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Colon cancer-associated B2 *Escherichia coli* colonize gut mucosa and promote cell proliferation

Jennifer Raisch, Emmanuel Buc, Mathilde Bonnet, Pierre Sauvanet, Emilie Vazeille, Amélie de Vallée, Pierre Déchelotte, Claude Darcha, Denis Pezet, Richard Bonnet, Marie-Agnès Bringer, Arlette Darfeuille-Michaud

Jennifer Raisch, Emmanuel Buc, Mathilde Bonnet, Pierre Sauvanet, Emilie Vazeille, Amélie de Vallée, Denis Pezet, Richard Bonnet, Marie-Agnès Bringer, Arlette Darfeuille-Michaud, Clermont Université, UMR1071 Inserm/Université d'Auvergne and INRA USC2018, 63000 Clermont-Ferrand, France
Emmanuel Buc, Pierre Sauvanet, Emilie Vazeille, Pierre Déchelotte, Claude Darcha, Denis Pezet, Richard Bonnet, Centre Hospitalier Universitaire, 63000 Clermont-Ferrand, France
Author contributions: Raisch J, Buc E, Bringer MA and Darfeuille-Michaud A conceived and designed the study, analysed data and drafted the manuscript; Bringer MA and Darfeuille-Michaud A contributed equally to the design and data analyses of this study; Raisch J, Buc E, Bonnet M, Vazeille E and Bringer MA performed experiments; Raisch J and Buc E contributed equally to this study; Buc E, Sauvanet P, de Vallée A, Pezet D and Bonnet R carried out the sample collection and the sample processing; Déchelotte P and Darcha C performed immunohistology analyses.

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Correspondence to: Marie-Agnès Bringer, PhD, Clermont Université, UMR1071 Inserm/Université d'Auvergne and INRA USC2018, 63000 Clermont-Ferrand, France. m-agnes.bringer@udamail.fr

Telephone: +33-4-7317 8371 Fax: +33-4-7317 8371

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determined by PCR. Adhesion and invasion experiments were performed with I-407 intestinal epithelial cells using gentamicin protection assay. Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression in T84 intestinal epithelial cells was measured by enzyme-linked immunosorbent assay and by Western Blot. Gut colonization, inflammation and pro-carcinogenic potential were assessed in a chronic infection model using CEABAC10 transgenic mice. Cell proliferation was analyzed by real-time mRNA quantification of *PCNA* and immunohistochemistry staining of Ki67.

RESULTS: Analysis of mucosa-associated *E. coli* from colon cancer and diverticulosis specimens showed that whatever the origin of the *E. coli* strains, 86% of cyclomodulin-positive *E. coli* belonged to B2 phylogroup and most harbored polyketide synthase (*pks*) island, which encodes colibactin, and/or cytotoxic necrotizing factor (*cnf*) genes. *In vitro* assays using I-407 intestinal epithelial cells revealed that mucosa-associated B2 *E. coli* strains were poorly adherent and invasive. However, mucosa-associated B2 *E. coli* similarly to Crohn's disease-associated *E. coli* are able to induce CEACAM6 expression in T84 intestinal epithelial cells. In addition, *in vivo* experiments using a chronic infection model of CEACAM6 expressing mice showed that B2 *E. coli* strain 11G5 isolated from colon cancer is able to highly persist in the gut, and to induce colon inflammation, epithelial damages and cell proliferation.

CONCLUSION: In conclusion, these data bring new insights into the ability of *E. coli* isolated from patients with colon cancer to establish persistent colonization, exacerbate inflammation and trigger carcinogenesis.

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Key words: B2 *Escherichia coli*; Carcinoembryonic antigen-related cell adhesion molecule 6; Cell proliferation;

Abstract

AIM: To provide further insight into the characterization of mucosa-associated *Escherichia coli* (*E. coli*) isolated from the colonic mucosa of cancer patients.

METHODS: Phylogroups and the presence of cyclomodulin-encoding genes of mucosa-associated *E. coli* from colon cancer and diverticulosis specimens were

Colon cancer; Polyketide synthase genomic island

Core tip: Tumors and mucosa of patients with colon cancer are abnormally colonized by *Escherichia coli* (*E. coli*) belonging to B2 phylogroup. The aim of the present study was to provide further insight into the characterization of colon cancer-associated *E. coli*. Despite their poor ability to adhere to and to invade intestinal epithelial cells *in vitro*, we showed that colon cancer-associated *E. coli* induce carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression, a receptor involved in adhesion of pathogenic *E. coli*. These bacteria were also able to persist and promote low grade inflammation and cell proliferation, in a chronic infection model of CEACAM6 expressing mice, highlighting their oncogenic potential.

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INTRODUCTION

Colorectal cancer (CRC) is the fourth leading cause of cancer death and is responsible for about 610000 deaths per year worldwide^[1]. Although many etiologic genetic changes are associated with progression from adenomatous lesions to invasive carcinoma^[2], the specific causative factors in the development of sporadic CRC remain unclear. Accumulating evidence supports that inflammation and gut microbial communities influence the development of colorectal carcinoma^[3-5]. Two theories have emerged to explain the contribution of bacteria in CRC: (1) the “alpha bug” concept, wherein select members of a microbial community with virulence and pro-carcinogenic features are capable of remodeling the microbiome as a whole to drive pro-inflammatory immune responses and colonic epithelial cell transformation leading to cancer^[6]; and (2) the “driver-passenger” concept, wherein certain indigenous intestinal bacteria, termed “bacteria drivers”, initiate CRC by inducing epithelial DNA damages: the resulting tumorigenesis induces intestinal niche alterations that promote the proliferation of passenger opportunistic bacteria with a growth advantage in the tumour microenvironment^[7].

Dysbiosis of the intestinal microbiota has been observed in CRC patients. Recent pyrosequencing data of CRC-associated bacterial microbiota have revealed, in particular, over-representation of some bacteria such as *Bacteroides/Prevotella*, *Faecalibacterium* and *Fusobacterium*^[8,9]. In addition, independent studies show that colonic adenomas, carcinomas and the mucosa of CRC patients are abnormally colonized by high numbers of adherent *Es-*

cherichia coli (*E. coli*) compared to controls^[10-12]. It has been suggested that the role of *E. coli* in CRC promotion and development is related to chronic inflammation. Inflammation can result from bacterial infection, *via* its effects on both the host and the microbiota, in particular by promoting the expansion of *E. coli*, which actively contribute to the accumulation of mutations resulting from DNA damages induced by genotoxins, or by downregulating host DNA mismatch repair proteins^[3,11,13]. In particular, *E. coli* strains harboring the polyketide synthase (*pks*) genotoxic island, which are found in a significantly high percentage of inflammatory bowel disease (IBD) and CRC patients, can promote invasive carcinoma in mono-colonized azoxymethane (AOM)-treated *I10^{-/-}* mice^[3]. In addition, certain pathogenic bacteria can also be involved in cancer development, like, for example enterotoxigenic *Bacteroides fragilis* (ETBF), a common human commensal bacterium that is associated with colon cancer^[14]. ETBF-induced chronic inflammation and tumorigenesis in *Ap^{c^{Min/+}}* mice (a mouse model of familial adenomatous polyposis) involve the induction of the polyamine catabolic enzyme spermine oxidase, which causes DNA damages and uncontrolled cell proliferation in intestinal epithelial cells^[15].

Patients with IBD have an increased risk of colon cancer and small bowel adenocarcinoma^[16,17]. As in colon cancer patients, dysbiosis toward selected micro-organisms and decreased complexity of commensal bacteria have been observed in patients with Crohn's disease (CD) and ulcerative colitis (UC), but it is not clear whether dysbiosis contributes to the development of IBD or is instead a consequence of the disease. Patients with IBD, compared to healthy controls, have fewer bacteria with anti-inflammatory properties and/or more bacteria with pro-inflammatory properties. Several metagenomic-based studies reported that members of the phyla Bacteroidetes and Firmicutes were reduced in patients with CD or UC^[18-20]. Among the Firmicutes, *Faecalibacterium prausnitzii* has anti-inflammatory properties; its numbers are reduced in patients with CD and associated with a risk of post-resection recurrence of ileal CD^[20]. In contrast, a greater relative abundance of *Enterobacteriaceae*, mostly *E. coli* belonging to the B2 phylogenetic group, has been reported in CD patients more notably on mucosa-associated microbiota than in fecal samples^[10,18,21-24]. Intestinal colonization by *E. coli* correlates with bacterial adhesion of CD-associated *E. coli* strains to intestinal epithelial cells^[10,25]. CD-associated *E. coli* share abilities to adhere to and to invade intestinal epithelial cells and to survive within macrophages^[26,27] and they are termed accordingly adherent-invasive *E. coli* (AIEC). The abnormal colonization of CD mucosa by AIEC involves abnormal expression of a host receptor, the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)^[28]. Interestingly, CEACAM6 is not only abnormally expressed in the ileum of patients with CD^[28] but expression of this molecule is also up-regulated in proliferating cells in adenomas and colorectal tumors^[29,30]. However, the origin

of CEACAM6 surexpression in colon cancer is not yet clearly understood.

The aim of the present study was to provide further insight into the characterization of mucosa-associated *E. coli* isolated from the colonic mucosa of cancer patients. We determined their ability to interact with intestinal epithelial cells, with a particular focus on biofilm formation and the presence of cyclomodulin-encoding genes, and to induce CEACAM6 expression in intestinal epithelial cells. Finally, using CEABAC10 transgenic mice expressing human CEACAMs, we assessed the effects of long-term chronic infection by the colon cancer-associated *E. coli* strain 11G5 for its ability to colonize the gut, to potentiate inflammation and to induce cell proliferation.

MATERIALS AND METHODS

Ethical considerations

Ethical approval for the study was granted by the Clermont-Ferrand research ethics committee. This IRB allowed for the waiver of written consent and approved the process of obtaining verbal consent from potential subjects, because the research involved no procedures for which written consent is normally required outside of the research context and presents no risk of harm to subjects. The biological samples were collected from colon resections, which were required for the treatment of patients. The investigators explained the study to the potential subject verbally, providing all pertinent information such as purpose, procedures and putative risks. Following this verbal explanation, the potential subject was provided with a study information sheet. After allowing the potential subject time to read the study information sheet, the investigators answered any additional questions the subject may have. A verbal agreement to participate in the research was obtained for all patients included in the study. The dates of verbal consent were tracked in a non-identifiable manner.

Patients

Eighty-one patients were studied between March 2007 and July 2010 at the University hospital of Clermont-Ferrand, France, 48 with colon cancer (adenocarcinoma), and 33 with diverticulosis. For ethical considerations no surgical specimens from healthy patients were included and diverticulosis specimens were used as non-neoplastic controls. Among patients with diverticulosis, we excluded those with acute or chronic inflammation at the time of surgery, and those with stenosis to avoid potential consequences of inflammation on gut microbiota. Sex ratio (M/F) was 1.22 and 0.74 for CRC and diverticulosis patients respectively. The age range was 35-95 years for cancer patients (median age, 70 years and average age, 67 years) and 34-81 years for controls (median age, 58 years and average age, 60 years). Biopsies were taken on non-involved mucosa near the site of malignant tumors in resected colon. Pathologic analysis confirmed the neoplastic features of the samples. Bowel preparation was

by oral sodium picosulfate or oral polyethylene glycol the evening before surgery. All resection patients had received cefoxitin (2 g intravenously) at the time of incision and none had received antibiotics in the 4 wk before sampling. Ethical approval for the study was granted by the Clermont-Ferrand Research Ethics Committee.

Biopsy treatment for determination of associated *E. coli* numbers

The mucosal biopsy specimens were transported on ice to the laboratory. The samples were weighed (50 to 100 mg each) and washed thoroughly three times in 10 mL PBS to remove most of the fecal bacteria. To determine the number of associated bacteria, samples were crushed (Ultra-Turrax, IKA) and incubated for 15 minutes in the presence of 0.1% Triton X-100. Ten-fold dilutions of the lysates were then plated on Drigalski agar and chromogenic agar chromID CPS3® (bioMérieux), which allow the identification of *E. coli* isolates. Colony forming units (CFUs) of *E. coli* isolates were collected after 24 h of incubation at 37 °C and the identification of bacteria was confirmed with the automated Vitek II® (bioMérieux) system. When possible a maximum of 96 *E. coli* isolates per sample were collected for molecular typing. The bacteria were subcultured for 24 h at 37 °C in 96-well plates in Luria Bertani medium, supplemented with 15% glycerol and then stored at -80 °C.

Molecular phylogenetic grouping and PCR assay for cyclomodulin and adhesin-encoding genes

Ten isolates per sample were typed with molecular methods to identify the *E. coli* isolates (*E. coli* genotypes) colonizing the samples. Two genotyping methods were used: an “Enterobacterial Repetitive Intergenic Consensus” sequence (ERIC)-PCR using primer ERIC2 (5’ AAGTAAGTGACTGGGGTGAGCG 3’) and a “Random Amplified Polymorphic DNA” (RAPD)-PCR using primer 1283 (5’ GCGATCCCCA 3’)^[31,32]. For each isolate, one representative isolate was subsequently analysed and stored at -80 °C in Luria-Bertani medium supplemented with 15% glycerol. *E. coli* isolates were then classified according to the *E. coli* Reference Collection system into phylogenetic groups A, B1, B2, and D using a multiplex PCR technique^[33]. Strain RS218, which harbors all the genes targeted by the multiplex PCR, was used as positive control. To investigate the presence of cyclomodulin (*pks* genomic island, *cdt*, *cnf*, and *cjf*)-, adhesin (*afa*, *afa/dr* and *aaf*)-, or intimin (*eae*)-encoding genes, PCR assays were performed using primers listed in Table 1.

Cell culture

The intestinal epithelial cell lines T84 (ATCC, CCL-248) and Intestine-407 (I-407; ATCC, CCL-6) were maintained in an atmosphere containing 5% CO₂ at 37 °C in the culture medium recommended by ATCC. For infection assays, cells were seeded in 24-well plates at a density of 2 × 10⁵/cm².

Table 1 List of primers used for PCR assays

Primer name	Sequence (5'-3')	Region specific for	Ref.
afa-f	CGGCTTTCTGCTGAACTGGCAGGC	<i>afaC</i>	[49]
afa-r	CCGTCAGCCCCACGGCAGACC		
afa1	GCTGGGCAGCAAACCTGATAACTCTC	<i>afaBC</i>	
afa2	CATCAAGCTGTTTGTTCGTCGCCCG		
afaE-f ¹	TTAGACCGTACTGTTGTGTACCCC		
	C		
afaE1-r	CATCGCCCGTCGCAGAGCCCAT	<i>afaE1</i>	
afaE2-r	GTTTCCAGTAGACTGGAATGAAG	<i>afaE2</i>	
	C		
afaE3-r	CCCTATTGTTGTCGCTGATCAGGAA	<i>afaE3</i>	
	G		
daaE-r	CGGCTAGTCATATATAGATTGTCTG	<i>daaE</i>	
	C		
afaE5-f	TCAACTCACCCAGTAGCCCCAG	<i>afaE5</i>	
afaE5-r	AGGAAGTGGTAGCACCGGTACG		
afaE7-f	GCTAAATCAACTGTTGATGTT	<i>afaE7</i>	
afaE7-r	GGACAATCCAAATGGCGAATTA		
afaE8-f	CTAACTTGCCATGCTGTGACAGTA	<i>afaE8</i>	
afaE8-r	TTATCCCTGCGTAGTTGTGAATC		
aggR1	CTAATTGTACAATCGATGTA	<i>aggR</i>	
aggR2	CTGAAGTAATCTGTGAA		[50]
pksORF9-10.1KJ	ATTTCGATAGCGTACCCCAAC	<i>clbK-clbJ</i>	
pksORF9-10.2KJ	TAAGCGTCTGGAATGCAGTG		[51]
CNF-1s	GGGGGAAGTACAGAAGAATTA	<i>cnf1</i>	
CNF-1as	TTGCCGTCCACTCTCACCAGT		
CNF-2s	TATCATACGGCAGGAGGAAGCACC	<i>cnf2</i>	
CNF-2as	GTCACAATAGACAATAATTTCCG		
CNF3-3D	TAACGTAATTAGCAAAGA	<i>cnf3</i>	
CNF-3as	GTCTTCATTACTACAGT		
CDT-s1	GAAAGTAAATGGAATATAAAATGTC	<i>cdtB-II</i> , <i>cdtB-III</i> , <i>cdtB-V</i>	[51]
	CG		
CDT-as1	AAATCACCAAGAATCATCCAGTTA	<i>cdtB-V</i>	
CDT-II as ²	TTTGTGTGCGCGCGCTGGTGAAA	<i>cdtB-II</i>	
CDT-III as ²	TTTGTGTGCGTGCAGCAGGGAAAA		
CDT-s2	GAAAATAAATGGAACACACATGTC	<i>cdtB-I</i> , <i>cdtB-IV</i>	
	CG		
CDT-as2	AAATCTCCTGCAATCATCCAGTTA		
CDT-I s	CAATAGTCGCCACAGGA	<i>cdtB-I</i>	
CDT-I as	ATAATCAAGAACACCACCAC		
CDT-IVs	CCTGATGGTTCAGGAGGCTGGTTC	<i>cdtB-IV</i>	
CDT-IVas	TTGCTCCAGAATCTATACCT		
P105	GTCAACGAACATTAGATTAT	<i>cdtC-V</i>	
c2767r	ATGGTCATGCTTTGTTATAT		
cif-int-s	AACAGATGGCAACAGACTGG	<i>cif</i>	
cif-int-as	AGTCAATGCTTTATGCGTCAT		[51]
clbQ-F	TTGTATAGTTACACAACATATTTC		
clbQ-R	CCTGTTAGCTTTCGTTTC		
			This study
MiclbQaa-dA7-F	CATTAAATCATCAAATTAAC-GAATTCTATTACACAACAAGGAGT-GGGACGCACTGGCATTTAATAAC-GCGTC		
MiclbQaa-dA7-R	GATGATGGAACAGCCATATCTATT-GCTCCTTGATAGTTACACAAC-TATTTTAAATCACTTTACTTTTATC		

¹The afa-f and afa-r primers detect all *afa*-positive strains, irrespective of the *afaE* subtype and the binding properties of the adhesins (Afa/Dr+ and Afa/Dr-). Specific detection of strains encoding Afa/Dr+ adhesins was performed using afa-1 and afa-2 primers; ²Primers used with CDT-s1 primer.

Adhesion and invasion assays

I -407 cells were infected at a multiplicity of infection (MOI) of 10 bacteria per cell. Adhesion and invasion assays were performed as previously described^[27]. For adhesion assays, monolayers were washed five times in PBS after 3 h of incubation at 37 °C. To determine the numbers of intracellular bacteria (invasion assay), cell culture medium containing gentamicin at a concentration of 200 µg/mL was added for 1 h to kill extracellular bacteria. The epithelial cells were then lysed with 1% Triton X-100 in deionized water. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 min. Samples were diluted and plated onto LB agar plates to determine the number of CFU.

Biofilm formation assays

Biofilm formation assays on abiotic surface were performed using a previously described method^[34]. Biofilm measurements were calculated using the formula SBF = (AB-CW)/G, in which SBF is the specific biofilm formation, AB the OD_{570nm} of the attached and stained bacteria, CW the OD_{570nm} of the stained control wells containing only bacteria-free medium (to eliminate unspecific or abiotic OD values), and G is the OD_{630nm} of cell growth in broth. Assays were performed in triplicate.

Biofilm formation assays were also performed using PFA-fixed I -407 cells^[34]. Briefly, confluent I -407 monolayers were fixed for 15 min in 3.7% PFA-PBS. The fixed cells were washed and infected with bacteria in M63 minimal medium and incubated overnight at 30 °C without shaking. For visualization, infected epithelial cells were fixed for 15 min in 3.7% PFA-PBS and permeabilized in PBS-0.1% Triton X-100. Coverslips were incubated with goat anti-*E. coli* polyclonal antibodies (dilution 1/100, AbD serotec) and Alexa 488-labeled anti-goat antibodies (dilution 1/300, Invitrogen). Actin cytoskeleton was stained using TRITC-labelled-phalloidin (Sigma). The slides were examined with a Zeiss LSM 510 Meta confocal microscope (ICCF platform, Clermont-Ferrand, France).

Mouse model infection

CEABAC10 transgenic mice (heterozygote^[35]) were housed in specific pathogen-free conditions in the animal care facility at Université d'Auvergne, Clermont-Ferrand, France). Mice from the same generation were used for experimentation. Animal protocols were approved by the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

A total of 22 female 10 wk-old CEABAC10 mice were divided into three groups: non-infected control group (*n* = 6), 11G5-infected group (*n* = 7) and AIEC LF82-infected group (*n* = 9). The animals were pretreated once before the first infection cycle by oral administration of the broad-spectrum antibiotic streptomycin (20 mg intragastric per mouse) to disrupt normal resident bacterial flora in the intestinal tract and received a dose of 0.25%

Table 2 Histological grading of intestinal inflammation

Symptoms	Characteristics
Infiltration of inflammatory cells	
0	Rare inflammatory cells in the lamina propria
1	Increased numbers of inflammatory cells, including neutrophils in the lamina propria
2	Confluence of inflammatory cells extending into the submucosa
3	Transmural extension of the inflammatory cell infiltrate
Infiltration of epithelium by polynuclear cells	
0	No infiltration
1	Surface
2	Inside the crypt
3	Cryptic abscess
Severity of epithelial damage	
0	Absence of mucosal damage
1	Lymphoepithelial lesions
2	Mucosal erosion/ulceration
3	Extensive mucosal damage and extension through deeper structures of the bowel wall
Surface of epithelial damage	
0	Normal
1	Focal
2	Wide

Table 3 List of primers used for RT qPCR assays

Primer name	Sequence (5'-3')	Region specific for	Ref.
mmu-26s-FW	TGTCATTTCGGAACATTGTAG	S26	This study
mmu-26s-RV	GGCTTTGGTGGAGGTC		
mmu-PCNA-FW	CCACATTGGAGATGCTGTTG	PCNA	
mmu-PCNA-RV	CAGTGGAGTGGCTTTTGTGA		

(wt/vol) of dextran sulfate sodium (DSS; molecular mass = 36000-50000 daltons; MP Biomedicals) in drinking water 3 d before infection to increase the accessibility of bacteria to the surface of the epithelial layer. The administration of 0.25% DSS did not affect the body weight of mice and did not induce clinical symptoms of colitis^[36]. The mice were subjected to 8 consecutive cycles of infection. For each infection cycle, they were orally challenged twice a week by intra-gastric gavage with 2×10^8 bacteria for a 3-wk period. This infection period was followed by a 1-wk recovery period without infection. For each cycle, 5 d after the last oral bacterial infection, fresh fecal pellets (100-200 mg) were collected and suspended in PBS to evaluate colonization. After serial dilution, bacteria were enumerated by plating on LB agar medium containing 50 µg/mL of kanamycin and 50 µg/mL of ampicillin isolate 11G5 bacteria or 100 µg/mL of ampicillin and 20 µg/mL of erythromycin to isolate LF82 bacteria, and incubated at 37 °C overnight.

Histological grading of intestinal inflammation and epithelial damages

After mouse sacrifice, the entire colon was excised and rolls of the proximal colon were fixed in buffered 4% formalin, paraffin-embedded, cut into 5-µm slices, and stained with hematoxylin/eosin/safranin. The histological severity of colitis was graded in a blinded fashion by a GI pathologist. The tissue samples were assessed for

the extent and depth of inflammation and the extent of epithelial damages, as presented in Table 2. The histology score corresponds to the sum of each item.

Immunohistochemistry

For immunohistochemical staining of mouse Ki-67, heat-induced epitope retrieval was performed using sodium citrate buffer (pH 6.0). Ki-67 antigen was detected using anti-mouse Ki-67 polyclonal antibodies (Leica) and revealed with Vectastain ABC kit (Vector) and DAB detection kit (Invitrogen). The sections were counterstained using Gill's hematoxylin (Vector).

Real-time mRNA quantification

Total RNAs were extracted from tissue using a Nucleospin® RNA/Protein extraction kit (Macherey-Nagel GmbH & Co). RNA samples were subjected to reverse transcription using High-Capacity cDNA Reverse Transcription Kit and non-specific random hexamer primers (Applied Biosystems) and quantification was performed using FastStart SYBR® Green Master kit (Roche Applied Science). The primer sequences used are given in Table 3. Gene expression values were calculated based on the $\Delta\Delta C_t$ method.

Enzyme-linked immunosorbent assay

T84 colon epithelial cells were infected with bacteria for 6h at a MOI of 100 bacteria per cell. The amount of CEACAM6 on whole cell protein extracts was determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (R and D systems).

Western immunoblotting

T84 colon epithelial cells were infected for 6h at a MOI of 100 bacteria per cell. Whole-cell protein extracts were prepared by adding NP-40 lysis buffer. Protein concen-

Table 4 Distribution of *Escherichia coli* strains producing various cyclomodulins according to phylogroups and specimen origins *n* (%)

Phylogroups		<i>E. coli</i> strains exhibiting cyclomodulin-encoding genes			
		<i>pks</i>	<i>cnf</i>	<i>cdt</i>	<i>cif</i>
Colon cancer-associated <i>E. coli</i> strains (<i>n</i> = 88) ¹	A (<i>n</i> = 20)	0 (0)	0 (0)	0 (0)	2 (2)
	B1 (<i>n</i> = 14)	0 (0)	1 (1)	1 (1)	1 (1)
	B2 (<i>n</i> = 38)	23 (26)	16 (18)	4 (11)	0 (0)
	D (<i>n</i> = 16)	0 (0)	1 (1)	1 (1)	0 (0)
Diverticulosis-associated <i>E. coli</i> strains (<i>n</i> = 46) ²	A (<i>n</i> = 17)	0 (0)	0 (0)	0 (0)	0 (0)
	B1 (<i>n</i> = 3)	0 (0)	0 (0)	0 (0)	0 (0)
	B2 (<i>n</i> = 15)	6 (13)	4 (9)	0 (0)	0 (0)
	D (<i>n</i> = 11)	0 (0)	0 (0)	0 (0)	0 (0)

¹Isolated from 48 colon cancer patients; ²Isolated from 33 patients with diverticulosis. *E. coli*: *Escherichia coli*.

Table 5 Phylogroup distribution of cyclomodulin-positive *Escherichia coli* strains according to specimen origins *n* (%)

	Phylogroups			
	A	B1	B2	D
Cyclomodulin-positive <i>E. coli</i> strains isolated from colon cancer patients	2 (6)	2 (6)	26 (84)	1 (3)
Cyclomodulin-positive <i>E. coli</i> strains isolated from patients with diverticulosis	0 (0)	0 (0)	6 (100)	0 (0)
Cyclomodulin-positive <i>E. coli</i> strains	2 (5)	2 (5)	32 (86)	1 (3)

E. coli: *Escherichia coli*.

trations were determined by Bradford assay. Total proteins were subjected to SDS-PAGE on 12% gels.

Proteins were electroblotted onto nitrocellulose membranes (Amersham International), and the membranes were immunoblotted for CEACAM6 (mouse anti-CEACAM6, dilution 1/1.000, Genovac) and GAPDH (rabbit anti-GAPDH; dilution 1/1.000, Cell Signaling). Immunoreactants were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibodies, ECL reagents (Amersham Biosciences) and autoradiography.

Statistical analysis

Where appropriate, nonparametric data were expressed by median value (range). Normally distributed data were expressed as means. Error bars represent SEM. Statistical analysis was done using ANOVA (Histopathological score and RT-qPCR), Mann Whitney (adhesion, invasion and biofilm assays) using GraphPad prism5 software. A *P* value of 0.05 was considered significant.

RESULTS

Most cyclomodulin-producing *E. coli* strains associated with colon cancer and diverticulosis belong to B2 phylogroup

The analysis of the presence of cyclomodulin-encoding genes, *pks* island coding for colibactin, and/or *cnf* and/or *cdt* and/or *cif* genes coding for cytotoxic necrotizing factor (CNF), cytolethal distending toxin (CDT), and cycle-inhibiting factor (Cif) indicated that, whatever the origin of the *E. coli* strains, either from colon cancer or diverticulosis samples, 86% of cyclomodulin-positive *E. coli* belonged to B2 phylogroup (Tables 4 and 5). Among *E. coli* strains isolated from colon cancer specimens, B2 *E. coli*

strains harboring *pks*, *cnf* and *cdt* genes represented 26%, 18% and 11% of the total strains isolated, respectively (Tables 4 and 6). Among *E. coli* strains isolated from patients with diverticulosis, *pks*-positive B2 *E. coli* strains represented 13%, and *cnf*-positive B2 *E. coli* strains 9% of the total strains isolated. Although a higher prevalence of B2 *E. coli* strains harboring *pks* or *cnf* genes was observed in colon cancer patients than in patients with diverticulosis, this was not significant (*P* = 0.06 for both *pks* and *cnf* genes). Of interest, all but two *cnf* positive strains also harbored *pks* and all *cnf*- and *pks*-positive *E. coli* strains belonged to the B2 phylogroup.

Low level of adhesion and invasion but high ability to form biofilm of B2 *E. coli* strains isolated from colon cancer or diverticulosis patients

The analysis of the ability of *E. coli* strains to adhere to and to invade intestinal epithelial cells was restricted to B2 *E. coli*, which were the main cyclomodulin producers in our study. Of note, due to cytolytic activity of hemolysin on cultured cells, hemolysin-positive *E. coli* strains were not tested. Results showed that B2 phylogroup *E. coli* strains isolated from colon cancer and from diverticulosis displayed low levels of adhesion to I-407 intestinal epithelial cells (Figure 1A). Compared to the adhesion level of the AIEC reference strain LF82, for which a mean adhesion index of 53.23 ± 6.63 was observed, the adhesion levels of all *E. coli* strains isolated from colon cancer (except *E. coli* strain 14H4, which had a mean adhesion level of 25.76 ± 5.06) or from diverticulosis ranged from 0.15 ± 0.02 to 4.04 ± 1.24 or from 0.10 ± 0.04 to 9.17 ± 3.40 , respectively. Microscopy examination after Giemsa staining showed a diffuse adhesion pattern (data not shown), and we therefore searched for adhesive factor-encoding genes associated with diffusely adhering

Table 6 Hemolysin expression and presence of cyclomodulin- and adhesin-encoding genes in B2 phylogroup *Escherichia coli* strains

<i>E. coli</i> strains	Haemolytic phenotype ¹	Cyclomodulin-encoding genes				Adhesin-encoding genes		
		<i>pks</i>	<i>cnf</i>	<i>cdtB</i>	<i>cif</i>	<i>afa</i>	<i>dra</i>	<i>aagR</i>
Colon cancer								
1C12	+	+	<i>cnf1</i>	-	-	-	-	-
1D2	-	+	-	-	-	-	-	-
1F8	-	-	-	-	-	-	-	-
2D5	-	-	-	-	-	-	-	-
2F8	+	+	<i>cnf1</i>	<i>cdtB-IV</i>	-	-	-	-
2G2	+	-	-	-	-	-	-	-
4A9	-	-	-	-	-	-	-	-
6A8	+	+	<i>cnf1</i>	-	-	-	-	-
6G8	+	+	<i>cnf1</i>	-	-	-	-	-
6G10	-	-	-	<i>cdtB-IV</i>	-	-	-	-
6G11	-	-	-	-	-	-	-	-
7G1	-	-	-	-	-	-	-	-
7G2	+	+	<i>cnf1</i>	-	-	-	-	-
8A9	+	+	-	-	-	-	-	-
8A10	-	-	-	-	-	-	-	-
8F1	+	-	<i>cnf1</i>	-	-	-	-	-
8G8	-	-	-	-	-	-	-	-
9G5	+	+	<i>cnf1</i>	-	-	+	-	-
10D12	-	+	-	-	-	-	-	-
10E9	+	+	<i>cnf1</i>	-	-	-	-	-
11F1	-	-	-	-	-	-	-	-
11G5	-	+	-	-	-	-	-	-
12B1	+	+	<i>cnf1</i>	-	-	-	-	-
13H2	+	+	<i>cnf1</i>	-	-	-	-	-
14H4	-	+	-	-	-	+	+ (<i>afaE5</i>)	-
15D1	+	-	-	-	-	-	-	-
15D3	+	+	<i>cnf1</i>	-	-	-	-	-
16C1	-	+	-	-	-	-	-	-
17G3	+	-	<i>cnf1</i>	-	-	-	-	-
18C3	+	+	<i>cnf1</i>	-	-	-	-	-
18C5	-	-	-	-	-	-	-	-
18H5	-	+	-	-	-	-	-	-
19D12	+	+	<i>cnf1</i>	-	-	-	-	-
19G1	-	+	-	-	-	-	-	-
19H2	+	+	<i>cnf1</i>	<i>cdtB-IV</i>	-	-	-	-
20B6	+	+	<i>cnf1</i>	-	-	-	-	-
20C3	-	+	-	<i>cdtB- I</i>	-	-	-	-
20D5	-	-	-	-	-	-	-	-
Diverticulosis								
1D5	-	-	-	-	-	-	-	-
4D5	+	+	<i>cnf1</i>	-	-	-	-	-
9D7	-	-	-	-	-	-	-	-
9F1	-	-	-	-	-	-	-	-
9F4	-	-	-	-	-	-	-	-
11D9	+	+	<i>cnf1</i>	-	-	-	-	-
12H1	-	+	-	-	-	-	-	-
13D1	+	+	<i>cnf1</i>	-	-	-	-	-
15C1	-	-	-	-	-	-	-	-
16A4	-	+	-	-	-	-	-	-
16A8	-	-	-	-	-	-	-	-
17C1	-	-	-	-	-	-	-	-
17F2	-	-	-	-	-	-	-	-
17E1	-	-	-	-	-	-	-	-
18E6	+	+	<i>cnf1</i>	-	-	-	-	-

¹Alpha-hemolysin expression was analysed after over-night growth of bacteria on agar plates containing sheep blood. *cnf*: Cytotoxic necrotizing factor; *cdt*: Cytolethal distending toxins; *cif*: Cycle-inhibiting factor; *afa*: Afimbrial adhesion. *E. coli*: *Escherichia coli*.

E. coli (DAEC) strains (*i.e.*, Afa and Afa/Dr adhesin-encoding genes). None of the B2 *E. coli* strains tested was positive for *afa* or *afa/dr* genes except the highly adherent *E. coli* strain 14H4 isolated from colon cancer (Table 6). Of note, none of the B2 *E. coli* strains tested was positive

for *eae* gene coding for intimin of enteropathogenic *E. coli* or for *aaf* gene coding for the adhesive factor AAF of enteroaggregative *E. coli*, indicating that B2 *E. coli* strains studied do not belong to these *E. coli* pathovars. Analysis of the ability of bacteria to invade I-407 cells showed

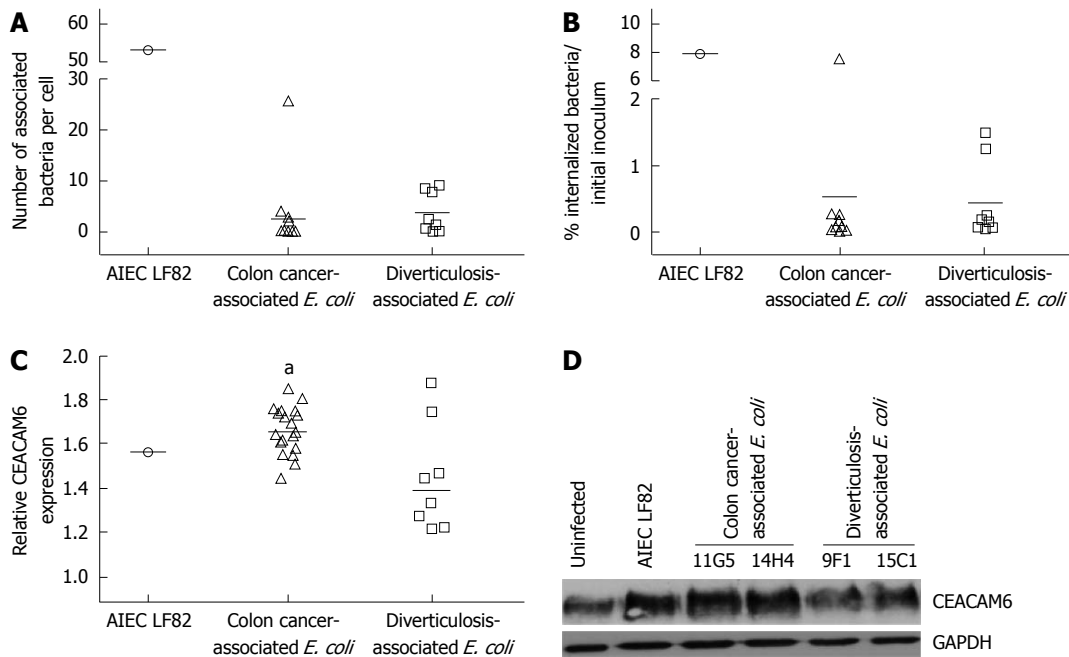


Figure 1 Adhesion, invasion and ability to induce carcinoembryonic antigen-related cell adhesion molecule 6 expression of B2 *Escherichia coli* strains. A and B: Ability of colon cancer- and diverticulosis-associated B2 *Escherichia coli* (*E. coli*) strains and AIEC strain LF82 to adhere to and to invade I-407 intestinal epithelial cells. A: Adhesion. Results are expressed as number of associated bacteria per cell after 3 h of infection; B: Invasion. Results are expressed as percentage of inoculum surviving after 3 h of infection and 1 h of gentamicin treatment; C and D: Induction of carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expression in colon epithelial T84 cells infected for 6 h with colon cancer- and diverticulosis-associated B2 *E. coli* strains and AIEC strain LF82. C: Quantitative dosage of CEACAM6 by ELISA. Results are expressed as amounts of CEACAM6 in stimulated or infected cells relative to untreated cells. ^a $P \leq 0.05$ vs diverticulosis associated *E. coli*. D: CEACAM6 expression analysis by Western Blot. *E. coli* strains 11G5 and 14H4 were isolated from colon cancer patients and *E. coli* strains 9F1 and 15C1 from patients with diverticulosis.

that whatever the origin of the B2 *E. coli* strains their invasion levels were very low, ranging from 0.02% to 1.49%, except strain 14H4, for which invasion level was similar to that of the AIEC strain LF82 (Figure 1B).

We also investigated the ability of B2 *E. coli* isolated from colon cancer to induce CEACAM6 expression as abnormal CEACAM6 expression was shown to promote gut colonization by AIEC^[36] and AIEC bacteria were reported to be able to induce increased CEACAM6 expression in intestinal epithelial cells^[28]. A quantitative analysis of the level of CEACAM6 expression by T84 intestinal epithelial cells in response to B2 *E. coli* infection was determined by ELISA (Figure 1C) and Western blot (Figure 1D). Interestingly, we observed that most of B2 *E. coli* strains isolated from colon cancer induced increased expression of CEACAM6 to a level similar to that of AIEC strain LF82. Of note, B2 *E. coli* strains isolated from diverticulosis induced no or very low expression of CEACAM6 in T84 cells.

Another important bacterial trait involved in the colonization of the intestinal mucosa by gut resident bacteria is their ability to form biofilm. This property was investigated both on abiotic and on fixed intestinal epithelial cells. The level of biofilm formation on abiotic surface was evaluated by calculating the specific biofilm formation index (SBF). An SBF index of 3.13 ± 0.23 was obtained for AIEC strain LF82 compared to 0.99 ± 0.22 for the non-pathogenic K-12 *E. coli* strain C600 (Figure 2A). We observed that 7/19 (37%) B2 *E. coli* strains isolated

from colon cancer and 2/8 (25%) B2 *E. coli* strains isolated from diverticulosis harbored SBF index similar to that of the biofilm producer AIEC strain LF82 ($P \geq 0.05$). Biofilm formation on fixed I-407 intestinal epithelial cells was evaluated by confocal microscopy (Figure 2B), which confirmed that 6/9 B2 *E. coli* strains having a high SBF index on abiotic surface were able to form a strong biofilm on fixed I-407 cultured cells. Combining the two methods of biofilm formation assessment, 16/27 B2 *E. coli* strains tested were able to form biofilm. This shows that even B2 *E. coli* strains have a low ability to adhere to intestinal epithelial cells, at least half of them were able to form biofilm to a level similar to that of CD-associated *E. coli* strain LF82 known to form a strong biofilm and no difference was observed between B2 *E. coli* strains isolated from colon cancer patients or from patients with diverticulosis.

Colonization of colon mucosa in CEACAM-expressing mice by colon cancer-associated B2 *E. coli* strain 11G5: induction of inflammation and enhanced epithelial intestinal cell proliferation

CEABAC10 mice harboring a bacterial artificial chromosome that contains part of the human CEA family gene cluster including the *CEACAM6* gene were infected with AIEC reference strain LF82 or B2 *E. coli* strain 11G5 isolated from colon cancer patient. To assess bacterial colonization, the levels of bacteria in the stools were determined 5 d after the last infection of each cycle, over the

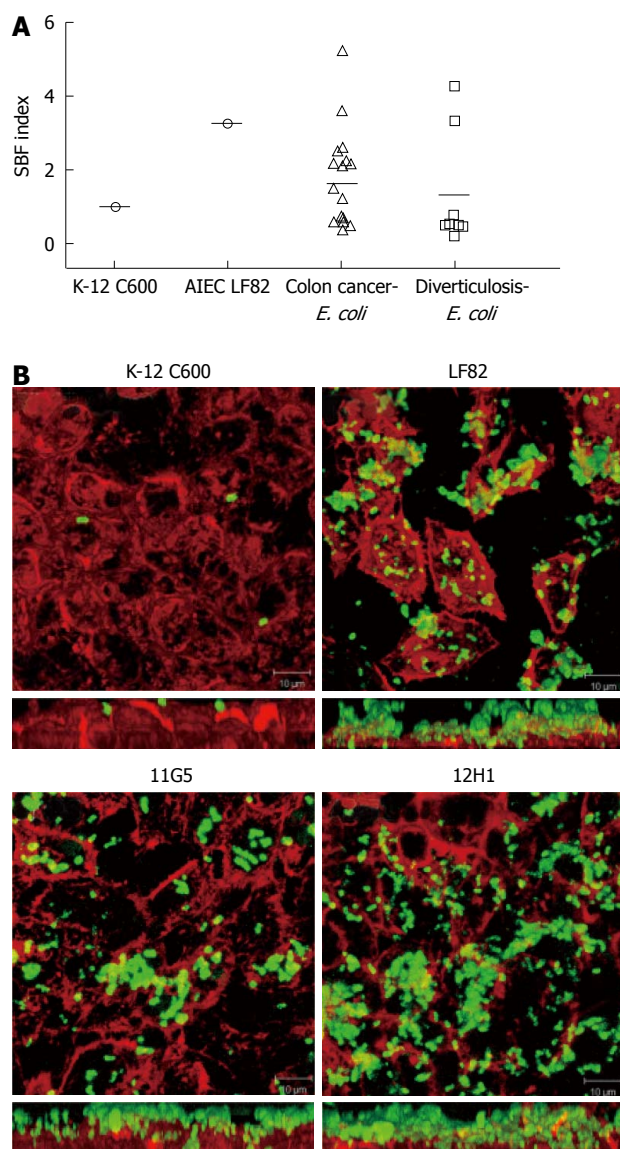


Figure 2 Ability of B2 *Escherichia coli* strains to form biofilm. Biofilm formation of colon cancer-associated and diverticulosis-associated B2 *Escherichia coli* (*E. coli*) were compared to that of the non-pathogenic K-12 *E. coli* strain C600 and the biofilm producer AIEC strain LF82. A: Biofilm formation on abiotic surface. Results are expressed as specific biofilm formation (SBF) index; B: Biofilm formation on human I-407 intestinal epithelial cells. *E. coli* strain 11G5 was isolated from a patient with colon cancer and *E. coli* strain 12H1 from a patient with diverticulosis. Bacteria were stained using goat anti-*E. coli* polyclonal antibodies (green) and I-407 cells were labeled for actin cytoskeleton using TRITC-labeled phalloidin (red). Y- and Z-stack projections are presented.

8 consecutive cycles of infection. Analysis of the number of bacteria recovered in the stools at cycle 4 and cycle 8 revealed similar ($P = 0.78$) colonization levels for mice infected with AIEC LF82 and colon cancer-associated 11G5 bacteria (Figure 3A).

On macroscopic examination, no sign of tumor development such as neoplasia or polyp was observed in the colon of mice infected with AIEC strain LF82 or colon cancer-associated 11G5 *E. coli* strain. Histological analysis showed a similar colonic histological score for inflammation and epithelial damages for mice infected with AIEC strain LF82 and *E. coli* strain 11G5 ($P \geq 0.05$)

(Figure 3B). Mice infected with *E. coli* strains LF82 and 11G5 exhibited infiltration of polynuclear cells in crypts, larger numbers of crypt abscesses and large and multifocal erosion plates (Figure 3C).

The level of *proliferating cell nuclear antigen* (PCNA) mRNA was measured in the colonic mucosa of infected mice to determine the proliferative index (Figure 3D). Significant ($P \leq 0.05$) 2.5-fold and 2.9-fold increases in PCNA mRNA levels were observed in the colonic mucosa of mice infected with the *E. coli* strain 11G5 compared to those of control mice or mice infected with AIEC strain LF82, respectively. This finding was confirmed by Ki67 immunostaining on colonic mucosa tissue. 11G5-infected mice had higher numbers of proliferative epithelial cells in crypts than control mice and mice infected with AIEC strain LF82 (Figure 3E). This indicates that colonic mucosa cells undergo accelerated proliferation in response to infection by B2 *E. coli* strain 11G5 associated with colon cancer.

DISCUSSION

Accumulating evidence supports the involvement of infectious agents in the development of cancer, especially in organs that are continuously exposed to microorganisms such as the colon. Remodeling of the colonic microbiota due to environmental changes is thought to contribute to the pathogenesis of colon cancer by suppressing the growth of cancer-protective bacterial species and allowing the emergence or expansion of bacterial species with oncogenic potential. It has been suggested that the role of *E. coli* in CRC promotion and development is linked to chronic inflammation, which can result from bacterial infection *via* its effects on both the host and the microbiota, in particular that of promoting the expansion of certain bacteria, such as pro-inflammatory *E. coli*^[37] or ETBF^[38,39]. In parallel, two different studies have reported that between 71% and 82% of patients with colonic adenoma or carcinoma^[10,12] are highly colonized by mucosa-associated *E. coli* compared to controls. The aim of the present study was to provide further insight into the characterization of the *E. coli* colonizing the mucosa of colon cancer patients.

It is well documented that B2 *E. coli* harbors genes coding for cyclomodulins such as colibactin, which is encoded by the *pks* genomic island, CDT, CNF or Cif, which can act as genotoxic agents and/or can modulate cellular differentiation, apoptosis, and proliferation^[13,40,41]. In the present study, we observed that 86% of cyclomodulin-positive *E. coli* isolated from colon cancer and diverticulosis specimens belonged to B2 phylogroup. Of interest, all but two *cnf* positive strains also harbored *pks* and all *cnf*- and *pks*-positive *E. coli* strains belonged to the B2 phylogroup. Our results are in good agreement with those reported by Arthur *et al.*^[3], who observed that 66.7% of patients with CRC and 20.8% of controls harbored *pks*-positive *E. coli*.

E. coli strains belonging to B2 phylogroup have a

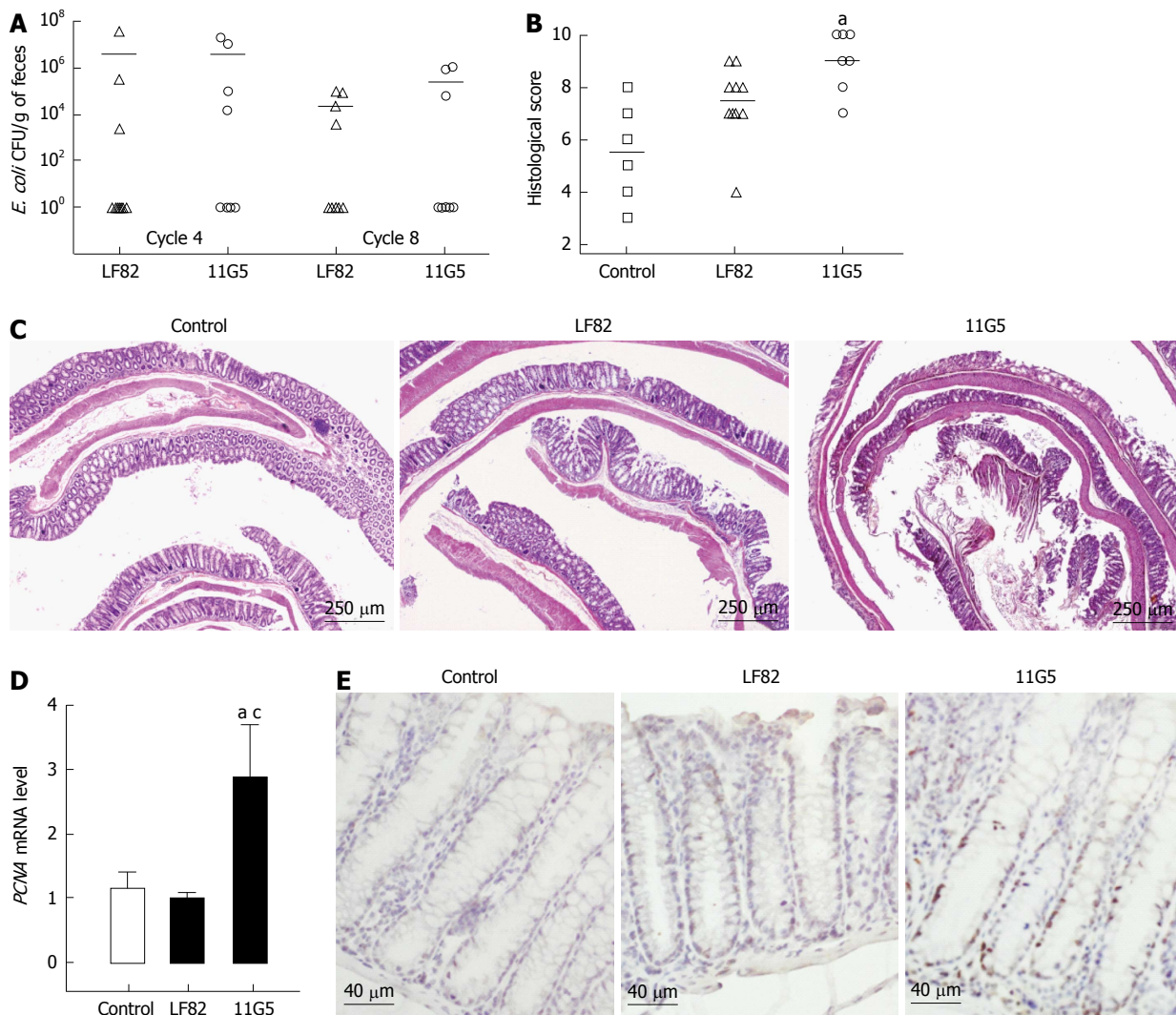


Figure 3 Impact of CEABAC10 mice colonization by B2 *Escherichia coli* strain on inflammation and cell proliferation. CEABAC10 mice transgenic for human CEACAMs, including CEACAM6, were subjected to 8 consecutive cycles of infection with AIEC LF82 or B2 phylogroup *Escherichia coli* (*E. coli*) strain 11G5. Control mice received PBS. A: Quantification of the number of bacteria in the feces of mice at cycle 4 and cycle 8; B: Histopathological scoring for several parameters of inflammation and epithelial damages (see Table 2) was performed at the end of the 8th cycle. ^a $P \leq 0.05$ vs control; $P = \text{NS}$ vs LF82; C: Hematoxylin/eosin/safran (HES) staining of colonic tissue sections; D: Total RNAs from colons were extracted at the end of the 8th cycle. PCNA and S26 mRNA levels were measured by RT-qPCR. PCNA amount relative to S26 is presented. ^a $P \leq 0.05$ vs control; ^c $P \leq 0.05$ vs LF82; E: Immunohistochemistry examination of Ki67 on colonic tissue sections. NS: Not significant.

greater ability to colonize the human gut, due, at least in part, to accumulation of genes encoding fitness factors such as pili and adhesins^[42,43]. In addition, an increased proportion of mucosa-associated *E. coli* expressing hemagglutinins was observed in CD patients (39%) and colon cancer patients (38%) compared to controls (4%), in correlation with the ability of bacteria to adhere to I-407 and HT-29 intestinal epithelial cells^[10]. However in our study, analysis of the adhesive abilities of B2 *E. coli* isolated from colon cancer or diverticulosis revealed that the strains were poorly adherent to I-407, even if the majority of them were able to form biofilm. However some B2 *E. coli* strains isolated from colon cancer induced increased expression of CEACAM6 to a level similar to that of AIEC strain LF82 associated with CD, indicating that colon cancer-associated *E. coli* could influence carcinogenesis, since CEACAM6 has been implicated in cel-

lular adhesiveness, invasiveness, and metastatic behavior of tumor cells^[30,44]. In addition, this result indicates that, in agreement with what we previously reported for AIEC strains isolated from CD patients^[28], colon cancer-associated *E. coli* strains could have the ability to promote their own colonisation since CEACAM6 serves as a receptor for mediating adherence and/or cell entry of pathogenic bacteria such as *Neisseria* bacteria^[45], diffusely-adhering *E. coli* (DAEC)^[46] or AIEC^[28].

Experiments of long-term colonization of CEABAC10 mice revealed that an *E. coli* strain isolated from colon cancer (strain 11G5) was able to persist in the gut of CEABAC10 transgenic mice expressing human CEACAMs, including CEACAM6 and to exacerbate colonic inflammation. Whether colonisation of the intestinal mucosa of colon cancer patients by B2 *E. coli* is a cause or a consequence of malignant transformation is a ques-

tion that has yet to be addressed. We show here that B2 phylogroup *E. coli* isolated from colon cancer increased the proliferative index of epithelial cells in crypts in the chronic infection model of CEABAC10 mice. This indicates that colonic mucosa cells undergo accelerated proliferation in response to infection by B2 *E. coli*. The ability to induce cell proliferation is a common trait of various pathogens involved in carcinogenesis. Indeed, *Bacteroides fragilis* enterotoxin induces *c-myc* transcription and translation and persistent cellular proliferation ensues, mediated in part by β -catenin/T-cell factor-dependent transcriptional activation^[47]. Another example is *Helicobacter pylori* (*H. pylori*), which increases the proliferation of gastric cancer cells. This process is dependent on the LPS-TLR4 pathway since *H. pylori* LPS induces the proliferation of gastric cancer cells and the use of neutralizing antibody against TLR4 almost completely abrogates the proliferative activities of cancer cells^[48]. Some cyclomodulins, such as CNF, which are mostly produced by B2 *E. coli*, induce epithelial cell proliferation^[49]. In our study the B2 *E. coli* strain 11G5 did not harbor the *cnf* genes and was able to promote cell proliferation as observed in infected CEABAC10 mice. This effect could be related to *E. coli*-derived LPS, which was previously reported to have a more remarkable cancer proliferative activity than *H. pylori*-derived LPS^[48]. Because *E. coli* inhabits the host colon as normal intestinal flora, owing to host tolerance toward *E. coli*, it is likely that *E. coli* LPS stimulates the host cellular immune response to prevent cancer progression. However, we can hypothesize that when too great a load of *E. coli* colonize the colonic mucosa, as observed in 11G5-infected CEABAC10 mice, potent tumor proliferative activity is no longer effectively repressed. The cell proliferation observed in 11G5-infected CEABAC10 mice could also result from the presence of colibactin. Colibactin with its genotoxic activity promotes DNA damages, which leads to carcinogenesis and cell proliferation.

In conclusion, B2 *E. coli* abnormally colonized the mucosa of colon cancer patients, indicating that microbiota remodeling had occurred promoting their expansion. Together with previous findings reported by Arthur *et al.*^[3], this study on a larger cohort of patients confirms the high prevalence of B2 *pks*-positive or *pks-cnf*-positive *E. coli* in colon cancer patients. The study also indicates that, these bacteria can promote low grade inflammation and cell proliferation, as shown in the CEABAC10 infected mouse model. Analyses to determine whether these bacteria take advantage of the tumor microenvironment to colonize the gut or promote their own colonization may be an important step in understanding their role in carcinogenesis and in the development of therapeutic strategies.

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COMMENTS

Background

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide, and is the fourth leading cause of cancer death worldwide. Inflammation and changes in composition and function of gut microbial communities are suspected to be causative factors in the development of sporadic CRC.

Research frontiers

The authors and other researchers have reported abnormal colonization of tumors and mucosa of colon cancer patients by *Escherichia coli* (*E. coli*) belonging to B2 phylogroup.

Innovations and breakthroughs

To date, there has been a limited number of studies analyzing interaction of colon cancer-associated *E. coli* to intestinal epithelial cells. The authors showed that colon cancer-associated *E. coli* induce expression of the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) receptor in intestinal epithelial cells, and that these bacteria were able to persist in a chronic infection model of CEACAM6 expressing mice and had oncogenic potential.

Applications

The authors have analyzed the ability of colon cancer-associated *E. coli* to colonize gut mucosa and influence carcinogenesis. Analyses to determine whether these bacteria take advantage of the tumor microenvironment to colonize the gut or promote their own colonization may be an important step in understanding their role in carcinogenesis and in the development of therapeutic strategies.

Terminology

CEACAM6 molecule serves as a receptor for mediating mucosa colonization by pathogenic bacteria.

Peer review

This study provides evidence supporting the hypothesis that colon cancer-associated *E. coli* are able to colonize gut mucosa and to induce cell proliferation in a mouse model with overexpression of human CEACAM6.

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