Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria

Christine M. Dejea,1,2,3 Payam Fathi,1,2,3,4 John M. Craig,4 Annemarie Boleij,1,5 Rahwa Taddesse,5 Abby L. Geis,1,2,4 Xinqun Wu,1,2,4 Christina E. DeStefano Shields,1,2 Elizabeth M. Hechenbleikner,5,6 David L. Hsu,7,6 Robert A. Anders,8 Francis M. Giardiello,2,3 Elizabeth C. Wick,6 Hao Wang,1,2 Shaoguang Wu,1,3 Drew M. Pardoll,1,2 Franck Houseau,1,2 Cynthia L. Sears1,2,3,8

Individuals with sporadic colorectal cancer (CRC) frequently harbor abnormalities in the composition of the gut microbiome; however, the microbiota associated with precancerous lesions in hereditary CRC remains largely unknown. We studied colonic mucosa of patients with familial adenomatous polyposis (FAP), who develop benign precursor lesions (polyps) early in life. We identified patchy bacterial biofilms composed predominantly of Escherichia coli and Bacteroides fragilis. Genes for colibactin (clbB) and Bacteroides fragilis toxin (bft), encoding secreted oncoproteins, were highly enriched in FAP patients’ colonic mucosa compared to healthy individuals. Tumor-prone mice co colonized with E. coli (expressing colibactin), and enterotoxigenic B. fragilis showed increased interleukin-17 in the colon and DNA damage in colonic epithelium with faster tumor onset and greater mortality, compared to mice with either bacterial strain alone. These data suggest an unexpected link between early neoplasia of the colon and tumorigenic bacteria.

Colorectal cancer (CRC) is very common globally and develops through accumulation of colonic epithelial cell (CEC) mutations that promote transition of normal mucosa to adenocarcinoma. Around 5% of CRC occurs in individuals with an inherited mutation (1). One hereditary condition, familial adenomatous polyposis (FAP), is caused by germline mutation in the APC tumor suppressor gene. Individuals with FAP are born with their first mutation in the transition to CRC, and as somatic mutations accumulate, develop hundreds to thousands of colonic polyps. The onset and frequency of polyp formation within families bearing the same APC gene mutation varies substantially (2), suggesting that additional factors contribute to disease onset, including components of the microbiome (3).

The colon contains trillions of bacteria that are separated from the colonic epithelium by a dense mucus layer. This mucus layer promotes tolerance to foreign antigens by limiting bacterial–epithelial cell contact and, thus, mucosal inflammatory responses. In contrast, bacterial breaches into the colonic mucosa layer with, in some, biofilm formation fosters chronic mucosal inflammation (4–6). We previously reported that biofilms on normal mucosa of sporadic CRC patients were associated with a pro-oncogenic state (6, 7), suggesting that biofilm formation is an important epithelial event influencing CRC. To test the hypothesis that biofilm formation may be an early event in the progression of hereditary colon cancer, we examined the mucosa of FAP patients at clinically indicated colectomy.

We initially screened surgically resected tissue preserved in Carnoy’s fixative from five patients with FAP and one with juvenile polyposis syndrome (table S1). Colon biopsies from individuals undergoing screening colonoscopy or surgical resections served as controls (n = 20, table S2). Polyps and macroscopically normal tissue were labeled with a panbacterial 16S rRNA (rRNA) fluorescence in situ hybridization (FISH) probe. Each FAP patient exhibited bacterial invasion through the mucus layer scattered along the colonic axis (Fig. 1A, table S3, fig. S1). Unlike the continuous mucosal biofilms in sporadic CRC patients, FAP tissue displayed patchy bacterial mucus invasion (average length, 150 μm) on ~70% of the surgically resected colon specimens collected from four of six hereditary tumor patients. Biofilms were not restricted to polyps, nor did they display right colon geographic preference as observed in sporadic CRC (table S3 and figs. S1 and S2). Biofilms were not detected in the mucus layer of the FAP patient who received oral antibiotics 24 hours before surgery (table S1 and fig. S2). Specimens with bacterial biofilms were further screened by additional 16S rRNA probes to recognize the major phyla detected in biofilms of sporadic CRC, namely, Bacteroides/Prevotella, Proteobacteria, Lachnospiraceae, and Fusobacteria (table S4). Notably, FAP biofilms were composed predominantly of mucus-invasive Proteobacteria (60 to 70%) and Bacteroides (10 to 32%) (table S3). Fusobacteria were not detected, and Lachnospiraceae were rare (<3%) by quantitative FISH analysis (table S3).

Additional probe sets (table S4) identified the predominant biofilm members as E. coli and B. fragilis (Fig. 1A, bottom panels; table S3). Invasion of the epithelial cell layer by biofilm community members was detected in all patients harboring biofilms (Fig. 1B), a finding similar to that in sporadic CRC patients. Further, FISH of mucosal biopsies from ileal pouches or anorectal remnants of additional, longitudinally followed, postcolectomy FAP patients revealed biofilms in 36% and mucosal-associated E. coli or B. fragilis in 50% (table S5). Thus, E. coli and B. fragilis are frequent, persistent mucosal colonizers of the FAP gastrointestinal tract. Of note, semiquantitative colon mucosa bacterial cultures of ApcMin/+ mice (truncation at the 716 codon of Apc), a murine correlate of FAP, displayed similar enrichment of Bacteroides and Enterobacteriaceae compared to wild-type (WT) littersmates, consistent with data reported for ApcMin/+ mice (fig. S3) (8). These results suggest that Apc mutations enhance mucosal bacterial adherence, altering the bacterial-host epithelial interaction.

Strong experimental evidence exists supporting the carcinogenic potential of molecular subtypes of both E. coli and B. fragilis (9, 10); the two dominant biofilm members identified in direct contact with host colon epithelial cells in our FAP patients. E. coli containing the polyketide synthase (pkss) genotoxic island (encodes the genes responsible for synthesis of the colibactin genotoxin) induces DNA damage in vitro and in vivo along with colon tumorigenesis in azoxymethane (AOM)-treated interleukin-10 (IL-10)-deficient mice (10), whereas, enterotoxigenic Bacteroides fragilis (ETBF) induces colon tumorigenesis in ApcMin/+ mice (9). Human epidemiological studies have associated ETBF and pks+E. coli with inflammatory bowel disease and sporadic CRC (10–13). Thus, we cultured banded frozen mucosal tissues from 25 FAP patients (two polyps and two normal tissues per patient when available, table S1) and 23 healthy individuals (mucosal sample from surgical resection or one ascending and one descending colon biopsy per colonoscopy subject, table S2) for the presence of pks+E. coli and ETBF. The mucosa of FAP patients was significantly associated with pks+E. coli (68%) and ETBF (60%) compared
reaction (PCR) analysis, indicating that the car-

bft

FAP patients (fig. S2 and table S1) contained both
dissection of mucosal biofilms from our initial

in healthy control subjects (22% observed versus

frequencies for the individual species (Fig. 1C).

Increased mucosal coassociation also occurred

expected to occur randomly (40.8%) given the

association occurred at a higher rate (52%) than

DELA @ eL et al. SCIENCE 359, 592–597 (2018) 2 February 2018

colon tumorigenesis is very low in both model

Specific pathogen-free wild-type mice were

with the carcinogen AOM and monoin-
oculated or coinoculated with canonical strains

of pks+ E. coli (the murine adherent and inva-

sive strain, NC101) and ETBF (strain 086-5443-2-2)

(9, 10). Fecal ETBF or pks+ E. coli colonization

was similar under monocolonization or cocolo-

nization conditions, persisting until colon tumor

formation was assessed at 15 weeks after colo-
nization (fig. S4). Monocolonized (pks+ E. coli or

ETBF) mice displayed few to no tumors. However,

pronounced tumor induction occurred in co-

olonized mice, including an invasive cancer,

suggesting the requirement for both bacteria
to yield oncogenesis (Fig. 2, A to C). Tumor-
genesis required the presence of BFT and the

colibactin genotoxin as in-frame deletions of

to healthy subject mucosa (22% pks+ E. coli and

30% ETBF) (Fig. 1C). There was no preferential

association of ETBF or pks+ E. coli with polyp

or normal mucosa from FAP patients. Typically,
mucosal samples from individual patients were

cordant for pks+ E. coli or ETBF (73% for

pks+ E. coli, 59% for ETBF), similar to results for

mucosal bft detection in sporadic CRC patients

(33). Notably, pks+ E. coli and ETBF mucosal co-

association occurred at a higher rate (52%) than

expected to occur randomly (40.8%) given the

frequencies for the individual species (Fig. 1C).

Increased mucosal coassociation also occurred

in healthy control subjects (22% observed versus

6.6% expected) (Fig. 1C). Laser capture micro-

dissection of mucosal biofilms from our initial

FAP patients (fig. S2 and table S1) contained both

bft and clbB as determined by polymerase chain

reaction (PCR) analysis, indicating that the car-

cinogenic subtypes of B. fragilis and E. coli, re-

spectively, were present in the mucus layer in
direct contact with the FAP epithelium (Fig. 1D).

In contrast, neither virulence gene was detected

in the mucus layer of control subject 3780 whereas

bft was detected in the mucus layer of control

subject 3730, consistent with our prior reported

culture analysis of this sample (Fig. 1D) (33).

The high frequency of pks+ E. coli and ETBF

cocolonization in FAP colons highlights the im-

portance of understanding the potential effects

of simultaneously harboring these two carcino-
genic bacteria. Consequently, we used two mu-

rine models, AOM treatment without dextran

sodium sulfate (see materials and methods) and

Ape−Min+/−/+ mice to test the hypothesis that pks+

E. coli and ETBF cocolonization enhances colon
tumorigenesis compared to monocolonization with either bacterium. The rate of spontaneous

Fig. 1. Fluorescent in situ hybridization (FISH) and microbiology
culture analysis of FAP mucosal tissues. (A) Top panels: Representative

FISH images of bacterial biofilms (red) on the mucosal surface of a

FAP polyp and paired normal tissues counterstained with DAPI (4',6-
diamidino-2-phenylindole) nuclear stain (blue). Middle panels: PAS (periodic acid–

Schiff)–stained histopathology images of polyp and paired normal

mucosal tissues demonstrating the presence of the mucus layer. Images

were obtained at 40× magnification; scale bars, 50 μm. Dotted lines

delineate the luminal edge of the colonic epithelial cells. Images are

representative of n = 4 to 23 tissue samples per patient screened (at least

10 5-μm sections screened per patient). (B) Enterobacteriaceae (yellow)

and E. coli (red) FISH probes on paired normal FAP tissue (100×
magnification) revealing invasion into the epithelial cell layer at the base

of a crypt (arrows). Bottom panels with insets of Enterobacteriaceae (bottom

left panel) in yellow, E. coli (bottom middle panel) in red, and overlay

(bottom right panel) confirming identification of the invasive species. Scale

bar, 20 μm. Images are representative of n = 5 to 16 tissue samples per

patient screened (at least 10 5-μm sections screened per patient). (C) FAP

and control prevalence of pks+ E. coli and enterotoxigenic Bacteroides

fragilis (ETBF). Chi-square P-values are shown that represent the difference

in probability of detection of each bacterium in FAP versus control

patients. (D) PCR detection of clbB (a gene in the pks island) and bft within

laser-captured biofilms containing E. coli and B. fragilis from designated

FAP patients (table S1) and controls (table S2; materials and methods).

Data show a representative image from two independent experiments with

two or three replicates per experiment performed.

Dejea et al., Science 359, 592–597 (2018) 2 February 2018
the bft gene and the pks virulence island significantly decreased tumors (Fig. 2A). ApcMin D716/+ mice cocolonized with ETBF and pks+ E. coli exhibited enhanced morbidity with rapid weight loss and significantly increased mortality (P < 0.0001) [loss of 80% of the mice (n = 8) and the remaining 20% (n = 2) at 12 weeks after colonization]. In contrast, 90% (n = 9) and 100% (n = 10) of mice monocolonized with ETBF or pks+ E. coli, respectively, survived 15 weeks after colonization (Fig. 2D). The robust tumorigenesis of ETBF alone (at 15 weeks) and cocolonized mice (majority deceased by 8 weeks after colonization) was similar, whereas tumor numbers were significantly increased in the cocolonized cohort compared to pks+ E. coli alone (Fig. S5). Notably, at early time points, inflammation was increased in the cocolonized cohort compared to either ETBF or pks+ E. coli alone (Fig. S5). Together these results suggest that the significant increase in colon inflammation and early tumorigenesis in the cocolonized mice contributed to their earlier mortality in the ApcMin/+ mouse model.

Consistent with enhanced tumorigenesis, histopathological analysis revealed significantly increased colon hyperplasia and mucosal microadenomas in cocolonized AOM-treated mice compared to monocolonized mice (Fig. 3A and fig. S6A). However, histopathology scoring revealed modest differences in inflammation over time (4 days to 15 weeks) in mono- and cocolonized AOM mice (Fig. 3B and fig. S6B). Thus, overall inflammation did not seem to explain differential tumor induction. To determine if the type of inflammation contributed to differences in tumorigenesis, we analyzed lamina propria immune-cell infiltrates of monocolonized and cocolonized wild-type AOM mice by flow cytometry. Our general lymphoid panel revealed a marked B cell influx across all colonization groups (Fig. 3C) but no significant differences in the proportion of infiltrating T cells (CD4, CD8, or γδ T cells) and myeloid populations between monocolonized and cocolonized AOM mice (Fig. 3C) either at the acute (1-week) or chronic (3-week) stage of infection.

Of particular interest was IL-17, as the tumorigenic potential of ETBF in ApcMinD716/+ mice has been attributed, in part, to IL-17 (9). Because bft was necessary for synergistic tumor induction
under cocolonization conditions (Fig. 2A), we tested the role of IL-17 in the cocolonized AOM model. Although IL-17 expression analysis by quantitative PCR revealed no significant difference in overall mucosal IL-17 mRNA levels between 15-week ETBF monocolonized (n = 11), or pks+ E. coli/ETBF cocolonized (n = 13) mice. Data represent mean ± SEM of three independent experiments. For (A) and (B), overall significance was calculated by using the Kruskal-Wallis test, and the overall P value is shown; Mann-Whitney U was used for two-group comparisons; **P = 0.01, ****P = 0.0001, NS, not significant. (C) Myeloid and lymphoid lamina propria immune cell infiltration plotted as percentage of live cells in AOM mice at day 7 (top panels) and day 21 (bottom panels) after colonization. Data represent mean ± SEM of three independent experiments (total three to five mice per group). (D) Total tumor numbers detected in IL-17–deficient AOM-treated mice (IL17−/−) versus wild-type AOM mice (WT). Both mouse strains were cocolonized with pks+ E. coli and ETBF and tumors assessed at 15 weeks. Data represent mean ± SEM of two or three independent experiments (total 6 to 13 mice per group). Significance calculated by the Mann-Whitney U test represents differences between the non-normally distributed colon tumors in the independent mouse groups. (E) IL-17–producing cell subsets and total number of IL-17–producing (IL-17tot) cells per colon harvested from germ-free C57BL/6 mice monocolonized with pks+ E. coli or ETBF or cocolonized with pks+ E. coli and ETBF for up to 60 hours. Data represent mean ± SEM of two independent experiments (total 3 to 5 mice per group). Overall significance across IL-17–producing cell types was calculated by using two-way analysis of variance testing based on log-transformed data (bold P value). For each cell subset and total number of IL-17–producing cells (gray dotted line box), the overall P value is shown and was calculated by using the Kruskal-Wallis test. Two-group cell subset and total number of IL-17–producing cell comparisons were analyzed by Mann-Whitney U test and are reported in table S7. Because our general lymphoid panel revealed a marked B cell influx across all colonization groups (Fig. 3C), we profiled the secretory immunoglobulin A (IgA) response by IgA enzyme-linked immunosorbent assay (ELISA) using stool collected 4 weeks after colonization from AOM mice. Cocolonized mice had a significantly more robust IgA response to pks+ E. coli than mice monocolonized with pks+ E. coli, whereas the fecal anti-ETBF IgA response was similar under mono- and cocolonization conditions (Fig. 4A). Thus, the increased fecal IgA response was specific to pks+ E. coli in mice cocolonized with ETBF,
suggesting that cocolonization enhanced mucosal exposure to \( \text{pks}^+ \) \( \text{E. coli} \).  

Although fecal colonization of both \( \text{pks}^+ \) \( \text{E. coli} \) and ETBF was equivalent under both mono- and cocolonization conditions (fig. S4), quantification of mucosal-adherent \( \text{pks}^+ \) \( \text{E. coli} \) and ETBF revealed a marked increase in mucosal-adherent \( \text{pks}^+ \) \( \text{E. coli} \) under cocolonization conditions compared to \( \text{pks}^+ \) \( \text{E. coli} \) monocolonization (Fig. 4B). Hence, under monocolonization conditions, \( \text{pks}^+ \) \( \text{E. coli} \) is largely cultivatable only from the colon lumen whereas in the presence of ETBF, \( \text{pks}^+ \) \( \text{E. coli} \) colonizes the mucosa at high levels (\( 10^7 \) to \( 10^9 \) colony-forming units per gram of tissue). Using Muc-2–producing HT29-MTX-E12 monolayers in vitro, we tested the impact of \( \text{pks}^+ \) \( \text{E. coli} \) and ETBF on mucus. Although \( \text{pks}^+ \) \( \text{E. coli} \) colonization alone had no impact on mucus depth, monolayer colonization with ETBF alone or cocolonized with \( \text{pks}^+ \) \( \text{E. coli} \) significantly reduced mucus depth similar to colonization with \( \text{A. muciniphila} \), a known human colonic mucin-degrading bacterium (Fig. 4C). These results suggest that mucus degradation by ETBF promotes enhanced \( \text{pks}^+ \) \( \text{E. coli} \) colonization. Such a shift in the bacterial niche of \( \text{pks}^+ \) \( \text{E. coli} \) would facilitate the delivery of colibactin, the DNA-damaging toxin released by \( \text{pks}^+ \) \( \text{E. coli} \), to colon epithelial cells. Consistent with this hypothesis, \( \gamma \text{-H2AX} \) immunohistochemistry revealed significantly enhanced DNA damage in the colon epithelial cells of AOM mice colonized with \( \text{pks}^+ \) \( \text{E. coli} \) and ETBF compared to monocolonized (\( \text{pks}^+ \) \( \text{E. coli} \) or ETBF) mice (Fig. 4D). Further, mice colonized with ETBF and \( \text{E. coli} \) did not enhance but rather reduced tumors and no increase in DNA damage or IL-17 (Fig. 2A and fig. S9, A and B, respectively). Lastly, persistent cocolonization of AOM-treated mice with the mucin-degrading \( \text{A. muciniphila} \) and \( \text{pks}^+ \) \( \text{E. coli} \) did not enhance, but rather reduced, the modest colon tumorigenesis (fig. S10, A and B) induced by \( \text{pks}^+ \) \( \text{E. coli} \) monocolonization. These results suggest that mucus degradation alone was insufficient to promote \( \text{pks}^+ \) \( \text{E. coli} \) colon carcinogenesis in AOM mice.
Taken together, these data suggest that co-colonization with ETBF and \( pks^+ \) \( E. coli \), found in more than half of FAP patients (in contrast to less than 25% of controls), promotes enhanced carcinogenesis through two distinct but complementary steps: (i) mucus degradation enabling increased \( pks^+ \) \( E. coli \) adherence, inducing increased colonic epithelial cell DNA damage by colibactin (Fig. 4D and fig S9); and (ii) IL-17 induction promoted, primarily, by ETBF with colibactin (Fig. 4D and fig S9); and (ii) IL-17 increased colonic epithelial cell DNA damage by ETBF and \( pks^+ \) \( E. coli \).

ETBF and \( pks^+ \) \( E. coli \) commonly colonize young children worldwide. Thus, our results suggest that persistent co-colonization in the colon mucosa from a young age may contribute to the pathogenesis of FAP and potentially even those who develop sporadic CRC because APC loss or mutation occurs in the vast majority of sporadic CRC. We note that \( pks^+ \) \( E. coli \) are phenotypic and genotypic adherent and invasive \( E. coli \) (AIEC) (14). Despite this designation, derived primarily from in vitro cell culture experiments, the canonical \( pks^+ \) \( E. coli \) strain (NC101) used in our experiments was only cultivatable from the colon lumen in the absence of concomitant ETBF colonization in our mouse model. This ETBF-dependent shift to marked mucosal \( pks^+ \) \( E. coli \) colonization is consistent with our observations that ETBF and \( pks^+ \) \( E. coli \) colonize FAP colon biopsies, where both bacteria invade and colonize the mucus layer throughout the FAP colon.

These findings suggest that analysis of coexpression of \( bft \) and \( clbB \) may have value in general screening and potential prevention of CRC.

**REFERENCES AND NOTES**


**ACKNOWLEDGMENTS**

We thank K. Kindler and B. Vogelstein for valuable discussions; K. Romans and L. Hylind for assistance with patient enrollment; and S. Besharati for assistance with histopathologic analyses. The data presented in this manuscript are tabulated in the main text and supplementary materials and methods. This work was supported by the Bloomberg Philanthropies and by NIH grants R01 CA151393 (to C.L.S., D.M.P.), K08 DK087856 (to E.C.W.), 5T2CA126607-05 (to E.M.H.); P30 DK095223 (Johns Hopkins University School of Medicine), P30 CA069673 (Johns Hopkins University School of Medicine), and P50 CA62904 (Johns Hopkins University School of Medicine). Funding was also provided through a research agreement with Bristol-Myers Squibb Co-International Immunology-Oncology Network-ION Resource Model, 300-2344 (to D.M.P.); Alexander and Margaret Stewart Trust (Johns Hopkins University School of Medicine); GSRIG-015 (American Society of Colon and Rectal Surgeons to E.M.H.); The Netherlands Organization for Scientific Research (NWO 825.11.03 and 016.166.089 to A.B.); and a grant from the Institute Mérix (to C.L.S. and D.M.P.). D.M.P. discloses consulting relationships with Aduro Biotech, Amgen, Astra Zeneca, Bayer, Compugen, DNAxix, Five Prime, GlaxoSmithKline, ImmuneXcite, Jounce Therapeutics, Neximmune, Pfizer, Rock Springs Capital, Sanofi, Tizona, Janssen, Merck, Astellas, Fix Bio, Envax, and DNAx. D.M.P. receives research support from Bristol-Myers Squibb, Compugen, Envaxx, and Potenza. D.M.P. is a scientific advisory board member for Immunomic Therapeutics. D.M.P. shares intellectual property with Aduro Biotech, Bristol-Myers Squibb, Compugen, and Immunomic Therapeutics. All other authors declare no competing interests. C.L.S., D.M.P., C.M.D., Myers Squibb, Compugen, and Immunomic Therapeutics. All other authors declare no competing interests. C.L.S., D.M.P., C.M.D., Einstein, and Potenza. D.M.P. is a scientific advisory board member for Immunomic Therapeutics. D.M.P. shares intellectual property with Aduro Biotech, Bristol-Myers Squibb, Compugen, and Immunomic Therapeutics. All other authors declare no competing interests. C.L.S., D.M.P., C.M.D., and E.C.W. are inventors on patent application PCT/US2014/055123 submitted by Johns Hopkins University that covers use of biofilm formation to define risk for colon cancer.

**SUPPLEMENTARY MATERIALS**

www.sciencemag.org/content/359/6375/592/suppl/DC1

Materials and Methods

Figs. S1 to S11

Tables S1 to S7

References (15-16)

15 June 2016; resubmitted 28 September 2017

Accepted 28 December 2017

10.1126/science.aah3648
Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria


*Science* 359 (6375), 592-597.
DOI: 10.1126/science.aah3648

Biofilms provide refuge for cancerous bacteria

Familial adenomatous polyposis (FAP) causes benign polyps along the colon. If left untreated, FAP leads to a high incidence of colon cancer. To understand how polyps influence tumor formation, Dejea *et al.* examined the colonic mucosa of FAP patients. They discovered biofilms containing the carcinogenic versions of the bacterial species *Escherichia coli* and *Bacteroides fragilis*. Colon tissue from FAP patients exhibited greater expression of two bacterial genes that produce secreted oncotoxins. Studies in mice showed that specific bacteria could work together to induce colon inflammation and tumor formation.

*Science*, this issue p. 592