Archives of Razi Institute, Vol. 78, No. 1 (2023) 445-452

DOI: 10.22092/ARI.2022.360307.2570

Original Article

Prevalence and Phylogenetic Analysis of *Fusobacterium nucleatum* and Virulent Factor *FadA* among Ulcerative Colitis Precancerous and Colorectal Carcinoma Patients in the Iraqi Kurdish Population

Taher, H. J[1 *], Kamel, F. H[2]

1. Department of Medical Laboratory Technology, Erbil Health and Medical Technical College, Erbil Polytechnic University, Erbil, Iraq
2. Department of Medical Laboratory Technology, Medical Technical Institute, Erbil Polytechnic University, Erbil, Iraq

Received 16 October 2022; Accepted 23 November 2022
Corresponding Author: hataw.taher@epu.edu.iq

Abstract

Fusobacterium nucleatum is considered one of the main risk factors that play a key role in the promotion and progression of colorectal carcinoma. The main goal of this study is to find out the association between the prevalence of various subtypes *of Fusobacterium nucleatum* with inflammation and colorectal cancer progression, in addition to screening the positive ratio of the possession of the *Fad*A gene. One hundred tissue samples were collected from healthy individuals and patients from colonoscopy and surgical operation biopsies. The patients were categorized into (Ulcerative colitis, precancerous colitis and colorectal carcinoma) according to their colonoscopy and histopathology examination reports. Molecular detection of *Fusobacterium nucleatum* and *Fad*A gene was performed via PCR and gel electrophoresis, and then phylogenetic analysis for *Fusobacterium nucleatum* was done using 16S rRNA partial sequencing based on specific primers. The results showed significant differences among the four groups regarding the prevalence of *Fusobacterium nucleatum.* The most prevalent subtype was *Fusobacterium nucleatum* subtype animalis, which constitutes 7 out of 17 samples. The ratio of *the Fad*A-positive gene was 20% among the *Fusobacterium nucleatum*-positive cases. This finding suggested a strong correlation between *Fusobacterium nucleatum* and colon inflammation and cancer progression steps, and *Fusobacterium nucleatum* subtype animalis was the most prevalent subtype.

Keywords: *Fusobacterium nucleatum*, *FadA*, Colorectal Carcinoma

1. Introduction

Colon cancer is one of the significant health problems, with an annual incidence of 1.7 million (1); nearly about 6.3 million people in the world have been diagnosed with CRC, that caused about 860000 death in 20018 (1, 2) all over the world, and this disease recorded the high morbidity in a year. In Iraq, colorectal cancer has increased in the last decades; its mortality rates increased by about 10% (3), and it has become the second and third most common cancer in women and men, respectively (4).

The microbiota that habituates the human intestinal tract form an ecosystem consisting of Archei, Bacteria, Fungi and Virus (5), including around 10^{14} bacteria. The highest amount is in the colon (6, 7) over 1000 species referring mainly to 4 identified phyla in human, of which each individual have 160 species (8). The investigations and evidence demonstrated that microbiota has a crucial role in maintaining intestinal

mucosal homeostasis and act as a protective epithelial barrier against other invasive pathogens, have an essential role in the promotion of wound repair (9, 10). Symbiotic relationship confirmed between commensal bacteria and healthy host, altering gut microbiota population, and irritable bowel disease leads to dysbiosis which contributed the CRC (11, 12).

*Fusobacterium nucleatum*is a gram-negative obligate anaerobic, non-motile, non-spore former bacteria spindle-shaped with pointed ends, and they are commensal microorganisms. Its natural habit is the oral cavity that employs the circulation system to reach the neoplastic cells in the intestine, where the Fap2 mediated in hemagglutination for transporting (13-17), although they act as opportunistic pathogens that can transmit from its natural habitat to other places through the bloodstream, e.g. from the oral cavity to gastrointestinal tract that leads to gastrointestinal carcinogenesis, or placenta and lead to preterm labour (18, 19) or urogenital tract (20, 21) then become pathogenic, also able to form biofilm as a structural supporting to other bacteria and associated with periodontal health and disease (22-25), from lung and pleural infection (26), bacteremia and liver abscesses, pancreatic cancer (27) appendicitis (28).

Fusobacterium nucleatum has several virulence factors that promote exploring the mechanism of CRC production. The most common basic biological characteristic associated with pathogenicity is; adhesin surface protein, which has a critical role in biofilm formation through the coaggregation of bacteria in the oral cavity and on the right side of a CRC patient's colon (29). It has invasive factors that influence the entrance of the bacteria to the host cells, like Fad A, which is selectively bound to E-cadherin and has a role in activating B- catenin signalling pathways, thus inducing oncogenic and inflammatory responses (30). *Fusobacterium nucleatum* possesses the most significant adhesion membrane protein, Fap2, which can inhibit natural killer cell cytotoxicity through binding to human immune inhibitory receptor T cell and ITIM domain. Hashemi Goradel, Heidarzadeh (31) recently showed that Fap 2 dependent invasions induce secretion of proinflammatory cytokines, ILK-8, and CXCL that promote CRC cell migration.

The main aim of this investigation is to determine the prevalence of detected subtypes of *Fusobacterium nucleatum* in ulcerative typical, colitis, Polyps and malignant CRC patients based on 16S rRNA sequencing. Then to detect the ratio of adhesive virulence factor FadA gene via PCR.

2. Materials and Methods

2.1. Subjects

The present study included four groups of subjects depending on their colonoscopy results and histopathological findings. The first group was regarded as control healthy people (25); the second group included patients who have ulcerative colitis (18); the third group were patients affected with precancerous polyps (19); and the last group were patients who have colorectal cancer (38). The studied subjects were collected in the OGD department from three hospitals within Erbil city from June 2021 until March 2023. The Ethical Committee approved this work of the College of Science at Salahaddin university. We obtained written informed consent from the patients who participated in this investigation before performing the study.

2.2. Colonoscopy Sample Collection

During this test, a flexible colonoscope and a viewing tube, a tool for removing tissue, were applied to check the colorectal region. Before starting the procedure, patients received sedative drugs. Then the observed abnormal noted growth was taken during a colonoscopy. The samples were collected during this process from the target region. All specimens were handled in phospho-buffer saline (PBS), directly transferred to the research center laboratory at Erbil polytechnic university, and stored at -80c.

2.3. Sample Collection from Surgical Operation

For malignant cases, surgical samples were obtained from CRC patients. Specimens were treated with the same procedure of colonoscopy sample collection. For

this study, biopsies were also taken for histopathological assessment.

2.4. Histopathology

Colon tissues were obtained immediately following surgery and directly fixed within formaldehyde. The process of histopathological slide preparation was made via embedding in paraffin. The staining process was performed using Hematoxylin and eosin stains added to the paraffin section (3–5 µm thickness) to identify the grade and the other histological findings for each sample.

2.5. DNA Extraction

Genomic DNA was extracted from tissue biopsies using the Favor Prep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech crop, Taiwan). Briefly, the tissue samples were cut up to 25 mg and ground into a microcentrifuge tube. The samples were processed according to the manufacturer's protocol. Each of provided buffers, Proteinase K and absolute ethanol, were added to the samples before applying the spin column. The samples underwent centrifugation and ran through column filters using a specific eluting buffer. Finally, the DNA quality and quantity of the extracted samples were measured using a NanoDrop spectrophotometer (Thermo Scientific, MA, USA).

2.6. The *F. Nucleatum* 16S rRNA-Based PCR and *Fad*A-Specific PCR

The extracted DNA samples were used for running conventional PCR and gel electrophoresis protocols. A specific sequence from 16S rRNA of *F. nucleatum* was used for a blast of primer design in the NCBI primer blast and Gene Runner program. Then the primer specificity was rechecked by nucleotide blast in the NCBI database. The primer 16S rRNA gene for *F. nucleatum* Forward was (F: 5′-AGA GTT TGA TCC TGG CTC AG -3′), and 16S rRNA Reverse was (R: 5′-GTC ATC GTG CAC ACA GAA TTG CTG-3′) with a product size of (380-bp) region, whereas *Fad*A primers Forward were (F:5′-CAC AAG CTG ACG CTG CTA GA -3′) and *Fad*A Reverse (R: 5′-TTA CCA GCT CTT AAA GCT TG -3′) with PCR band size of (232-bp) region.

The conventional PCR was performed on the extracted DNA samples using amplification reaction in a Thermal Cycler with 25 µl solution prepared as follows: (12.5 µl Master mix +0.5 µl F primer +0.5 µl R primer +3 µl DNA extracted from colon tissue samples +8.5 µl PCR-Garde DW).

The PCR protocol for *F. nucleatum* was performed with an initial 5 min at 94 °C, then 30 cycles repeated; each cycle consists of a denaturation process at 94 °C for 30 sec, annealing at 58 °C for 30 sec and extension at 72 °C for 1 min, with 10 min at 72 °C as a final extension. *Fad*A PCR protocol was performed with an initial 4 min at 94 °C, then 30 cycles repeated; each cycle consists of denaturing process at 94 °C for 30 sec, an annealing process at 55.8 °C for 30 sec and an extension at 72 °C for 40 sec, with 6 min at 72 °C as a final extension.

The PCR products of both protocols underwent the electrophoresis process by using 1.5% agarose gel in Tris-borate buffer (pH=8.0). The bands were visualized with safe stain via a gel documentation system.

2.7. 16S rRNA Sequencing and Phylogenetic Analysis

The F. nucleatum-positive PCR products were sent to the Molecular genetics Laboratory in Zheen international hospital, Erbil, for 16S rRNA sequencing using the same primer. The obtained data were compared to the reference 16S rRNA sequences at the NCBI database via applying nucleotide BLAST. Later, sequence alignment and Phylogenetic analysis were conducted using the bootstrap method. Then the phylogenetic tree was constructed based on the 16S rRNA nucleotide sequences using Molecular Evolutionary Genetic Analysis (MEGA11) software.

2.8. Statistical Analysis

Chi-squared test was applied to find the difference regarding the detection of *F. nucleatum* and *Fad*A among all groups. All statistical

analyses were performed using GraphPad Prism version 9. The values of $P<0.05$ were considered to be statistically significant.

3. Results

Out of one hundred studied patients, 54% were male, and 46% were female. The colonoscopy results were obtained, and the tissue biopsies were classified according to them. Molecular screening for F. nucleatum using conventional PCR technique reported highly significant differences among the studied groups ($P<0.001$). Approximately half of the patients (49%) suffering from CRC were positive for F. nucleatum, while the other groups, including normal, Ulcerative colitis and Precancerous polyps, recorded 16%, 23% and 40%, respectively (Figure 1).

The molecular identification process started with amplifying a specific region using a specific primer. Then, the electrogram bands with 380 PB were confirmed according to the DNA ladder measure (Figure 2a). Among the 34 positive samples, only 18 samples were sent for DNA sanger sequencing. The 16S rRNA partial sequencing revealed the various subtypes of F. nucleatum (Figure 2c).

The results of phylogenetic construction via the MEGA 11 software program reported 10 strains of *F. nucleatum* according to the NCBI blast for 16S r RNA partial sequencing. The most prevalent subtype was *F. nucleatum* subtype animalis strain KCOM 3229 with 7 taxa. Then 2 taxa belonged to the uncultured *Fusobacterium* clone MS055A1 (Figure 3).

The screening for *Fad*A positivity among *F. nucleatum*-positive patients showed that only 7 cases (20.59%) possessed the *Fad*A gene (Figure 4a). Among these 7 cases, 3 were CRC patients, while 2 were suffering from precancerous polyps (Figure 4b). The positive results were confirmed through gel electrophoresis, which resulted in 232 bp bands (Figure 5).

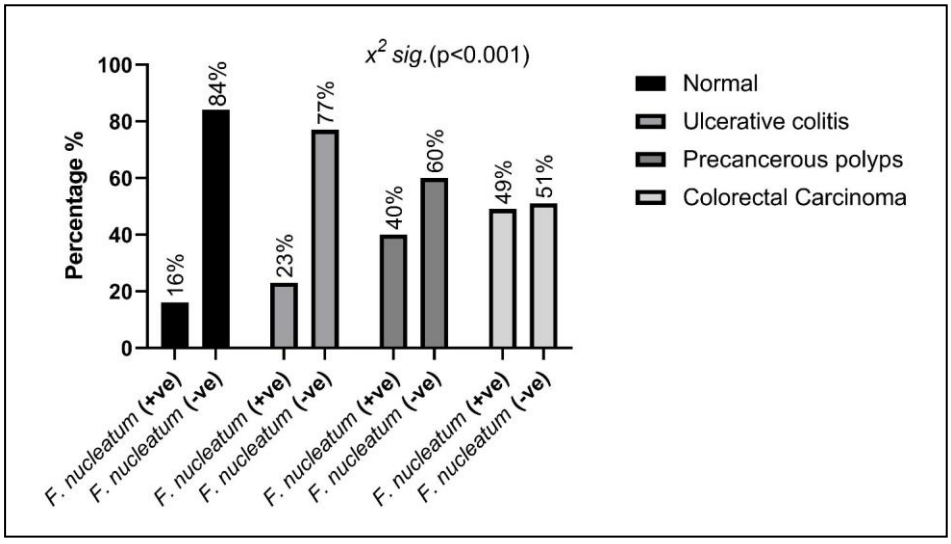

Figure 1. Percent values for detection of F. nucleatum among typical, ulcerative colitis, precancerous polyps and colorectal carcinoma samples using specific primer. Chi-square analysis revealed a significant difference between the studied group regarding the percent positive values for F. nucleatum at $P<0.001$

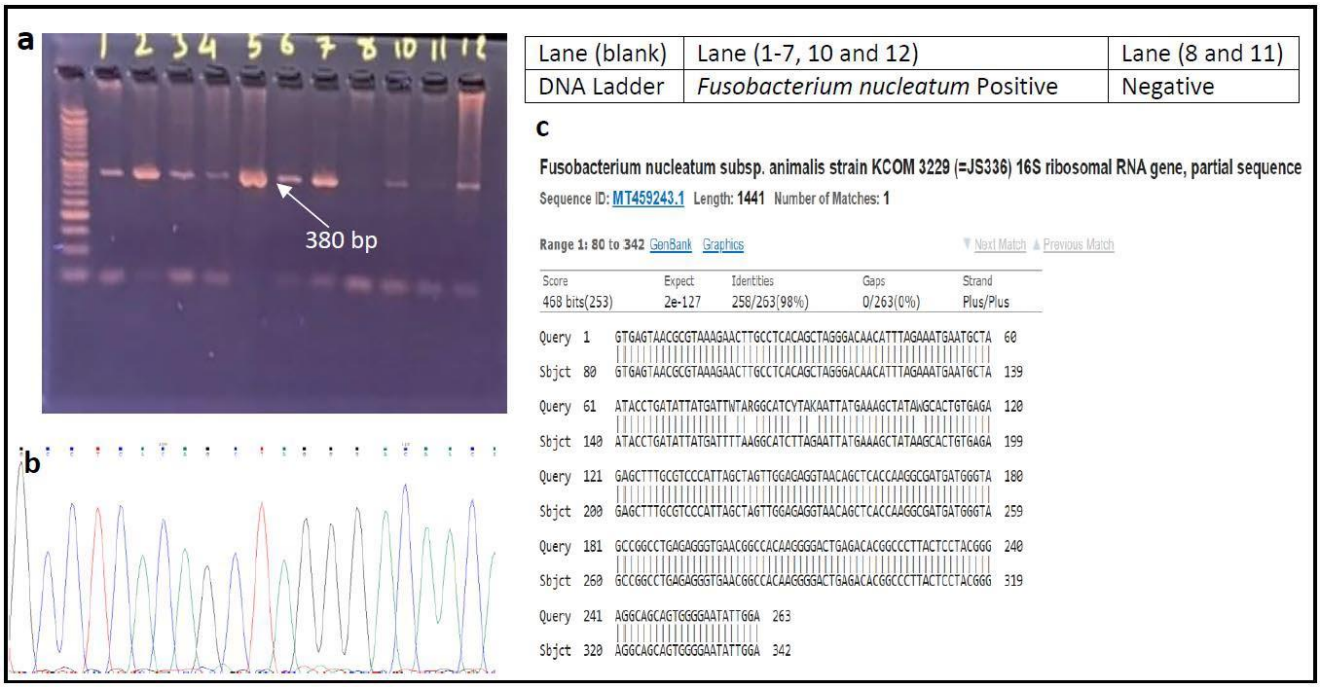

Figure 2. Molecular identification of F. nucleatum in clinically studied biopsies. **a:** Gel electrogram showing positive lanes and opposing lanes. The positive lane (1-7, 10 and 12) recorded 380 pb band size. **b:** Chromatogram sample showing the sequencing result. **c:** NCBI nucleotide blast result for partial sequencing of 16S rRNA gene

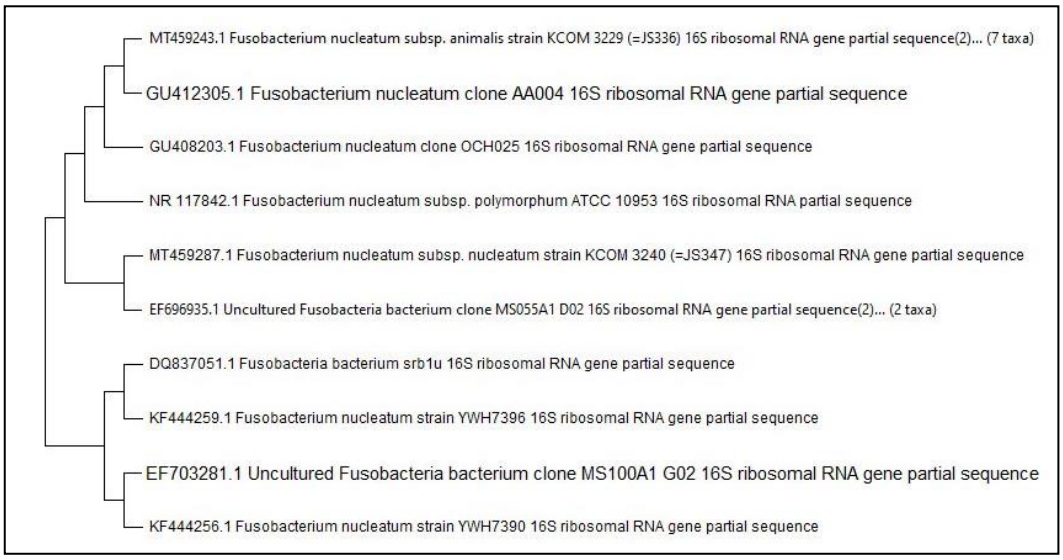

Figure 3. Phylogenetic tree analysis in human colon cancer tissues detected various *F. nucleatum* sub-sp. strains were obtained during 16S rRNA alignment. The most prevalent subtype was *F. nucleatum* subsp. animalis with 7 taxa

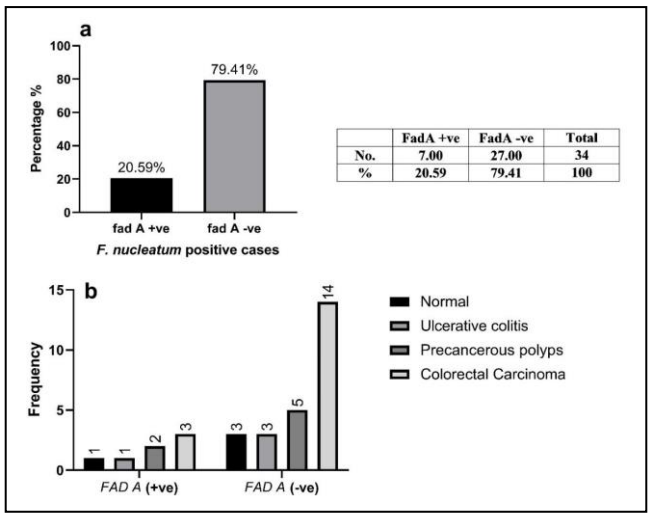

Figure 4. Detection of *Fad*A positive gene among studied clinical biopsies. **a:** Percent values for detecting *F. nucleatum* with positive and negative FadA using specific primer. **b:** FadA-positive and negative bacteria are frequent among typical ulcerative colitis, precancerous polyps and colorectal carcinoma samples

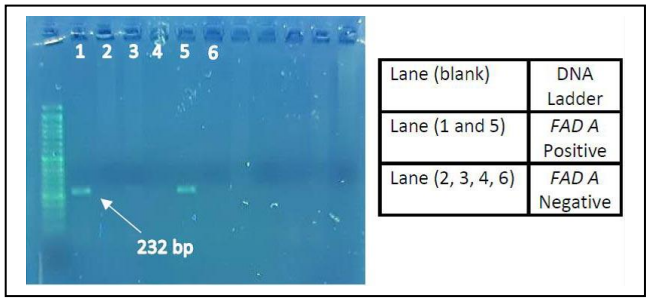

Figure 5. Molecular identification of *Fad*A gene among clinical samples which recorded positive *F. nucleatum*. Gel electrogram showing positive lanes and negative lanes. The positive lane (1 and 5) recorded 232 pb band size

4. Discussion

The present study determined the prevalence of various subtypes of *F. nucleatum* in healthy individuals and patients with ulcerative colitis, precancerous polyps and colorectal cancer in the Iraqi-Kurdish population. In addition to detecting the ratio of *F. nucleatum*, which carries *the Fad*A gene.

Several pieces of evidence indicated the association between the alteration of the gastrointestinal microbiome and with carcinogenesis mechanisms of colorectal carcinoma (32). It becomes one of the critical risk factors for tumorigenesis, tumour metastasis (33) and immune modulation against cancer (34). Our findings regarding our samples supported the relationship between the rate of *F. nucleatum* infection and inflammation and cancer progression status.

Our findings suggested a strong relationship between CRC and *F. nucleatum* subtype animalis (about half of the phylogenetic tree based on 16S rRNA sequencing results belonged to *F. nucleatum* subtype animalis). This result was supported by Borozan, Zaidi (35), who concluded that *F. nucleatum*, especially subtype animalis, strongly correlated with a higher colorectal cancer-specific mortality and specific somatic mutated genes. The possible reason might be due to the immunomodulatory action of *F. nucleatum* subtype animalis (36) or the induction of inflammatory cytokines that promote colorectal carcinogenesis (37).

Molecular detection of *Fad*A results showed that only 20% of *F. nucleatum* cases were positive for *the Fad*A gene. A similar study observed that *Fad*A gene positive was prevalent among CRC *F. nucleatum* positive cases (38). It has been demonstrated that *the Fad*A gene may play a key role in colorectal carcinogenesis via modulating E-cadherin/β-catenin signalling (39).

This finding suggested that *F. nucleatum* subtype animalis strain KCOM 3229 is the most prevalent strain in colorectal cancer patients based on specific primer for 16S rRNA partial sequence; the *Fad*A gene was found in lesser than a quarter of the *F. nucleatum* positive cases.

Authors' Contribution

Study concept and design: H. J. T.
Taher, H. J1 *, Kamel, F. H2
Acquisition of data: H. J. T.
Analysis and interpretation of data: H. J. T.
Drafting of the manuscript: F. H. K.
Critical revision of the manuscript for important intellectual content: F. H. K.
Statistical analysis: F. H. K.
Administrative, technical, and material support: F. H. K.

Ethics

The Ethical Committee approved this work of the College of Science at Salahaddin University. We obtained written informed consent from the patients who participated in this investigation before performing the study.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: Cancer J Clin. 2018;68(6):394-424.
2. Degett TH, Dalton SO, Christensen J, Søgaard J, Iversen LH, Gögenur I. Mortality after emergency treatment of colorectal cancer and associated risk factors—a nationwide cohort study. Int J Colorectal Dis. 2019;34(1):85-95.
3. Schreuders EH, Wieten E, Kuipers EJ, Spaander MC. Optimizing fecal immunochemical testing for colorectal cancer screening. Clin Gastroenterol Hepatol. 2017;15(10):1498-9.
4. Al-Saigh TH, Al-Bayati SA, Abdulmawjood SA, Ahmed FA. Descriptive Study of Colorectal Cancer in Iraq, 1999-2016. ACMM. 2019;41(1):81-5.
5. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. N Engl J Med. 2016;375(24):2369-79.
6. Yu LC-H, Wang J-T, Wei S-C, Ni Y-H. Host-microbial interactions and regulation of intestinal epithelial barrier function: From physiology to pathology. World J Gastrointest Pathophysiol. 2012;3(1):27.
7. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. PLoS biology. 2016 Aug 19;14(8):e1002533.
8. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
9. Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. The resilience of the intestinal microbiota influences health and disease. Nat Rev Microbiol. 2017;15(10):630-8.
10. Nagao-Kitamoto H, Kitamoto S, Kuffa P, Kamada N. Pathogenic role of the gut microbiota in gastrointestinal diseases. Intest Res. 2016;14(2):127.
11. Schwabe RF, Jobin C. The microbiome and cancer. Nature Reviews Cancer. 2013;13(11):800-12.
12. Abreu MT, Peek Jr RM. Gastrointestinal malignancy and the microbiome. Gastroenterology. 2014;146(6):1534-46. e3.
13. Rich JN. Cancer stem cells in radiation resistance. Cancer Res. 2007;67(19):8980-4.
14. Moore W. Moore LV-1994-. The bacteria of periodontal diseases. Periodontol 2000. 2000;5:66-77.
15. Fragkioudakis I, Riggio MP, Apatzidou DA. Understanding the microbial components of periodontal diseases and periodontal treatment-induced microbiological shifts. J Med Microbiol. 2021;70(1):001247.
16. Han YW, Ikegami A, Rajanna C, Kawsar HI, Zhou Y, Li M, et al. Identification and characterization of a novel adhesin unique to oral fusobacteria. J Bacteriol. 2005;187(15):5330-40.
17. Datorre JG, de Carvalho AC, Guimaraes DP, Reis RM. The Role of Fusobacterium nucleatum in Colorectal Carcinogenesis. Pathobiology. 2021;88(2):127-40.
18. Payne MS, Bayatibojakhi S. Exploring preterm birth as a polymicrobial disease: an overview of the uterine microbiome. Front Immunol. 2014;5:595.
19. Payne MS, Newnham JP, Doherty DA, Furfaro LL, Pendal NL, Loh DE, et al. A specific bacterial DNA signature in the vagina of Australian women in midpregnancy predicts high risk of spontaneous preterm birth (the Predict1000 study). Am J Obstet Gynecol. 2021;224(2):206.
20. Ribot S, Gal K, Goldblat MV, Eslami HH. The role of anaerobic bacteria in the pathogenesis of urinary tract infections. J Urol. 1981;126(6):852-3.
21. Xu R, Deebel N, Casals R, Dutta R, Mirzazadeh M. A New Gold Rush: A Review of Current and Developing Diagnostic Tools for Urinary Tract Infections. Diagnostics. 2021;11(3):479.
22. Signat B, Roques C, Poulet P, Duffaut D. Role of Fusobacterium nucleatum in periodontal health and disease. Curr Issues Mol Biol. 2011;13(2):25-36.
23. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. Nat Rev Microbiol. 2018;16(12):745-59.

24. Zhang Y, Wang X, Li H, Ni C, Du Z, Yan F. Human oral microbiota and its modulation for oral health. Biomed Pharmacother. 2018;99:883-93.
25. Nair V. Oral bacteria and colorectal pathology. Ann Colorectal Res. 2021;9(2):47-50.
26. Maitre T, Ok V, Calin R, Lassel L, Canestri A, Denis M, et al. Pyogenic lung abscess in an infectious disease unit: a 20-year retrospective study. Ther Adv Respir Dis. 2021;15:17534666211003012.
27. Mitsuhashi K, Nosho K, Sukawa Y, Matsunaga Y, Ito M, Kurihara H, et al. Association of Fusobacterium species in pancreatic cancer tissues with molecular features and prognosis. Oncotarget. 2015;6(9):7209.
28. Yoshida A, Ikegami A. Genetic Transformation of Fusobacterium nucleatum. Periodontal Pathogens: Springer; 2021. p. 43-50.
29. Fan X, Jin Y, Chen G, Ma X, Zhang L. Gut microbiota dysbiosis drives the development of colorectal cancer. Digestion. 2021;102(4):508-15.
30. Casasanta MA, Yoo CC, Udayasuryan B, Sanders BE, Umaña A, Zhang Y, et al. Fusobacterium nucleatum host-cell binding and invasion induces IL-8 and CXCL1 secretion that drives colorectal cancer cell migration. Sci Signal. 2020;13(641).
31. Hashemi Goradel N, Heidarzadeh S, Jahangiri S, Farhood B, Mortezaee K, Khanlarkhani N, et al. Fusobacterium nucleatum and colorectal cancer: A mechanistic overview. J Cell Physiol. 2019;234(3):2337-44.
32. Kong C, Yan X, Zhu Y, Zhu H, Luo Y, Liu P, et al. Fusobacterium Nucleatum Promotes the Development of Colorectal Cancer by Activating a Cytochrome P450/Epoxyoctadecenoic Acid Axis via TLR4/Keap1/NRF2 Signaling. Cancer Res. 2021;81(17):4485-98.
33. Xu C, Fan L, Lin Y, Shen W, Qi Y, Zhang Y, et al. Fusobacterium nucleatum promotes colorectal cancer metastasis through miR-1322/CCL20 axis and M2 polarization. Gut microbes. 2021;13(1):1980347.
34. Nosho K, Sukawa Y, Adachi Y, Ito M, Mitsuhashi K, Kurihara H, et al. Association of Fusobacterium nucleatum with immunity and molecular alterations in colorectal cancer. World J Gastroenterol. 2016;22(2):557-66.
35. Borozan I, Zaidi SH, Harrison TA, Phipps AI, Zheng J, Lee S, et al. Molecular and Pathology Features of Colorectal Tumors and Patient Outcomes Are Associated with Fusobacterium nucleatum and Its Subspecies animalis. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2022;31(1):210-20.
36. Lamprinaki D, Garcia-Vello P, Marchetti R, Hellmich C, McCord KA, Bowles KM, et al. Siglec-7 Mediates Immunomodulation by Colorectal Cancer-Associated Fusobacterium nucleatum ssp. animalis. Front Immunol. 2021;12:744184.
37. Ye X, Wang R, Bhattacharya R, Boulbes DR, Fan F, Xia L, et al. Fusobacterium Nucleatum Subspecies Animalis Influences Proinflammatory Cytokine Expression and Monocyte Activation in Human Colorectal Tumors. Cancer Prev Res. 2017;10(7):398-409.
38. Kashani N, Bezmin Abadi AT, Rahimi F, Forootan M. FadA-positive Fusobacterium nucleatum is prevalent in biopsy specimens of Iranian patients with colorectal cancer. New Microbes New Infect. 2020;34:100651.
39. Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. Cell Host Microbe. 2013;14(2):195-206.