were not overexpressed (Table 2).

**EGFR effectors and targets are simultaneously up-regulated or activated in a subset of hyperproliferative ACF.** We used PCNA to assess proliferation by Western blotting in ACF. PCNA produces a sharper, more accurately quantified band than Ki-67 for Western blotting. We also examined the protein expression levels of

**Table 1. Clinical characteristics of patients for ACF study**

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Age</th>
<th>Male/female</th>
<th>Family history of colon cancer (%)</th>
<th>History of adenomas or carcinomas (%)</th>
<th>NSAID use (%)</th>
<th>Smoking (pack-years)</th>
<th>No. patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>64.2</td>
<td>0.94/1</td>
<td>31</td>
<td>53</td>
<td>52</td>
<td>19</td>
<td>73</td>
</tr>
<tr>
<td>Patients with hyperproliferative ACF*</td>
<td>69 ± 20</td>
<td>0.7/1</td>
<td>31</td>
<td>64</td>
<td>52</td>
<td>17.2</td>
<td>34</td>
</tr>
<tr>
<td>Patients without hyperproliferative ACF†</td>
<td>60 ± 16</td>
<td>1.2/1</td>
<td>30</td>
<td>43</td>
<td>52</td>
<td>20.6</td>
<td>39</td>
</tr>
<tr>
<td>( P^c )</td>
<td>&lt;0.01</td>
<td>0.31</td>
<td>0.95</td>
<td>0.11</td>
<td>0.99</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

*Patients with large hyperproliferative ACF (PCNA transcript abundance or protein ≥ 2-fold of adjacent mucosa).
†Patients without hyperproliferative ACF (patients with no ACF or small or nonhyperproliferative ACF).
\( P^c \) Comparing patients with hyperproliferative ACF with those without hyperproliferative ACF.
EGFR, phosphoactive ErbB2, phosphoactive ERK, cyclin D1, and COX-2 by Western blotting. As in mRNA studies, ACF with increases in PCNA protein expression levels ≥2-fold of adjacent mucosa were defined as hyperproliferative. By this criterion, 46% of ACF analyzed for protein expression were hyperproliferative (PCNA, 3.2 ± 1.2; P < 0.05) compared with 38% of ACF with increased PCNA mRNA (P = 0.60).

Among ACF that were hyperproliferative by protein criteria, 43.8% had simultaneously and significantly increased levels of four EGFR signaling components: EGFR, pErbB2, pERK, and cyclin D1. This subgroup of hyperproliferative ACF represents ~20% of large ACF. Shown in Fig. 2A are representative blots of these hyperproliferative ACF that simultaneously expressed increased levels of multiple EGFR signaling components. Shown in Fig. 2B are the quantitative expression levels of PCNA, EGFR, pErbB2, pERK, and cyclin D1 that were significantly increased in this group of hyperproliferative ACF.

The percentages of ACF with elevated PCNA, EGFR, or pERK as well as the mean increases in these proteins are summarized in Fig. 3. In these respective groups, PCNA was increased 3.2 ± 1.2–fold (P < 0.05), EGFR was increased 8.7 ± 3.4–fold (P < 0.05), and pERK was increased 2.7 ± 1.1–fold (P < 0.05). A Venn diagram showing the overlapping and nonoverlapping distributions of ACF with increased EGFR, PCNA, and/or pERK is shown in Fig. 3. Fully 90% of ACF with increased PCNA, EGFR, and/or activated ERK (pERK) could be assigned to one of four major groups. Group I included ACF with simultaneous increases in EGFR, pERK, and PCNA (Fig. 3, group I). This group constituted nearly 44% of the hyperproliferative ACF. Group II were hyperproliferative ACF with increased EGFR but normal pERK (Fig. 3, group II). Group III included ACF with increased EGFR but normal pERK and PCNA (Fig. 3, group III). Group IV included ACF with increased EGFR and pERK but normal PCNA (Fig. 3, group IV). Although ACF in groups III and IV possessed increased EGFR, they were not hyperproliferative, presumably reflecting the heterogeneous nature of ACF (7).

**K-ras and B-Raf analyses.** We also analyzed Ras activation in ACF. Ras was immunoprecipitated and the abundance of Ras-GTP and Ras-GDP was quantified. This procedure required separate ACF because there was insufficient protein available for immunoprecipitation and quantitative Western blotting. Ras was significantly activated in 6 of 13 (46%) large ACF (see Supplementary Table S2). K-ras mutations were identified in only 7% (2 of 30) of ACF both in codon 12. In contrast, we identified K-ras mutations in 29% of colon cancers. As positive controls, we also confirmed a K-ras mutation in codon 12 of SW480 cells and a K-ras mutation in codon 13 of HT29-116 cells. Interestingly, the proportion of ACF with activated Ras was comparable with the fraction with hyperproliferation as assessed by increased PCNA protein, suggesting that Ras plays an important role in ACF growth. We infer that the observed activations of wild-type Ras were caused by upstream signals, including EGFR and ErbB2. EGFR was up-regulated in 84% of hyperproliferative ACF (73.8% of all ACF; Fig. 3). Activating B-Raf mutations could also contribute to increases in pERK levels. We did not detect any B-Raf mutations in 14 ACF examined, however, in agreement with a previous report (37). As a positive control, in RKO cells, we did confirm a mutation in B-Raf with the codon 599.

**COX-2 mRNA, but not protein, is overexpressed in hyperproliferative ACF.** Although COX-2 levels are elevated in colon cancer (19), expression levels of this inducible COX were not detected by immunostaining in human ACF (38). Because COX-2 is a target of EGFR signaling in colon cancer cells (17), we examined COX-2 transcripts and Western blotting protein expression in ACF. We found that COX-2 mRNA was significantly overexpressed 9.3 ± 4.2–fold in hyperproliferative but not in nonhyperproliferative ACF (Table 2). In contrast, COX-2 protein levels were not significantly increased in ACF compared with adjacent mucosa (2.0 ± 0.9 versus 1.0 ± 0.5; Fig. 4), in agreement with prior studies (38). To investigate potential causes of increased COX-2 mRNA, we measured the expression of HuR and CUGBP2 in ACF. HuR and CUGBP2 are mRNA-regulating proteins that bind to AU-rich sequences in the 3’-untranslated region of the COX-2 transcript and stabilize mRNA (39, 40). Whereas HuR could not be detected in hyperproliferative ACF, CUGBP2 mRNA was increased 3.6 ± 0.3-fold (P < 0.01) compared with adjacent mucosa (Table 2). Furthermore, CUGBP2 protein was increased in more than half of hyperproliferative ACF assayed by Western blotting (Fig. 4). CUGBP2 binding stabilizes COX-2 message but inhibits mRNA translation (40). These results suggest that CUGBP2 might stabilize COX-2 mRNA while concomitantly inhibiting translation of COX-2 mRNA in human ACF.

**Discussion**

In agreement with Otori et al. (11), we confirmed that a subset of large ACF are hyperproliferative as assessed by Ki-67 immunostaining and PCNA expression levels. We believe that many hyperproliferative ACF in our study were likely dysplastic because they exhibited endoscopic features of dysplastic ACF (5, 7, 31).
have investigated a possible mechanism to drive this increased proliferation and showed that EGFR signaling is up-regulated in human ACF. Specifically, 38% of large ACF, with endoscopic features suggesting dysplasia, were hyperproliferative as assessed by increased PCNA mRNA. In these hyperproliferative ACF, transcript levels of EGFR ligands, receptors, and targets were also up-regulated. At the protein level, 46% of large ACF were hyperproliferative by PCNA criteria. The percentage of ACF with elevated PCNA mRNA and the percentage with increased PCNA protein were in close agreement. Among the group of hyperproliferative ACF, 44% had simultaneous increases in EGFR protein, activated ErbB2 and ERK, and up-regulated cyclin D1. To our knowledge, this is the first study to show an important role for EGFR signaling in ACF proliferation.

In our patient population, age was a significant predictor of the presence of hyperproliferative ACF. We also found a trend toward hyperproliferative ACF in patients with a history of colonic adenomas or carcinomas, in agreement with others (5–7). We did not find that aspirin or other NSAIDs altered the proliferative state of ACF. Other studies have also suggested that NSAIDs influence apoptosis more than proliferation and do not require COX-2 expression for chemopreventive efficacy (41–43).

By real-time PCR, all the hyperproliferative ACF had increased EGFR transcripts (Table 2), whereas by Western blotting 84% of hyperproliferative ACF had increased EGFR protein (Fig. 3). Furthermore, nearly 44% of hyperproliferative ACF (increased PCNA protein) had concomitant elevations in EGFR, pErbB2, pERK, and cyclin D1 (Figs. 2 and 3, group I). Based on these findings, up-regulated EGFR signaling seems to drive mitogenesis in nearly half of hyperproliferative ACF. The remaining ACF with up-regulated EGFR were heterogeneous in other downstream components (Fig. 3, groups II-IV). Group II was composed of hyperproliferative ACF with normal levels of pERK. Alternative EGFR-dependent, ERK-independent pathways, such as phosphatidylinositol 3-kinase/AKT or signal transducers and activators of transcription 3, might drive increased mitogenesis in this group. Presumably, preneoplastic lesions accumulating transforming genetic changes could be promoted by mechanisms that stimulate cellular proliferation, such as EGFR signaling. Groups III and IV were composed of nonhyperproliferative ACF with increased EGFR, but only group IV had elevated pERK. Perhaps ACF in the latter groups grow by a mechanism that inhibits normal apoptosis (38). Many patients with hyperproliferative ACF also had nonhyperproliferative ACF, indicating that local factors also regulate their proliferation and apoptosis (38).

Because of limited protein yields from individual ACF and the low abundance of phosphorylated EGFR in our samples, we were unable to quantify activated EGFR. We have shown, however, that EGFR signaling is activated in ACF as assessed by increases in phosphoactive ErbB2. This orphan receptor has no identified ligands and requires an activating ErbB heterodimeric partner (13). In addition to ErbB2 activation, the up-regulation we observed in EGFR ligands (transcripts) and receptors (transcripts and protein) as well as activations of EGFR downstream effectors Ras and ERK and increases in EGFR-inducible cyclin D1 support our conclusion that EGFR is activated in human ACF. In preliminary studies in mice, activation of only 20% of colonic EGFRs is sufficient for maximal ERK activation (44). Further supporting the role of EGFR signaling in ACF formation, we have shown in other preliminary studies in azoxymethane-treated mice that EGFR antagonist gefitinib inhibited crypt cell hyperproliferation and decreased ACF growth while concomitantly blocking EGFR signaling (29). Interestingly, pErbB2 and pERK signals were stronger than pEGFR signals in azoxymethane premalignant mucosa (29). More recently, we showed that gefitinib significantly inhibited tumor incidence in azoxymethane-treated rats (30). In Min mice, a genetic model of intestinal tumorigenesis, EGFR mutations also significantly reduced adenoma growth (28). We believe, therefore, that low levels of EGFR activation are amplified to produce measurable changes in pErbB2 and effectors Ras and

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**Figure 2.** EGFR signaling is up-regulated in hyperproliferative human ACF. Methylene blue–stained ACF and adjacent mucosa were identified by image magnification chromoendoscopy. Large dysplastic-appearing ACF and normal-appearing crypts were collected by biopsy. Western blots of lysates (80 μg protein) were probed consecutively for PCNA, total EGFR, phosphoactive ERK (pERK), phosphoactive ErbB2 (pErbB2), and cyclin D1. Separately, 5 μg of each sample were run for β-actin to confirm comparable protein loading. Protein expression levels were measured by quantitative Western blotting as described in Materials and Methods. A, representative Western blot of hyperproliferative ACF (A) with increased EGFR, phosphoactive ErbB2, and phosphoactive ERK and up-regulated cyclin D1 compared with adjacent normal-appearing mucosa (N). B, quantitative Western blotting. For adjacent normal-appearing mucosa (controls), values were normalized to 1. ACF samples were run with matched controls on the same gel and protein expression levels were expressed as fold of control. Columns, mean; bars, SD. Note EGFR, pErbB2, pERK, and cyclin D1 were significantly increased in ACF with elevated PCNA (n = 4 patients, 13 ACF). *, P < 0.05; †, P < 0.005, compared with adjacent normal-appearing mucosa.
ERK and downstream targets cyclin D1 and PCNA that contribute to aberrant crypt cell proliferation. Our analysis showed that wild-type Ras was activated in 46% of ACF assayed, but activating K-\textit{ras} mutations were present in only 7% ACF. Previous investigators have reported K-\textit{ras} mutations in human ACF with widely varying frequencies, ranging from 13% to 95% (3). Whereas K-\textit{ras} mutations occur in ~50% of colon carcinomas and large adenomas, these mutations are present in only 9% of adenomas under 1 cm in size (45). Because we postulate that ACF are precursors of small adenomas, we would predict few mutations in our sample size. Furthermore, K-\textit{ras} mutations seem to be rare in dysplastic or large ACF (9, 11, 46), the population targeted by endoscopic features in this study. The occurrence of activated wild-type Ras in nearly half of ACF indicates that upstream signals, including ErbB2, stimulate this proto-oncogene product in ACF without K-\textit{ras} mutations. With neoplastic progression, development of K-\textit{ras} mutations would be expected to drive increasing autonomy and reduce the requirement for persistent EGFR signaling in tumors acquiring this mutation. Because B-Raf mutations were reported in only 2% of ACF (37), it was not surprising that we did not detect B-Raf mutations.

We also examined COX-2 expression in ACF. COX-2 is a target of EGFR signaling and causally involved in colonic carcinogenesis (17, 19). We found that COX-2 mRNA, but not protein, was overexpressed in hyperproliferative ACF. Furthermore, CUGBP2, a mRNA-binding protein that stabilizes COX-2 mRNA and prevents mRNA translation (40), was up-regulated in ACF, perhaps contributing to the dissociation between COX-2 mRNA and protein expression in ACF. We have summarized these EGFR signaling pathways, including COX-2 regulation in ACF, in Fig. 5. With progression to the adenoma stage, additional alterations presumably facilitate translation of the up-regulated COX-2 transcripts. As a corollary to these observations, the chemopreventive effects of NSAIDs at the ACF stage do not seem to require COX-inhibitory activity and perhaps involve apoptosis rather than proliferation (41, 43). Further studies are warranted to identify NSAID targets in ACF.

Although we have shown EGFR up-regulation in ACF, the mechanisms that control EGFR gene expression are complex and involve transcriptional and post-transcriptional regulation. Several transcription factors that regulate EGFR gene expression have been identified, including activating factors Sp1, \textit{h}-catenin, activator protein-1, early growth response-1 gene, IFN-regulatory factor-1, and p53 (47–52) and inhibitory factors GC binding transcriptional...
repressor factor and VDR (53, 54). Moreover, recent studies have identified an important mechanism that could up-regulate EGFR expression in human ACF through enhanced Wnt/β-catenin signaling (55, 56).

In summary, we found EGFR up-regulation in a substantial fraction of hyperproliferative human ACF by both RNA and protein criteria. The mRNA levels of EGFR, TGF-α, HB-EGF, cyclin D1, and COX-2 were significantly increased in these ACF. Among hyperproliferative ACF, 44% exhibited EGFR and cyclin D1 protein up-regulation and ErbB2 and ERK activation. Whereas transcripts of EGFR downstream effectors cyclin D1 and COX-2 were overexpressed in hyperproliferative ACF, translation of COX-2 mRNA seemed to be blocked, perhaps by the mRNA-binding protein CUGBP2. This study also highlights the potential importance of activated wild-type Ras as an early event in colonic carcinogenesis. We conclude that dysregulated EGFR signaling contributes to the hyperproliferative state of large ACF.

Because ACF are the first identifiable preneoplastic lesions in the colon, elucidation of mechanisms that drive their proliferation is important for understanding early colonic carcinogenesis. Furthermore, with an increasing array of selective EGFR inhibitors in the clinical arena, these results suggest potential new strategies in colon cancer chemoprevention in patients at increased risk for this malignancy.

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