Research Article

Cancer Prevention Research

Fusobacterium Nucleatum Subspecies *Animalis* Influences Proinflammatory Cytokine Expression and Monocyte Activation in Human Colorectal Tumors

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Abstract

Chronic infection and associated inflammation have long been suspected to promote human carcinogenesis. Recently, certain gut bacteria, including some in the *Fusobacterium* genus, have been implicated in playing a role in human colorectal cancer development. However, the *Fusobacterium* species and subspecies involved and their oncogenic mechanisms remain to be determined. We sought to identify the specific *Fusobacterium* spp. and ssp. in clinical colorectal cancer specimens by targeted sequencing of *Fusobacterium* 16S ribosomal RNA gene. Five *Fusobacterium* spp. were identified in clinical colorectal cancer specimens. Additional analyses confirmed that *Fusobacterium nucleatum* ssp. *animalis* was the most prevalent *F. nucleatum* subspecies in human colorectal cancers. We also assessed inflammatory cytokines in colorectal cancer specimens using immunoassays and found that expression of the cytokines IL17A and TNFα was markedly increased but IL21 decreased in the colorectal tumors. Furthermore, the chemokine (C-C motif) ligand 20 was differentially expressed in colorectal tumors at all stages. In *in vitro* coculture assays, *F. nucleatum* ssp. *animalis* induced CCL20 protein expression in colorectal cancer cells and monocytes. It also stimulated the monocyte/macrophage activation and migration. Our observations suggested that infection with *F. nucleatum* ssp. *animalis* in colorectal tissue could induce inflammatory response and promote colorectal cancer development. Further studies are warranted to determine if *F. nucleatum* ssp. *animalis* could be a novel target for colorectal cancer prevention and treatment. *Cancer Prev Res; 10(7); 398–409. ©2017 AACR.*

Introduction

The gastrointestinal tract has a high density of commensal microbes that are not only important to digestive physiology but also critical to immune system development and function. However, an altered intestinal microbiota is believed to be a potential risk factor for colorectal carcinogenesis (1, 2). Early studies linked certain enteric bacterial infections with inflammatory bowel disease and colorectal cancer, particularly pathogenic bacterial species such as *Streptococcus gallolyticus* (3), *Enterococcus faecalis* (4), adherent *Escherichia coli* (5), and enterotoxigenic *Bacteroides fragilis* (6). However, the role of these species as causal factors for colorectal carcinogenesis remains to be determined.

Recent discoveries using next-generation sequencing and functional studies established an association between *Fusobacterium* spp. infection and colorectal cancer development (7, 8). *Fusobacterium* is a genus of anaerobic Gram-negative bacteria previously known to be oral pathogens. They are mainly associated with dental plaque biofilms and

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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doi: 10.1158/1940-6207.CAPR-16-0178

inflammatory periodontal diseases but are also found in individuals with extraoral infections (9). *Fusobacterium* encompasses at least 15 species (10, 11), of which *Fusobacterium nucleatum* was one of the most frequently identified species in periodontal and bowel diseases (9, 12). Moreover, the taxonomy of *F. nucleatum* consists of five subspecies—*nucleatum*, *polymorphum*, *fusiforme*, *animalis*, and *vincentii*—with *fusiforme* and *vincentii* indicated to be the same subspecies according to genomic analysis (13, 14). However, the etiological relationship between the *F. nucleatum* subspecies and colorectal cancer development remains to be determined.

It is known that colorectal mucosal immunity plays an important role in maintenance of mucosal symbiosis with the gut microbiota (15). Chronic relapse and remission of inflammation can result in repeated epithelial injury and DNA damage, leading to colorectal carcinogenesis (15). Inflamed mucosa and neoplasia secrete proinflammatory cytokines, including IL17A, IL1B (IL1β), IL6, and TNF, all of which are implicated to promote colorectal tumorigenesis (16, 17). The recruitment of myeloid-derived cells to the inflamed mucosa is partly mediated by chemokine signaling. Chemokine (C-C motif) ligand 20 (CCL20), also known as macrophage inflammatory protein-3α or liver activation-regulated chemokine, is one of upregulated chemokines in the inflammatory microenvironment (18, 19). CCL20 protein expression can be stimulated by lipopolysaccharide (LPS) and TNF via the NF-κB pathway (20). It is the sole high-affinity ligand for chemokine (C-C motif) receptor 6 (CCR6; ref. 21). The CCL20/CCR6 axis regulates recruitment of $CCR6^+$ immune cells, including subsets of IL17-expressing T helper cells (Th17), regulatory T cells, and dendritic cells, to neoplastic lesions (22–25). Interestingly, colorectal cancer can hijack CCL20/CCR6 function to promote hepatic metastasis of colorectal cancers (19, 26–28). However, the mechanisms involving the tripartite relationships among specific bacteria, tumor cells, and immune cells in the tumor microenvironments have yet to be fully understood.

We hypothesized that specific *Fusobacterium* subspecies prevalent in colorectal cancer patients play critical roles in promoting proinflammatory response and neoplastic development. To test this hypothesis, we profiled the differential microbiomes in human colorectal cancers and adjacent mucosal tissue by high-throughput bacterial 16S rDNA sequencing. Then, using the *Fusobacterium* 16S gene-targeted sequencing, we identified the specific *Fusobacterium* spp. and subspecies associated with colorectal tumors. Moreover, we examined Th17 proinflammatory cytokine/chemokine expression in the paired colorectal tissue specimens using cytokine array analysis and specific ELISA. Finally, we assessed the phenotypes of colorectal cancer cells and monocytes responding to *Fusobacterium* infection in coculture systems. The results demonstrated that *F. nucleatum* ssp. *animalis* and dysregulated CCL20 protein expression are important factors associated with human colorectal cancer progression.

Materials and Methods

Cell lines and bacterial strains

The human colon epithelial cell line CCD841CoN, colorectal cancer cell lines (SW480, HT29, HCT116, and RKO), and monocyte THP-1 were obtained from the ATCC. The human primary colorectal cancer cell line HCP1 was generated in our laboratory under a protocol approved by the Institutional Review Board (IRB) of The University of Texas MD Anderson Cancer Center (UTMDACC; ref. 29). Cell lines were routinely cultured in minimum essential medium or RPMI 1640 complete medium containing 10% FBS under standard human cell culture conditions at 37°C. The normoxic culture was performed in an incubator with 5% CO_2 and 21% O_2, whereas the hypoxic culture was carried in 5% CO_2 and 2% O_2. Cells were free of mycoplasma as confirmed using a MycoAlert mycoplasma detection kit (Lonza Group).

The *F. nucleatum* ssp. *animalis* Gharbia and Shah (ATCC 51191) type strain NCTC 12276 was obtained from the ATCC. The strain was cultured in CDC anaerobe 5% sheep blood agar plates (Becton Dickinson and Company) under anaerobic conditions using an AnaeroGen Compact system (Oxoid) with a 37°C incubator.

Human tissue specimens

Surgical residual colorectal adenoma or adenocarcinoma and adjacent normal tissue specimens were collected from patients by members of the Department of Pathology at UTMDACC. Informed consent for use of the paired frozen specimens was obtained from the patients according to an IRB-approved protocol. Clinical characteristics of the patients in this study are listed in Supplementary Table S1. Aliquots of each of the tissue specimens were utilized for protein extraction and cytokine panel analysis; the tissue aliquots were also used in DNA extraction and bacterial taxonomic analyses (assays described below).

DNA isolation and bacterial 16S rDNA sequencing

Total DNA was extracted from the frozen tissue specimens using a QIAamp kit (QIAGEN) according to the manufacturer's instructions and stored at −20°C prior to sequencing analysis. Microbial 16S ribosomal RNA (rRNA) gene sequencing was performed as described previously (30) with minor modifications. Briefly, the V4 region of 16S rRNA gene was amplified using PCR and sequenced on the MiSeq platform (Illumina) using a 2 × 250-bp paired-end protocol yielding pair-end reads. The primers used for amplification contained adapters for MiSeq sequencing and single-end barcodes, allowing for pooling and direct sequencing of PCR products (30). The read pairs were demultiplexed based on the unique molecular barcodes, and reads were merged using the USEARCH software program

(version 7.0.1090; ref. 31) with no mismatches and a minimum overlap of 50 bases. In addition, a quality filter was applied to the resulting merged reads, and reads with more than 0.05 expected errors were discarded. Custom analytic software packages and pipelines developed at the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine were used with the 16S rRNA gene profiling pipeline to provide summary statistics and quality control measurements for the sequencing run. 16S rRNA gene sequences were clustered into operational taxonomic units (OTU) at a similarity cutoff value of 97% using the UPARSE algorithm (32). OTUs were mapped to classification data in an optimized version of the SILVA database (33) containing only the 16S V4 region to determine taxonomies of bacteria. Abundances of OTUs were recovered by mapping the de-multiplexed reads to the UPARSE OTUs. A custom script was used to construct an OTU table from the output files for downstream analyses of alpha-diversity, beta-diversity, and phylogenetic trends in bacteria.

To further examine the 16S V4 reads corresponding to the *Fusobacterium* genus, we used a *Fusobacterium* 16S V4 consensus sequence as a query sequence for the data search. The reads containing the matched sequence were mapped to the nonredundant nucleotide collection (nr/nt) database from NCBI to determine the closest species identities. In addition, we performed *Fusobacterium* genus 16S rDNA–targeted PCR and Sanger sequencing to confirm the *Fusobacterium* spp. and subspecies in the specimens. Briefly, the *Fusobacterium* 16S rDNA was first amplified using PrimeSTAR high-fidelity DNA polymerase (Clontech) using outer primers (Univ-8F′ 5′-AGAGTTTGATCHTGGCTCAG-3′; FUSO-v5r 5′-GACCCCCAACACCTAGTAAT-3′) and then followed with inner primers for a nest PCR (FUSO-v2df 5′-GGGACAACATYTRGAAAYGRATGC-3′; FUSO-v4r 5′-GCYYACCTCTCCAGTACTCTAG-3′). The resulting 16S V2–V4 region amplicons (508 nucleotides length) were sequenced bidirectionally at CMMR. The entire sequences were used for mapping in the SILVA database to determine species identities. The PCR amplicons were also cloned and sequenced individually in a confirmatory study. In addition, we used a different set of inner primers (FUSO-v2f 5′-GCCTCACAGNTAGGGACAACAT-3′; FUSO-v4r 5′-GCYYACCTCTCCAGTACTCTAG-3′) to produce 16S V2–V4 amplicons in an independent study. The PCR products were gel-purified and sequenced directly using a V4 internal consensus primer (FUSO-cv4r: 5′-CCRCCTAGACGCGCTTTAC-3′) at the UTMDACC Sequencing and Microarray facility. This method can identify approximate 437 nucleotides length sequence of V2–V3 regions. The PCR amplicons were also cloned and sequenced individually for confirmation. The sequence data were analyzed using the Basic Local Assignment Search Tool, and sequence alignment was performed using the Clustal Omega software program to compare with the reference sequences in the Ribosomal Database Project database (http://rdp.cme.msu.edu/index.jsp).

Quantitative PCR

qPCR analysis of 16S rRNA gene was carried out using HotStart-IT SYBR Green qPCR master mix (Affymetrix) with *Fusobacterium*-specific quantitative PCR primers (FUSO-v2f 5′-GCCTCACAGNTAGGGACAACAT-3′; FUSO-v4r 5′-GCYYACCTCTCCAGTACTCTAG-3′). Also, analysis of an internal control gene HNRNPK was performed using specific primers (HNRNPK-f2: 5′-GTCTCCCATCAAAGGACGTG-3′; HNRNPK-r1: 5′-GTCTCCAGGTCTCCCTCTTC-3′). The reactions and data processing were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems).

Cytokine panel assay and ELISA

Soluble proteins were extracted from frozen tissue specimens in cold PBS (pH 7.4) containing a protease inhibitor cocktail (Roche) using a Polytron homogenizer protocol (Kinematica). Quantitative Luminex cytokine assays were performed using a MILLIPLEX Human Th17 cytokine panel kit (HT17MG-14K-PX25; EMD Millipore) according to the manufacturer's instructions at the Functional Genomics and Microbiome Core in the Texas Medical Center-Digestive Diseases Center. Human CCL20 protein ELISA was performed using the extracted tissue protein solution with a Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocol.

Cell migration assay

A Boyden chamber assay was used to assess cell migration *in vitro*. Briefly, human colorectal cancer cells and monocytes were prepared and suspended in antibiotic-free RPMI 1640 medium containing 1% FBS. Cells (2×10^5) were then seeded in the upper chamber (filter pore size, 8 μm; Corning). A CCL20-neutralizing antibody (AF360; R&D Systems) or control IgG was added to the upper chamber at a final concentration of 0.4 μg/mL in the medium. *F. nucleatum* ssp. *animalis* (ATCC 51191) suspension was prepared by washing and diluting of the bacterium in PBS. An aliquot of bacterial suspension (1×10^4 cells) or PBS (control) was added to the bottom chamber containing antibiotic-free RPMI 1640 medium with 1% FBS. After incubation under standard mammalian cell culture conditions for 24 hours, the upper chamber filters were fixed and stained using the PROTOCOL Hema 3 stain set (Fisher Scientific). Migrated cells on the bottom side of filter were counted in five random fields per filter. The ImageJ software program (National Institutes of Health, Bethesda, MD) was used for cell quantification.

Statistical analysis

Differences in values for the paired colorectal tumor and adjacent normal tissue specimens were assessed using the

Wilcoxon matched-pairs signed rank test. Differences in the means of the cell line *in vitro* assays were assessed using a two-tailed unpaired Student *t* test. A P value < 0.05 was considered significant.

Results

Fusobacterium species are relatively abundant in colorectal tumors

To determine profiles of the microbiome associated with human colorectal tumors and normal colorectal mucosa, we conducted Illumina MiSeq analysis of bacterial 16S rDNA in the frozen tissue specimens obtained from patients with colorectal cancer. This analysis yielded a total of 638,542 high-quality sequence reads, with an average of 15,963 reads per sample. Comparison of the microbial taxonomic compositions at the phylum level demonstrated that *Fusobacteria* was one of the most abundant phyla in the colorectal tissue specimens. The distribution between the tumor and normal mucosa was approximately an average of 15.3% *Fusobacteria* OTUs in the tumors versus 7.7% *Fusobacteria* OTUs in the normal tissue. We observed a similar result at the genus level of increased *Fusobacterium* in the tumor compared with mucosa ($P < 0.05$, Fig. 1). In addition, *Gemella* as a low abundant genus in the tissues was also increased in the tumor ($P < 0.05$). In contrast, *Bacteroides*, *Lachnospiraceae*, and *Blautia* were relatively overrepresented in the adjacent normal mucosa compared with tumor ($P < 0.05$).

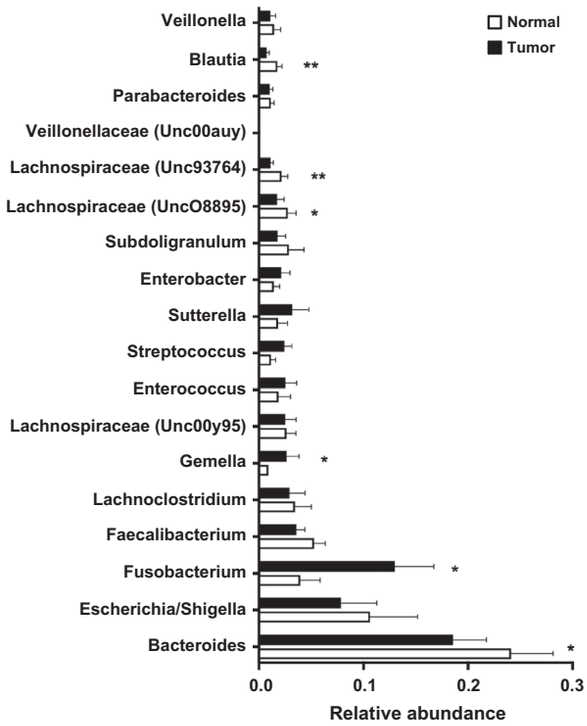

Figure 1.
Relative abundance of classified and unclassified genera at an average abundance of >1% across all paired colorectal tumor and normal mucosa. Of the genera associated with the tissue specimens ($n = 25$ pairs), *Fusobacterium* was the genus most commonly increased in the tumor. Bars represent means and SEM. Wilcoxon matched-pairs signed rank test was used to determine the abundance of OTUs in the tumors vs. adjacent normal mucosa (*, $P < 0.05$ and **, $P < 0.01$).

F. nucleatum ssp. *animalis* predominates in the *Fusobacterium* population associated with colorectal tumors

In order to determine the *Fusobacterium* spp. and subspecies (ssp.) composition in the colorectal tumor and normal mucosa specimens, we first analyzed the Illumina MiSeq 16S sequencing data that mapped the *Fusobacteria* OTUs in the specimens. Using a *Fusobacterium* 16S V4 consensus sequence as a query sequence for the data search, we found reads mapping to *F. nucleatum* with higher hits in the tumor specimens (Supplementary Fig. S1). We then performed an independent experiment using the *Fusobacterium* genus–specific PCR and deep sequencing of 16S rDNA V2–V4 regions to determine *Fusobacterium* spp. and ssp. in the specimens. The results demonstrated that the *Fusobacterium* population in the specimens was composed of five species: *Fusobacterium periodonticum*, *Fusobacterium canifelinum*, *Fusobacterium varium*, *Fusobacterium simiae*, and *F. nucleatum*. Of these species, *F. nucleatum* was the most abundant (Fig. 2A). The sequence analysis also demonstrated reads matching four *F. nucleatum* subspecies—*F. nucleatum* ssp. *nucleatum*, *F. nucleatum* ssp. *polymorphum*, *F. nucleatum* ssp. *fusiforme/vincentii*, and *F. nucleatum* ssp. *animalis*—of which *F. nucleatum* ssp. *animalis* was unambiguously predominant (Fig. 2B).

To further validate the presence of *F. nucleatum* ssp. *animalis* in colorectal tumors, we performed additional confirmatory sequencing of the *Fusobacterium* 16S V2-V4 at a different sequencing core facility with cloning or without cloning of PCR amplicons. We found the cloned amplicons were all *F. nucleatum* ssp. *animalis* sequences (Supplementary Fig. S2A) but could not exhaust the possibility of finding other *Fusobacterium* species or subspecies in clone pools. We then used a *Fusobacterium* genus 16S consensus primer to sequence the amplicons without cloning. Results demonstrated that all sequences in four colorectal cancer specimens were again identical to that of *F. nucleatum* ssp. *animalis* (Fig. 3A; Supplementary Fig. S2A) except for one that contained a minor overlapping sequence aligned to potentially *F. nucleatum* ssp. *nucleatum* or *F. nucleatum* ssp. *fusiforme/vincentii* (Supplementary Fig. S2B). In a parallel experiment, we also sequenced the *F. nucleatum* ssp. *animalis* Gharbia and Shah (ATCC 51191) type strain as a positive control. Figure 3A shows the representative sequence alignments of the V2

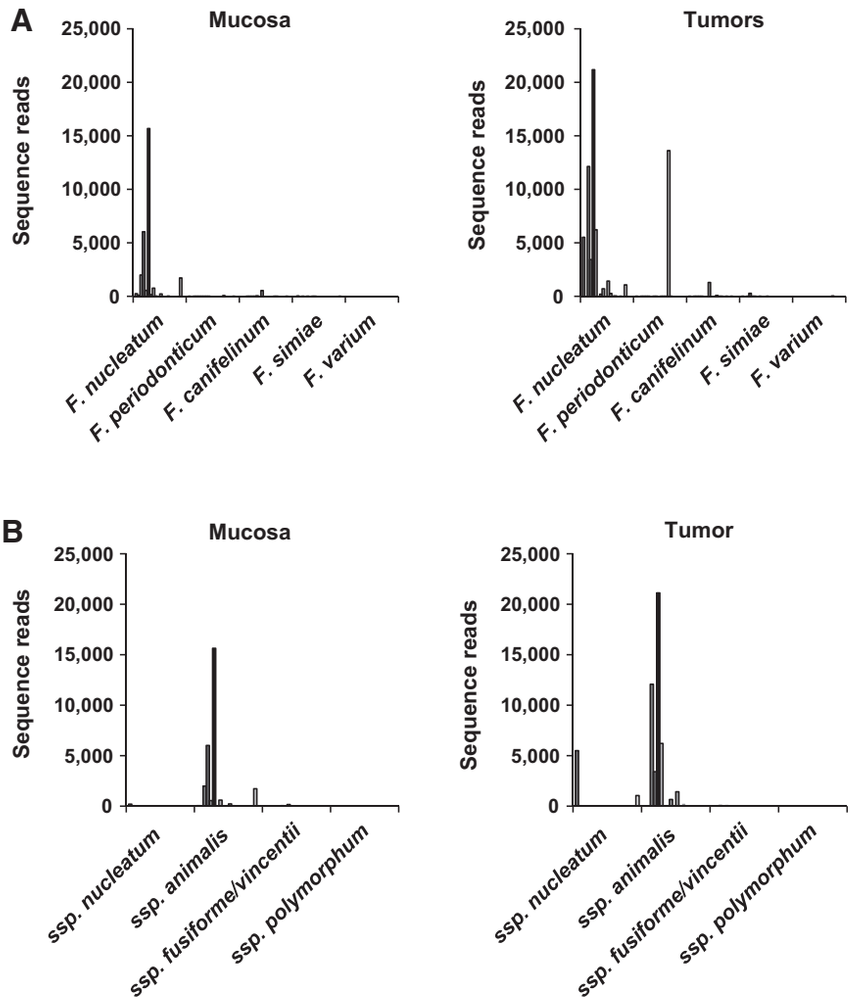

Figure 2.
The predominant enriched *Fusobacterium* species in colorectal tumors is *F. nucleatum* ssp. *animalis*. **A,** The composition of *Fusobacterium* in colorectal tumors and mucosal tissue specimens ($n = 18$ pairs) as assessed using 16S rRNA gene V2–V4 region–targeted Sanger sequencing. *F. nucleatum* was shown with the highest sequence reads in five *Fusobacterium* spp. identified. **B,** In-depth taxonomic analysis further identified four subspecies of *F. nucleatum*, with *F. nucleatum* ssp. *animalis* as the most dominate subspecies in colorectal tumors and mucosal tissue specimens ($n = 18$ pairs).

hypervariable region of 16S rRNA gene. These sequences were derived from four specimens and compared with the type strain sequence and the *Fusobacterium* spp. reference sequences from the Ribosomal Database Project database. Taken together, these results confirmed that *F. nucleatum* ssp. *animalis* was the predominant *Fusobacterium* subspecies associated with colorectal cancer in the samples evaluated.

We then established a qPCR assay based on the confirmed 16S rDNA sequences of *Fusobacterium* spp., including *F. nucleatum*, *F. simiae*, *F. periodonticum*, and *F. canifelinum*, in the colorectal tumors. However, the qPCR primer specificity excludes *F. varium*. In addition, the qPCR also detects four *F. nucleatum* subspecies: *animalis*, *fusiforme/vincentii*, *nucleatum*, and *polymorphum*. Analysis of this subpopulation of *Fusobacterium* spp. and ssp. in the colorectal specimens showed that their relative abundance was significantly higher in the tumor than in the normal mucosa in the paired analysis ($P < 0.05$; Fig. 3B).

Proinflammatory cytokines are differentially expressed in colorectal tumors

F. nucleatum is known for its invasive and inflammatory pathogenicity in cases of periodontal disease. We hypothesized that its prevalence with colorectal tumors likely induces a similar inflammatory response via activation of colorectal mucosal immune cells. To examine the cytokine expression in colorectal tissue obtained from our study patients, we performed two independent Luminex multiplex analyses of Th17 cytokines to compare the expression in colorectal tumor and adjacent mucosal tissue specimens. The analyses of first 16 pairs of specimens (Exploratory Set) and then additional 12 pairs of specimens (Confirmatory Set) demonstrated the significant increase of cytokines CCL20, IL17A, and TNF, but decrease of IL21, in the tumor (Table 1). The finding of differential expression of CCL20, IL17A, and TNF proteins supports the suspected inflammatory statuses of the human colorectal tumors. More importantly, the differential expression of CCL20 protein was not only significantly higher but also

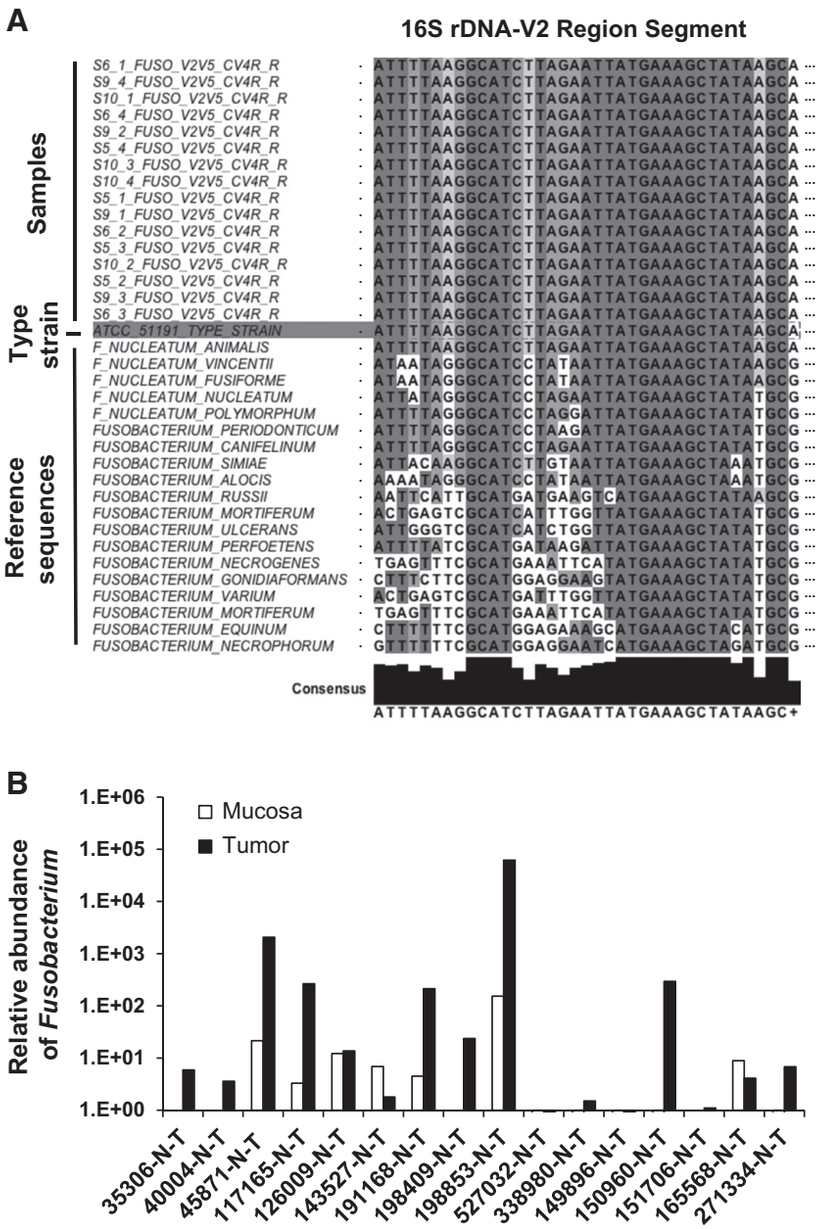

Figure 3.
Validation of *F. nucleatum* ssp. *animalis* in colorectal tissue specimens. **A,** Alignment of *Fusobacterium* 16S rRNA gene sequences. The sample sequences were derived from four independent colorectal tumors and an ATCC type strain of *F. nucleatum* ssp. *animalis* using 16S rDNA amplicon–targeted sequencing. They are compared with the reference sequences of known *Fusobacterium* spp. and *F. nucleatum* subspecies in the Ribosomal Database Project database. A representative segment of the V2 hypervariable region is shown. The whole 16S V2–V4 sequence alignment of representative samples can be found in Supplementary Fig. S2A. **B,** qPCR analysis of the relative *Fusobacterium* abundance in 16 pairs of colorectal tumor and normal mucosal tissue.

present at all stages of tumors (Fig. 4), suggesting that CCL20 protein is probably involved in the tumor initiation and progression throughout their entire courses. In addition to the Luminex assay, we also used a human CCL20-specific ELISA that confirmed the differential expression of CCL20 protein levels in the colorectal tumor specimens (Supplementary Fig. S3).

F. nucleatum ssp. *animalis* induces CCL20 protein expression in colorectal cancer cells and monocytes

It is unclear whether *Fusobacterium* infection directly contributes to CCL20 protein upregulation in colorectal tumors. To determine the effect of *Fusobacterium* on CCL20 protein expression, we set up a human cell–bacterium coculture system under normoxic (optimal for human cell growth) and hypoxic (favorable for anaerobic *Fusobacterium* proliferation) conditions. *F. nucleatum* ssp. *animalis* (ATCC 51191 strain) was used to represent the predominant subspecies found in the colorectal tumors. Conditioned media were collected from cocultures and measured for the CCL20 protein concentrations by ELISA, which demonstrated that the CCL20 protein expression in HCP1 cells was markedly induced in the presence of *F. nucleatum* ssp. *animalis* under normoxic conditions (Fig. 5A). Note that HCP1 is a low-passage colorectal cancer cell line (29). However, we observed a very subtle increase

Table 1. Th17 cytokine concentrations in colorectal tissue protein extracts

	Exploratory set (16 pairs)			Confirmatory set (12 pairs)		
Cytokine[a]	**Normal**	**Tumor**	***P* value**	**Normal**	**Tumor**	***P* value**
IL17F	122.26 ± 3.94	163.9 ± 17.56	0.04260	156.2 ± 4.54	167.9 ± 7.69	0.05858
CSF2	26.76 ± 6.05	211.0 ± 79.74	0.03944	45.6 ± 11.58	137.9 ± 38.70	0.05431
IFNG	10.18 ± 0.51	12.2 ± 0.94	0.08538	30.7 ± 12.94	100.2 ± 27.02	0.04850
IL10	23.28 ± 11.41	31.0 ± 10.78	0.64706	97.1 ± 43.28	48.4 ± 12.02	0.23779
CCL20	1589.38 ± 296.38	8025.6 ± 1091.38	0.00005	1063.7 ± 280.37	6222.8 ± 989.85	0.00013
IL12B	35.03 ± 3.60	37.2 ± 2.31	0.63484	62.3 ± 5.42	62.8 ± 4.01	0.87171
IL13	84.54 ± 5.64	82.8 ± 6.73	0.84114	84.9 ± 15.80	49.9 ± 8.30	0.08219
IL15	112.43 ± 13.32	147.6 ± 15.65	0.17259	154.6 ± 33.06	202.2 ± 19.92	0.26086
IL17A	86.80 ± 2.50	153.5 ± 20.86	0.00744	32.6 ± 10.93	113.2 ± 33.87	0.01359
IL22	107.40 ± 7.41	126.7 ± 9.07	0.10175	140.3 ± 11.91	147.8 ± 5.54	0.47986
IL9	59.10 ± 2.51	54.7 ± 3.39	0.27660	38.7 ± 7.06	47.7 ± 9.70	0.47586
IL1B	45.98 ± 6.82	1993.8 ± 1103.84	0.09737	28.9 ± 5.20	1618.5 ± 1100.05	0.17671
IL33	2377.08 ± 369.51	2992.5 ± 683.67	0.38270	1500.9 ± 202.02	2365.1 ± 602.96	0.13696
IL2	87.01 ± 2.78	88.6 ± 3.18	0.70469	70.4 ± 11.64	65.1 ± 7.92	0.65633
IL21	137.60 ± 16.36	73.8 ± 8.09	0.00117	166.4 ± 14.46	129.9 ± 8.14	0.02706
IL4	208.68 ± 202.25	61.1 ± 39.25	0.48816	457.8 ± 250.77	105.7 ± 40.47	0.15920
IL23A	1885.19 ± 212.50	2219.2 ± 194.68	0.25823	1825.8 ± 609.55	2373.5 ± 560.11	0.33497
IL5	31.23 ± 3.18	41.0 ± 4.84	0.13295	11.2 ± 5.04	6.5 ± 1.70	0.36160
IL6	161.88 ± 119.04	1346.6 ± 675.51	0.10300	16.9 ± 10.60	507.6 ± 308.94	0.14098
IL25	97.71 ± 3.54	107.1 ± 6.22	0.23201	80.4 ± 28.04	184.5 ± 110.04	0.34049
IL27	797.39 ± 131.93	907.6 ± 115.36	0.57615	1405.0 ± 207.12	1492.6 ± 117.56	0.64230
IL31	176.84 ± 50.04	189.9 ± 31.48	0.82849	692.8 ± 114.76	641.7 ± 53.97	0.61297
TNF	24.80 ± 4.17	118.2 ± 42.26	0.04036	38.5 ± 11.62	187.6 ± 30.42	0.00053
LTA	255.99 ± 22.00	254.1 ± 16.76	0.94290	137.0 ± 51.79	98.8 ± 25.43	0.40572
IL28A	427.57 ± 21.46	438.4 ± 24.16	0.70711	431.4 ± 42.59	467.1 ± 35.38	0.49168

Abbreviations: CSF2 (GM-CSF, granulocyte-macrophage colony-stimulating factor); IFNG, interferon gamma; LTA, lymphotoxin alpha (TNF-beta).

[a]Cytokine concentration (pg/mg tissue protein, mean ± standard error).

of CCL20 protein expression in high-passaged colorectal cancer cell lines (SW480, HT29, HCT116, and RKO) but no induction in a noncancerous colonic cell line CCD841 after coculture with *F. nucleatum* ssp. *animalis* (Supplementary Fig. S4A). In contrast, THP-1 monocytes were highly responsive to presence of *F. nucleatum* ssp. *animalis*, which not only markedly induced CCL20 protein expression (Fig. 5B; Supplementary Fig. S4B) but also apparently induced cellular morphologic changes from unanchored round cells to anchored spiky cells (Supplementary Fig. S5). This phenotypic change suggests that the monocytes were activated by interaction with *F. nucleatum* ssp. *animalis*. This is similar to a previous finding that LPS isolated from *F. nucleatum* or *Porphyromonas gingivalis* can stimulate the THP-1 cells to express activation antigens and macrophage phenotype (34). Furthermore, we observed that hypoxic stress conditions effectively increased basal expression of CCL20 protein in HCP1 and THP-1 cells, with an additive effect on the CCL20 protein expression in THP-1 cells by *F. nucleatum* ssp. *animalis* (Fig. 5B). These results suggested that both *Fusobacterium* infection and a hypoxic microenvironment can induce upregulation of CCL20 protein expression in cancerous epithelial cells and immune cells and may together contribute to aberrant CCL20 protein expression in colorectal tumors.

F. nucleatum ssp. *animalis* stimulates monocyte activation and migration

On the basis of the observations described above, we hypothesized that *F. nucleatum* ssp. *animalis* infection-triggered cellular responses in both colorectal cancer cells and monocytes/macrophages can lead to downstream effects, such as increased chemokine-mediated cell migration. To test this hypothesis, we performed a cell migration assay using a Boyden chamber system in which we seeded HCP1 or THP-1 cells in the top chambers and placed *F. nucleatum* ssp. *animalis* suspension or control medium in the bottom chambers. In addition, to assess the effect of CCL20 protein expression on cell motility, we used a CCL20-neutralizing antibody to block the chemokine function in the Boyden chamber system. The results demonstrated that the THP-1–monocyte migration was markedly enhanced by incubation with *F. nucleatum* ssp. *animalis* (Fig. 5C). We also observed that migration of THP-1 cells was partially inhibited by the CCL20-neutralizing antibody, suggesting that CCL20 signaling is indeed involved in regulation of monocyte/macrophage motility. However, the HCP1 colorectal cancer cell migration was not affected by the presence of *F. nucleatum* ssp. *animalis* or CCL20-neutralizing antibody (Supplementary Fig. S6). These observations warrant further investigation to better understand

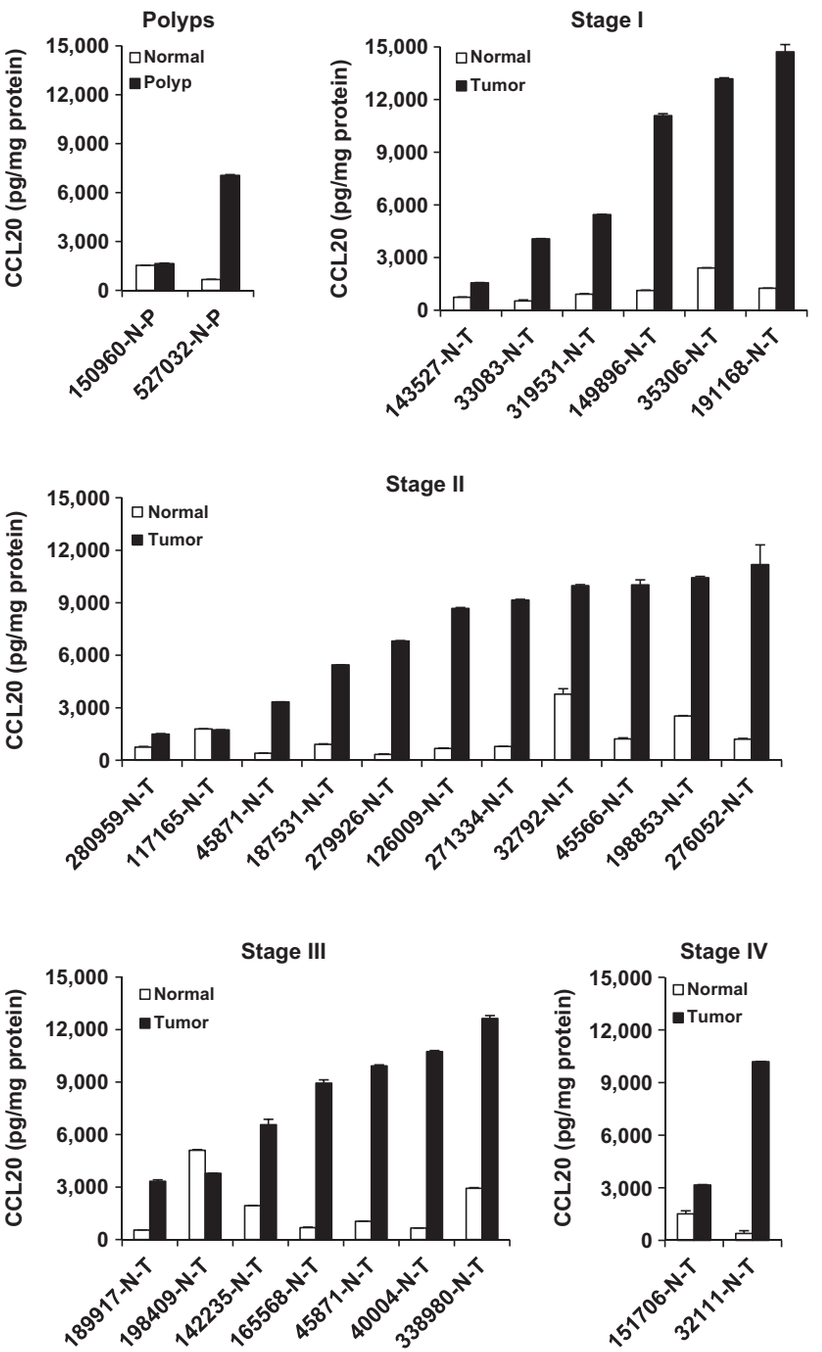

Figure 4.
CCL20 expression is markedly increased in colorectal tumors. Luminex cytokine analysis of CCL20 expression in colorectal adenomas and carcinomas of different stages is shown. CCL20 expression was significantly higher in the tumors than in paired mucosa specimens ($P < 0.01$).

the molecular mechanisms that regulate the interactions between *F. nucleatum* subspecies and human cells.

Discussion

Fusobacterium infection has been implicated as one of possible etiological factors in human inflammatory bowel disease (35, 36) and colorectal adenoma and carcinoma (1, 7, 8, 37–39). However, the specific pathogenic/oncogenic species of *Fusobacterium* and mechanism are largely unclear. In this study, we used 16S rDNA sequencing analysis to profile microbiota in the paired colorectal tumor and mucosal tissue specimens and found that *Fusobacterium* OTUs were relatively enriched with colorectal tumor, a result that is in line with previous reports (7, 8, 40). However, the relative abundance of *Fusobacterium* in the specimens was quite variable as also observed by others (7), suggesting many other factors (e.g., antibiotic therapy and bowel preparation) potentially affecting bacterial

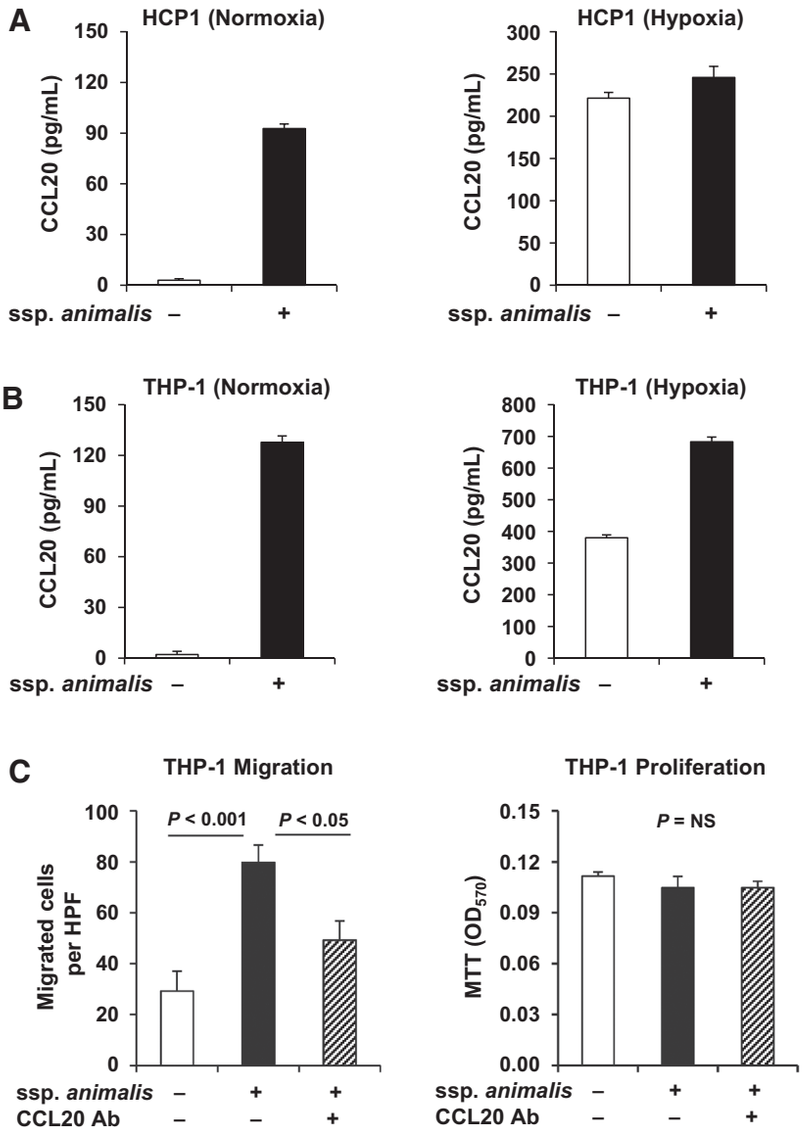

Figure 5.

F. nucleatum ssp. *animalis* induces CCL20 expression and monocyte migration in coculture. **A,** ELISA-based measurement of the CCL20 concentrations in the conditioned media from coculture of HCP1 colorectal cancer cells with or without *F. nucleatum* ssp. *animalis* under normoxic or hypoxic conditions. **B,** The CCL20 concentrations in the conditioned media from coculture of the THP1 monocytes with or without *F. nucleatum* ssp. *animalis* under normoxic or hypoxic conditions. **C,** *F. nucleatum* ssp. *animalis* stimulates monocyte migration that can be partially blocked by a CCL20-neutralizing antibody. The migration of THP-1 monocytes was assessed in Boyden chamber systems with or without *F. nucleatum* ssp. *animalis* or a CCL20-neutralizing antibody under normoxic conditions. The cell proliferation of THP-1 monocytes under the same treatments was measured by MTT assay. The growth rate was not significantly different between the control and treated cells.

dynamics in the specimens. In addition to the sequencing analysis, we established a qPCR assay and demonstrated differential distribution of *Fusobacterium* in the colorectal specimens. Further improvement of the assay specificity for detecting the disease-relevant species will help resolve some inconsistencies regarding the role of *Fusobacterium* in colorectal cancer (41–43).

Fusobacterium spp. and ssp. are very heterogeneous (10, 11). In our in-depth taxonomic analysis, we found that *F. nucleatum* was the prevalent species present in the tumor specimens we profiled. Our analysis further revealed that *F. nucleatum* ssp. *animalis* was the most dominant subspecies associated with colorectal cancer in the same specimens. Interestingly, a recent study of the fecal samples from colorectal cancer patients showed that *F. nucleatum* ssp. *vincentii* and *F. nucleatum* ssp. *animalis* were the top-ranked gut microbial species associated with colorectal cancer (44). Although we agree that the predominance of *F. nucleatum* sp. *animalis* was associated with colorectal cancer, our data did not support *F. nucleatum* ssp. *vincentii* as an abundant species in the tissue specimens of colorectal tumors (Fig. 3A; Supplementary Fig. S2). Further validation is critical for the identification of the specific *Fusobacterium* pathogen and its oncogenic mechanism(s) that, in turn, may theoretically be targets for prevention and possibly even treatment of colorectal cancer.

F. nucleatum ssp. *animalis* infections associated with human diseases have been documented in the literature. Studies have demonstrated an association of *F. nucleatum* ssp. *animalis* virulence with inflammatory periodontal diseases and adverse pregnancy outcomes (45, 46). Interestingly, *F. nucleatum* ssp. *animalis* was found more

frequently in the isolates from gastrointestinal tract than in the isolates from oral sites (47). A draft genome sequence of *F. nucleatum* ssp. *animalis* was reported (48), but information of its unique genetic features is lacking. In addition, *F. nucleatum* ssp. *animalis* has two bacteriophage variants (49). Because bacteriophages are known to contribute to the host strain's virulent phenotype, the *F. nucleatum* ssp. *animalis* bacteriophage may be another layer of the complexity of this subspecies' virulence.

Investigators have explored the function of *Fusobacterium* spp. in carcinogenesis. Early studies demonstrated *Fusobacterium* strains that are invasive and proinflammatory in the oral mucosa (50). The strains can also invade colorectal cancer cells and induce cytokine secretion (51–53). In colorectal adenomas and carcinomas, invasive *Fusobacterium* spp. can be visualized using FISH (8, 54). In an $Apc^{Min/+}$ mouse model of colorectal cancer, *F. nucleatum* promoted intestinal carcinogenesis via the bacterial virulence factor FadA, which binds to E-cadherin on the host cell surface and activates β-catenin signaling and inflammatory response (52). On the other hand, *F. nucleatum* increased tumor multiplicity and selectively recruited tumor-infiltrating myeloid cells, predominantly myeloid-derived suppressor cells, with potent immunosuppressive activity (40). *F. nucleatum* infection has also been associated with NF-κB activation and proinflammatory gene expression (40, 53). Similarly, in the present study, we found that expression/secretion of proinflammatory cytokines IL17A and TNF was markedly higher in colorectal tumors than in adjacent mucosal tissue. In addition, we found that CCL20 protein was increased in the colorectal adenomas and carcinomas compared with mucosa.

CCL20 and its receptor CCR6 are known for their important roles in the recruitment of immune cells and their paradoxical functions in regulation of both immunological tolerance and inflammation (55). Clinical studies have established that overexpression of CCL20 and CCR6 is associated with colorectal cancer progression (19, 26, 27, 56). In preclinical studies, the CCL20/CCR6 axis has proven to be critical for intestinal tumorigenesis in mice. For example, using the mutagen MNU plus *Helicobacter pylori* to induce colorectal tumorigenesis in wild-type C57BL mice, investigators showed that tumor-associated macrophages recruited $CCR6^+$ regulatory T cells to the tumor microenvironment via CCL20 signaling and promoted tumor growth (24). However, in $Apc^{Min/+}$ mice, knockout of CCR6 decreased spontaneous intestinal tumorigenesis via reduction of macrophage recruitment to the inflamed intestinal mucosa (57). Taking our own data into consideration for the *F. nucleatum* ssp. *animalis* interaction with colorectal cancer cells and monocytes, the response was stronger in the monocytes and showed a macrophage activation phenotype with increased migration and CCL20 protein expression, particularly under a hypoxic condition mimicking the tumor microenvironment. In a recent study of *Fusobacterium* and T-cell density in colorectal cancer, investigators showed that *Fusobacterium*-high cases were inversely associated with the density of $CD3^+$ pan-T cells (58). Future studies need to address whether the *Fusobacterium*-induced cytokine/chemokine signaling, including CCL20/CCR6 axis, selectively regulates the inflammation and immune suppression in tumor microenvironment, thus promoting the colorectal tumor growth and progression.

In summary, we identified five species of *Fusobacterium* in clinical colorectal cancer specimens, of which *F. nucleatum* was the most predominant. We also identified and confirmed that *F. nucleatum* ssp. *animalis* was the most prevalent subspecies of *F. nucleatum* in colorectal tumors. Moreover, we found that colorectal tumors differentially expressed the proinflammatory cytokines IL17A, IL21, TNF, and CCL20, with CCL20 being highly expressed at every stage of CRC. In *in vitro* assays, coculture with *F. nucleatum* ssp. *animalis* markedly induced CCL20 protein expression in certain colorectal cancer cells. Similarly, the coculture strongly stimulated CCL20 protein expression in monocytes and induced monocyte/macrophage activation and migration, suggesting that *F. nucleatum* ssp. *animalis* interacts directly with monocytes, which may recruit other immunoregulatory cells via CCL20 signaling and subsequently promote colorectal cancer progression. These findings provide unique insight into the tumorigenic mechanism mediated by *Fusobacterium* spp. and mucosal immunity. More importantly, we identified a specific subspecies of tumor-associated *F. nucleatum* ssp. *animalis* that may be useful for early-stage detection of colorectal cancer and for antibiotic therapy or vaccination against for colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Ye, R. Bhattacharya, N.J. Ajami, M.C. Wong, D.P. Smith, J.F. Petrosino, W. Qiao, V. Baladandayuthapani
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Study supervision: L.M. Ellis

Acknowledgments

We thank Drs. James Versalovic and Jennifer K. Spinler of the Department of Pathology and Texas Children's Microbiome Center at Baylor College of Medicine, and Drs. Diane L.S. Hutchinson and

Suzanne Kennedy of the Alkek Center for Metagenomics and Microbiome Research at Baylor College of Medicine for their help and discussion during this study. The authors also thank Donald R. Norwood of the UTMDACC Department of Scientific Publications for editorial assistance.

Grant Support

This work was supported in part by NIH Cancer Center Support Grant CA016672, NIH grant R01CA157880 (to L.M. Ellis), Department of Defense grant CA100879 (to L.M. Ellis), the William C. Liedtke, Jr., Chair in Cancer Research (to L.M. Ellis), and a Texas Medical Center-Digestive Disease Center (TMC-DDC) Pilot Award (to X. Ye). In addition, this work used the TMC-DDC core facility that was supported in part by PHS grant P30 DK56338 (to principal investigators including J.F. Petrosino).

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Received July 14, 2016; revised December 2, 2016; accepted May 3, 2017; published OnlineFirst May 8, 2017.

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