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Detection of genetic determinants of potentially oncogenic representatives of the intestinal microbiota as biomarkers of colorectal cancer

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Abstract

Relevance. Colorectal cancer (CRC) is the second leading cause of cancer mortality worldwide. Non-invasive diagnostic methods based on the determination of hidden blood in the stool (fecal immunochemical test, guaiac test), which have been proven to be effective in clinical studies, are used for CRC screening. However, a significant disadvantage of the available non-invasive diagnostic methods is the low sensitivity in detecting the oncological process at the early stages. A number of recent studies discuss the relationship between the disease and various potentially oncogenic microorganisms in the human intestinal tract, which can be used to expand the arsenal of non-invasive methods for diagnosing CRC based on molecular genetic examination of a stool sample to identify oncogenic microorganisms.

The aim of this study was to evaluate the possibility of using genetic determinants of potentially oncogenic microorganisms as markers for colorectal cancer, based on a comparison of their prevalence in groups of patients with colorectal cancer, facultative precancerous diseases and patients without intestinal pathology.

Materials and methods. 215 participants were included in the "case–control" study: 70 patients with newly diagnosed colorectal cancer, 70 patients with inflammatory bowel disease, 75 participants without diagnosed intestinal pathology. Polymerase chain reaction (PCR) was used to identify and detect genes of potentially oncogenic microorganisms.

Results and discussion. An association was found between CRC and the presence of the *Bacteroides fragilis* fragilisin gene (OR 7.00; 95% CI: 2.55–22.50; $p < 0.001$), species-specific genes of the periodontal pathogenic microorganisms *Fusobacterium nucleatum* (OR 5.61; 95% CI: 2.87–11.30; $p < 0.001$) and *Porphyromonas gingivalis* (OR 16.3; 95% CI: 4.33–106.00; $p < 0.001$), the *clbB* gene of *pks* pathogenicity island of the enterobacteria (OR 3.44; 95% CI: 1.39–8.51; $p = 0.010$).

Conclusion. The presence of genetic markers of potentially oncogenic bacterial species and genotypes in the gut microbiome is associated with colorectal cancer. The results obtained support the possibility of using molecular genetic detection of the studied potentially oncogenic microorganisms as a method for non-invasive diagnosis of CRC.

Keywords: *colorectal cancer, microbiota, microbial markers, potentially oncogenic microorganisms, non-invasive diagnosis, screening*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the North-Western State Medical University named after I.I. Mechnikov (protocol No. 10, November 3, 2021).

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Оригинальное исследование
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Детекция генетических детерминант потенциально онкогенных представителей микробиоты кишечника в качестве биомаркеров колоректального рака

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Аннотация

Введение. Колоректальный рак (КРР) является второй по значимости причиной смертности от рака в мире. Для скрининга КРР применяются неинвазивные методы диагностики, основанные на определении скрытой крови в стуле (фекальный иммунохимический тест, гваяковый тест), хорошо зарекомендовавшие себя в клинических исследованиях. Однако существенным недостатком неинвазивных методов диагностики является невысокая чувствительность при выявлении онкологического процесса на ранних стадиях. В ряде современных работ обсуждается связь заболевания с различными потенциально онкогенными микроорганизмами (МО) в кишечном тракте человека, которые могут быть использованы для расширения арсенала неинвазивных методов диагностики КРР на основе молекулярно-генетического исследования образца кала для идентификации онкогенных МО.

Цель исследования — оценка возможности использования генетических детерминант потенциально онкогенных МО в качестве маркеров КРР, основанная на сопоставлении их распространённости в группах пациентов с КРР, факультативными предраковыми состояниями и пациентов без патологии кишечника.

Материалы и методы. В исследование, организованное по дизайну «случай–контроль», было включено 215 участников: 70 пациентов с впервые выявленным КРР, 70 пациентов с воспалительными заболеваниями кишечника, 75 участников без диагностированной патологии кишечника. Детекцию генов потенциально онкогенных МО осуществляли с помощью метода полимеразной цепной реакции.

Результаты и обсуждение. Установлена связь между КРР и наличием гена фрагилизина *Bacteroides fragilis* (ОШ = 7,00; 95% ДИ 2,55–22,50; $p < 0,001$), видоспецифических генов пародонтопатогенных МО *Fusobacterium nucleatum* (ОШ = 5,61; 95% ДИ 2,87–11,30; $p < 0,001$) и *Porphyromonas gingivalis* (ОШ = 16,3; 95% ДИ 4,33–106,00; $p < 0,001$), гена *clbB* острова патогенности *pks* энтеробактерий (ОШ = 3,44; 95% ДИ 1,39–8,51; $p = 0,010$).

Заключение. Наличие в составе кишечного микробиома генетических маркеров потенциально онкогенных видов бактерий ассоциировано с КРР. Полученные результаты обосновывают возможность применения молекулярно-генетической детекции изученных потенциально онкогенных МО в качестве метода неинвазивной диагностики КРР.

Ключевые слова: *колоректальный рак, микробиота, микробные маркеры, потенциально онкогенные микроорганизмы, неинвазивная диагностика, скрининг*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов и было одобрено на заседании Локального этического комитета СЗГМУ им. И.И. Мечникова (протокол № 10 от 03.11.2021).

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Colorectal cancer (CRC) is the 3rd most common type of cancer and the 2nd leading cause of cancer mortality in the world [1]. It is noteworthy that the incidence of colorectal cancer is increasing among people under 50 years of age [2]. Noninvasive (stool blood test, guaiac test and fecal immunochemical test (FIT)) and invasive (flexible rectoromanoscopy, colonoscopy) diagnostic methods are used to detect colorectal cancer. The obvious advantages of non-invasive diagnostic methods are their simplicity and availability for screening examinations of persons at risk.

The use of noninvasive diagnostic methods, such as FIT, in screening examinations can achieve a 27% reduction in mortality from CRC [3]. A meta-analysis of the results of 4 randomized controlled trials demonstrated that the use of guaiac test and flexible rectoromanoscopy contributes to the reduction of CRC mortality by 18% and 26%, respectively [4].

However, a significant disadvantage of noninvasive methods is their low diagnostic sensitivity at early stages of the disease. Thus, the sensitivity of FIT at stage I of CRC is 68% (95% CI 57–78%), at stage II — 92% (95% CI 87–96%), at stage III — 82% (95% CI 73–89%) [5]. Thus, there is a need to expand the arsenal of available non-invasive methods for CRC screening.

Specific features of the quantitative and qualitative composition of the microbiota can act as diagnostic markers of CRC, which is discussed in detail in reviews devoted to the role of the intestinal microbiota in the development and diagnosis of intestinal cancer [6, 7]. Numerous studies have revealed changes in the composition of the intestinal microbiome associated with the development of CRC, which suggests the possibility of using the identification of individual representatives of the intestinal microbiome as an independent method of noninvasive diagnosis of CRC or as an addition to existing noninvasive methods.

The first classification model based on intestinal microbial markers and allowing to distinguish patients with CRC from controls was proposed by G. Zeller et al. [8]. The classification algorithm included data on the relative abundance of 22 microorganism species (MOs), but at least half of the predictive power of the model was determined by only 4 species: two *Fusobacterium* species, *Porphyromonas asaccharolytica* and *Peptostreptococcus stomatis*, the presence of each of which was elevated in CRC.

Authors from the Chinese University of Hong Kong developed a diagnostic model demonstrating specificity of 81.2% and sensitivity of 93.8% when combining FIT and 4 bacterial markers (the *"m3"* marker gene of *"Lachnoclostridium"* sp., *Fusobacterium nucleatum*, *Hungatella hathewayi* (baseonym: *Clostridium hathewayi*) and *Bacteroides clarus*) [9]. In an earlier study, an experimental model based on 23 MOs from the *Oscillospiraceae* (heterotypic synonym: *Ruminococcaceae*) and *Lachnospiraceae* families, the *Bacteroides*, *Porphyromonas*, *Parabacteroides*, *Collinsella* genera and the *Enterobacteriaceae* family detected 91.7% of CRC cases in a sample of 490 patients, compared to 75.0% of cases detected by FIT [10].

The MOs most closely associated with CRC include both some oral pathobionts and intestinal bacteria. The former, in addition to the already mentioned representatives of the genera *Fusobacterium*, *Porphyromonas* and *Peptostreptococcus*, include *Parvimonas micra*, *Gemella morbillorum*, *Tannerella forsythia* and some other species [11]. Intestinal bacteria are most often represented by enterotoxigenic strains of *Bacteroides fragilis*, pathogenic and opportunistic bacteria of the *Enterobacteriaceae* family (*Escherichia coli*, *Salmonella spp.*, *Klebsiella pneumoniae*, *Citrobacter rodentium*), as well as *Campylobacter jejuni*, *Morganella morganii*, *Enterococcus faecalis*, *Clostridioides difficile* and others [12].

Based on the results of the literature review, we selected the MOs most frequently associated with CRC according to research data. We included colibactin-producing *Enterobacteriaceae* carrying the *clbA* and *clbB* genes as part of the *pks* pathogenicity island, fragilysin-producing *Bacteroides fragilis* carrying the *bft* gene, periodontal pathogenic bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis* among the potentially oncogenic MOs [6]. An additional factor in favor of including these MOs in the list of potential oncogenes was their association with the stage (progression) of CRC, prognosis/survival and resistance to therapy in patients with CRC [13, 14].

Taking into account probable regional, ethnic and other peculiarities of quantitative and qualitative composition of intestinal microbiota, it is urgent to assess the prevalence of the above mentioned potentially oncogenic MOs among the Russian population and their role in the development of CRC.

The aim of this study is to evaluate the possibility of using genetic determinants of potentially oncogenic MIs as markers of CRC, based on the comparison of their prevalence in groups of patients with CRC, facultative precancerous conditions and patients without intestinal pathology.

Materials and methods

On the basis of clinical departments of the Peter the Great Clinic of the I.I. Mechnikov NWSMU and the City Oncologic Dispensary of St. Petersburg in 2022–2024 a case-control study was conducted. A case-control study was conducted in 2022–2024 at the Peter the Great Clinic of the I.I. Mechnikov NWSMU and the City Oncologic Dispensary of St. Petersburg. The study included 215 participants: a group of 70 patients with diagnosed CRC (CRC group); a group of 70 patients with inflammatory bowel diseases (ulcerative colitis, Crohn's disease — IBD group); 75 participants without

Table 1. Nucleotide sequences of primers for identification of potentially oncogenic microorganisms

Target gene	GenBank Accession number	Forward primer/reverse primer	Source
The *clbA pks+* (polyketide synthase) gene of *E. coli*	CP155641.1	5'-CTCCACAGGAAGCTACTAAC-3' 5'-CGTGGTGATAAAGTTGGGAC-3'	[15]
The *clbB pks+ (*polyketide synthase) gene of *E. coli*	CP155641.1	5'-GCAACATACTCGCCCAGACT-3' 5'-TCTCAAGGCGTTGTTGTTTG-3' probe FAM (5'-CAAGGTGCGCGCTAGGCTGT-3');	[16]
The fragilisin (*bft*) gene synthesized by enterotoxigenic *B. fragilis*	AF103902.1	5'-GAACCTAAAACGGTATATGT-3' 5'-GTTGTAGACATCCCACTGGC-3'	[15]
fadA adhesion protein gene of *F. nucleatum*	DQ012973.1	5'-GCAGTTTCTGCTTCAGCATTT-3' 5'-TGCTTGAAGTCTTTGAGCTCTTT-3'	[17]
fimA gene for biofilm formation of *P. gingivalis*	AB195793.1	5'-TGCGACGCTATATGCAAGAC-3' 5'-TCTTCAAAACCACGCTGATG-3'	[17]

diagnosed bowel pathology (control group). The study was conducted with voluntary informed consent of the patients and was approved at the meeting of the Local Ethical Committee of the I.I. Mechnikov NWSMU (protocol No. 10 of 03.11.2021).

In the group of patients with CRC the distribution by stages of oncologic process taking into account TNM classification was as follows: stage 0 — 1 patient; stage 1 — 17 patients; stage 2 — 13 patients; stage 3 — 37 patients; stage 4 — 12 patients.

Inclusion criteria for patients from the CRC group: age over 18 years; diagnosis of CRC established for the first time on the basis of anamnesis, physical examination, morphological examination of tumor material, data of instrumental and laboratory methods of examination; receipt of clinical material (feces) from the patient.

Inclusion criteria for patients from the IBD group: age over 18 years; diagnosed IBD.

Inclusion criteria for patients from the control group: age over 18 years; no diagnosed IBD and CRC.

Exclusion criteria for all study groups: taking an antibacterial drug for the last 30 days and/or undergoing endoscopic examination (colonoscopy, rectomanoscopy) for the last 14 days before the submission of clinical material (feces).

Samples of clinical material obtained from study participants were stored at –20ºC until molecular genetic studies were performed.

DNA from fecal samples for polymerase chain reaction (PCR) was isolated by magnetic particle sorption using the MetaFec reagent kit (Raissol). The primer sequences used earlier for identification of potentially oncogenic MOs were applied in PCR (**Table 1**).

To identify potentially oncogenic MOs, quantitative PCR with hybridization-fluorescence detection (Bio-Rad CFX96 Thermal Cycler) was performed for *clbB pks*$^+$ and qualitative PCR (Bio-Rad T100 Thermal Cycler) with electrophoretic detection for other markers.

No cases of nonspecific amplification were detected.

The results of marker genes amplification were validated by capillary sequencing of amplicons on the GenomeLab GeXP device (Beckman Coulter, Inc.), and in all cases the sequences corresponding to the sequences of target fragments of marker genes presented in GenBank were obtained (**Table 1**). Aliquots of total DNA from the first 5 samples of biological material, in which positive PCR results were obtained, were used as positive controls after confirmation of amplification specificity by sequencing, and sterile deionized water served as a negative control.

R program (RStudio) was used for statistical data processing. To quantify the association of potentially oncogenic MOs with CRC, odds ratios (ORs) and 95% confidence intervals (CI) to them were determined. Sensitivity, specificity and CI were calculated using the Epitools[1] program. The results were considered statistically significant at $p < 0.05$.

Results

The study of clinical material samples by PCR allowed us to determine the detection rates of genetic markers of potentially oncogenic MOs in patients with CRC at different stages of the disease and in the comparison groups (**Fig. 1**). A higher prevalence of potentially oncogenic markers was detected in patients with CRC compared to patients with CRC and control group participants. There was a direct correlation between the stage of cancer and the prevalence of potentially oncogenic markers, in particular, *F. nucleatum* DNA, and a higher frequency of detection of periodontal pathogenic MOs at late stages of the cancer process. Colibactin-producing bacteria carrying the *clbB* gene of the *pks* pathogenicity island prevail at early tumor stages.

The association of some potentially oncogenic representatives of the intestinal microbiome with CRC was observed in the course of the case-control study (**Table 2**). Thus, the presence of the *bft* gene of entero-

[1] Epitools — Epidemiological Calculators. URL: http://epitools.ausvet.com.au

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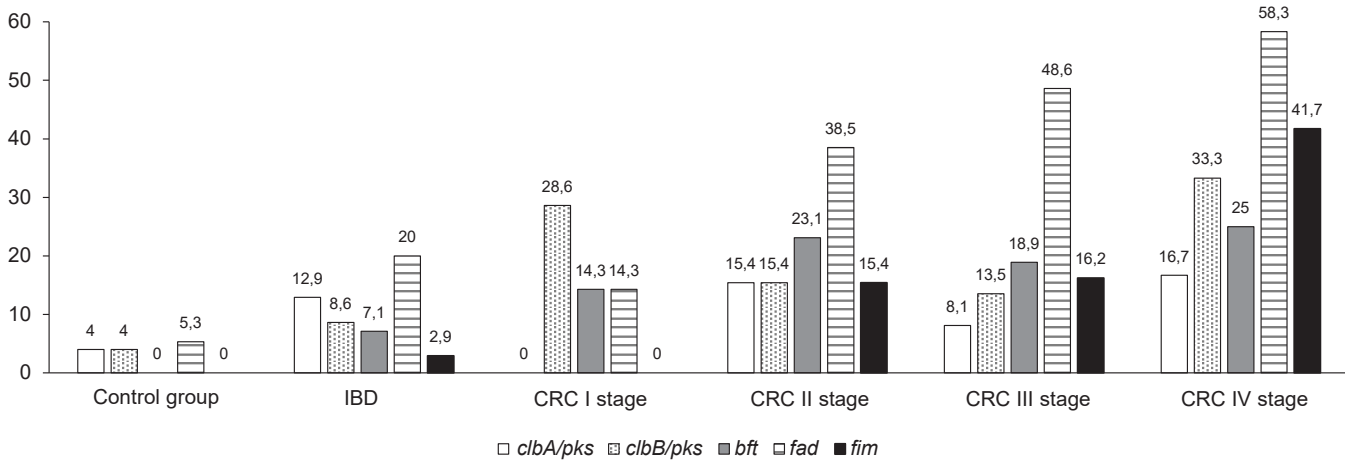

The prevalence of potentially oncogenic microorganisms in patients with CRC at various stages of the disease and in comparison groups.

toxigenic *B. fragilis* was significantly different between the groups of patients with IBD and patients with CRC and associated with the microbiome of patients with CRC (OR = 3.25; 95% CI 1.16–10.6; $p = 0.033$). Furthermore, a higher prevalence of this gene was found in patients with CRC compared to patients with IBD, while the enterotoxigenic *B. fragilis* gene was not detected in samples from participants in control group.

According to the data of this study, the presence of DNA of the periodontal pathogenic MO *F. nucleatum* was found to be associated with CRC, and its detection rate differed between the case and control groups. In patients with IBD, CRC was associated with the presence of DNA from another periodontal pathogenic MO, *P. gingivalis* (OR = 7.75; 95% CI 2.03–50.9; $p = 0.009$). The frequency of *F. nucleatum* DNA identification was higher in patients with CRC (44%) compared to patients with IBD (22%) and control group participants (5.3%). A high prevalence of *P. gingivalis* DNA was found in CRC patients (in 19% of samples) compared to IBD patients (in 2.9% of samples), *P. gingivalis* DNA was not detected in control group participants.

This study found that the presence of the *clbB* gene of the pathogenicity island of *Enterobacteriaceae pks* (OR = 5.47; 95% CI 1.49–20.14; $p = 0.015$) was associated with CRC and allowed us to separate the microbiome of patients with this pathology from that of control participants. The detectable *Enterobacteriaceae pks* pathogenicity island *clbB* gene was found in 19% of samples from patients with CRC, in 8.6% from patients with IBD, and in 4% from control participants. In contrast, the frequency of *clbA* gene identification did not differ between the comparison groups. The *clbA* gene was identified in 10% of samples from patients with CRC, in 13% of samples from patients with IBD, and in 4% of samples from control group participants.

In patients with CRC, the frequency of detection of markers of potentially oncogenic MOs did not depend on tumor localization: detection of *F. nucleatum* in tumors of distal location in 14 cases, proximal location — in 15 cases; *P. gingivalis* — in 5 and 7 cases; *clbB* gene of the *Enterobacteriaceae pks* pathogenicity island — in 6 and 7 cases; *clbA* gene — in 2 and 5 cases; fragilisin *bft* gene of enterotoxigenic *B. fragilis* – in 6 and 7 cases, respectively. No significant differences related to the morphological type of the tumor were revealed (detection of *F. nucleatum* in 18 cases of highly differentiated tumor, in 13 cases of low-differentiated tumor; *P. gingivalis* — in 6 and 7 cases; *clbB* gene of the *Enterobacteriaceae pks* pathogenicity island — in 7 and 6 cases; *clbA* gene — in 4 and 3 cases; fragilisin *bft* gene of enterotoxigenic *B. fragilis* — in 6 and 8 cases, respectively). Thus, the detection of markers of potentially oncogenic MOs allows to distinguish CRC regardless of the localization and morphological type of tumor.

Taking into account the obtained data, we calculated the sensitivity and specificity of the diagnostic test, which allows us to distinguish the microbiomes of patients with CRC from the microbiomes of patients with IBD and control group participants in case of separate and joint detection of genetic determinants of potentially oncogenic MOs (**Table 3**). The optimal (in terms of combination of sensitivity and specificity) variant of testing seems to be the one that takes into account the fact of DNA identification of at least 1 out of 5 potential microbial onco-markers.

Discussion

In the present study, we evaluated the prevalence of potentially oncogenic CRC-associated MOs among residents of a Russian megacity with diagnosed CRC, IBD and individuals without intestinal tract pathology.

It is known that *B. fragilis* is a commensal representative of the intestinal microbiota. Both non-toxigenic strains of *B. fragilis* (not associated with CRC) [18] and enterotoxigenic *B. fragilis* synthesizing fragilisin,

Table 2. Representatives of the intestinal microbiome associated with CRC

Target gene	CRC, *n* (%)	IBD, *n* (%)	Control, *n* (%)	OR (95% CI)			
				CRC/IBD	CRC/Control	CRC + IBD/Control	CRC/IBD + Control
Fragilisin *bft* gene of enterotoxigenic *B. fragilis*	14 (20%)	5 (7,1%)	0 (0%)	3,25 (1,16–10,6)	∞	∞	7 (2,55–22,5)
fadA adhesion protein gene of *F. nucleatum*	31 (44%)	14 (20%)	4 (5,3%)	3,18 (1,52–6,9)	14,1 (5,13–50,1)	8,41 (2,89–24,46)	5,61 (2,87–11,3)
fimA gene for biofilm formation of *P. gingivalis*	13 (19%)	2 (2,9%)	0 (0%)	7,75 (2,03–50,9)	∞	∞	16,3 (4,33–106)
The gene *clbA* of the pks pathogenicity island of *E. coli*	7 (10%)	9 (13%)	3 (4,0%)	0,75 (0,25–2,15)	2,67 (0,71–12,8)	3,09 (0,87–10,99)	1,23 (0,44–3,21)
The gene *clbB* of the pks pathogenicity island of *E. coli*	13 (19%)	6 (8,6%)	3 (4,0%)	2,43 (0,9–7,31)	5,47 (1,49–20,14)	3,77 (1,08–13,18)	3,44 (1,39–8,51)

Note. ∞ — due to the zero value of the denominator, it is not possible to calculate the OR indicator.

a toxin that cleaves the cell adhesion protein E-cadherin, disrupting the intestinal barrier and contributing to the development of diarrhea, are present in the gastrointestinal tract [19]. In addition, this toxin can activate the implementation of the Wnt/β-catenin signaling pathway, promoting cell proliferation, induction of inflammatory mediator production, and carcinogenesis [20]. The toxin of *B. fragilis* is encoded by the *bft* gene with 3 isotypes (*bft 1*, *bft 2*, *bft 3*), which is located in the pathogenicity island (PAI) and flanked by genes encoding mobilization proteins and representing CTn86 and CTn9343 conjugative transposon sequences. Non-toxigenic strains of *B. fragilis* do not possess a pathogenicity island, but in the presence of conjugative transposons in some strains PAI can transfer from enterotoxigenic *B. fragilis* to non-toxigenic strains of *B. fragilis* [21]. The role of enterotoxigenic *B. fragilis* as a "driver" in the "driver-passenger" model has been established, which consists in the damage of epithelial DNA by "driver" bacteria, which leads to the development of carcinogenesis and changes in the microbial community; further in the process of oncogenesis, "drivers" are displaced by commensal bacteria - "passengers" with tumor-promoting properties [22]. Enterotoxigenic strains of *B. fragilis* can inhibit the exosomal microRNA miR-149-3p, which mediates intercellular interactions by modulating the differentiation of Th17 cells, contributing to inflammation and carcinogenesis in the intestine [23].

The association of enterotoxigenic *B. fragilis* with CRC identified in our study was confirmed in different ethnic cohorts. For example, in the Iranian population, the frequency of *bft* gene detection in colorectal biopsy specimens of patients with CRC ranged from 30.5 to 47% compared with control group participants — up to 6.25% of biopsy specimens [24, 25]. Moreover, a higher prevalence of enterotoxigenic *B. fragilis* in biopsy specimens was found among patients from Tehran with ulcerative colitis compared to individuals without intestinal pathology [26]. In Canadian and French cohorts of patients with CRC, a high prevalence of enterotoxigenic *B. fragilis* was found (up to 32% of samples) compared to control subjects [15, 19]. The results of the European Prospective Investigation into Nutrition and Cancer (EPIC) showed that in the European cohort IgA- and IgG-seropositivity to enterotoxigenic *B. fragilis* and genotoxic *E. coli* was significantly associated with the development of CRC [27].

F. nucleatum is a Gram-negative non-spore-forming obligate anaerobic MO of the family Fusobacteriaceae and is the dominant MO in dental plaque biofilms [28]. *F. nucleatum* promotes carcinogenesis and metastasis through multiple mechanisms: promotes proliferation of myeloid suppressor cells; accelerates T cell apoptosis, suppresses T cell proliferation, thereby orchestrating a tumor microenvironment that promotes oncogenesis and metastasis; induces expression of the

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Table 3. Characterization of sensitivity and specificity of identification of potentially oncogenic microorganisms for the diagnosis of CRC

Parameter of the diagnostic method	CRC, *n*	IBD + control, *n*	Sensitivity, % (95% CI)	Specificity, % (95% CI)
Detection of the *clbA* gene	7	11	10 (5–19)	92 (86–95)
Detection of the fragilisin *bft* gene of enterotoxigenic *B. fragilis*	14	5	20 (12–31)	97 (92–99)
Detection the *fadA* adhesion protein gene of *F. nucleatum*	31	18	44 (33–56)	88 (81–92)
Detection the *fimA* gene for biofilm formation of *P. gingivalis*	13	2	19 (11–29)	99 (95–99)
Detection of the *clbB* gene	13	9	19 (11–29)	94 (89–97)
Detection of 1 or more microorganisms	36	27	51 (39–64)	81 (74–87)
Detection of 2 or more microorganisms	22	10	31 (21–44)	93 (88–97)
Detection of 3 or more microorganisms	12	4	17 (9–28)	97 (93–99)
Detection of 4 or more microorganisms	6	3	9 (3–18)	98 (94–100)
Detection of 5 or more microorganisms	2	2	3 (0,35–10,00)	99 (95–100)

molecular structure protein S100A9 and triggers activation of M2 macrophages via nuclear factor-κB, thereby activating tumor cell proliferation and migration; stimulates the proliferation of Foxp3+ regulatory T cells and inhibits the proliferation and function of effector T cells, impeding the antitumor immune response; induces the secretion of circulating exosomes, enhancing tumor invasion; furthermore, a possible role of *F. nucleatum* in resistance to tumor immunotherapy and chemotherapy [29]. A key virulence/oncogenicity factor of *F. nucleatum* is the adhesin FadA, which regulates annexin A1 expression via E-cadherin. Induction of annexin A1, which is a modulator of Wnt/β-catenin, specifically stimulates colorectal carcinoma cells, contributing to the progression of CRC [30]. Furthermore, *F. nucleatum* stimulates inflammatory and antiapoptotic responses in CRC cells through the release of ADP-heptose and activation of the ALPK1/TIFA axis [31]. Recently, a distinct Fna C2 clade of *F. nucleatum* associated with CRC has been characterized, which exhibits increased virulence [32].

According to a study conducted by J. Jones et al., it is *F. nucleatum* and enterotoxigenic *B. fragilis* represent two key pathobionts that promote oncogenic reprogramming of intestinal epithelial cells [11].

P. gingivalis is an anaerobic oral bacterium that causes chronic periodontitis. Over the past decade, the mechanisms by which *P. gingivalis* promotes tumor progression and stimulates cell invasion and metastasis of tumor cells have been identified. These mechanisms include increased expression of proinflammatory factors and matrix metalloproteinases that degrade the basal membranes and extracellular matrix of the intestinal epithelium [33].

According to these studies, an association was found between the presence of periodontopathogenic bacteria in fecal samples and biopsy material from CRC. A case-control study using metagenomic sequencing revealed that the detection rate of *Fusobacterium* was higher in CRC patients (31.9% vs. 11.7% in controls) and the development of CRC was associated with the presence of *F. nucleatum* (OR = 4.11; 95% CI 1.62–10.47; $p = 0.004$) and *P. gingivalis* (OR = 5.17; 95% CI 1.75–15.25; $p = 0.001$) [34]. A study conducted in France revealed that the prevalence of *F. nucleatum* was higher among patients with CRC (70.4%) compared to individuals without intestinal pathology [19].

P. gingivalis and *F. nucleatum*, oral bacteria belonging to the so-called red and orange complexes, can not only induce chronic inflammation but also promote oncogenesis in both the oral cavity and intestine, possibly having a synergistic effect [35].

It should also be noted that all three oncogenic bacteria (*F. nucleatum*, *B. fragilis* and *P. gingivalis*) are potentially associated not only with the development of CRC, but also with a worse prognosis for patients (lower survival rate) [13, 14]. Furthermore, the results of our study indicate a direct relationship between the stage of the cancer process and the prevalence of periodontal pathogenic MOs *F. nucleatum* and *P. gingivalis*.

Various authors have also found colibactin-producing genotypes of *Enterobacteriaceae* to be associated with CRC. Colibactin is a genotoxin that causes double-stranded DNA breaks, cell cycle arrest, and chromosomal instability in eukaryotic cells. It is synthesized by an assembly line of non-ribosomal polyketide synthase (*pks*) consisting of 19 genes (*clbA* to *clbS*) located on a 54 bp genomic island [36]. The *pks* island is present in the genomes of *K. pneumoniae*, *K. aerogenes* (basonym: *Enterobacter aerogenes*), *Citrobacter koseri*, and in the phylogenetic groups of *E. coli* [19]. Infection may occur at early stages of ontogenesis: it is known that transmission of colibactin-producing genotypes

of *Enterobacteriaceae* occurs in the perinatal period during passage through the birth canal (the *clbB* gene was detected in 87.5% of children born naturally, in 12.5% — by cesarean section) and as a result of breastfeeding [37]. The frequency of *clbN* gene detection was higher in CRC patients (49.4%) compared to control participants (24%; $p < 0.005$), with a high prevalence (72.2%) found in the last stage IV compared to stage I/II CRC (42.3%; $p < 0.05$) and stage III CRC (43.2%; $p < 0.05$) [19]. In a Japanese cohort study (543 colorectal neoplasia cases (22 CRC and 521 adenomas), 425 control participants), *pks*$^+$-*E. coli* was found in 32.6% of fecal samples from patients with colorectal neoplasia and in 30.8% from control participants [38]. Also among the Canadian cohort, the frequency of colonization with *pks*$^+$ bacteria was found not to differ between control group participants (42%) and CRC patients (46%), notably, *pks*$^+$ bacteria were common in advanced stages of CRC (40/79; 52%) compared to early tumor stages (3/15; 20%; $p < 0.05$) [15]. In a cohort study, *pks*$^+$-*E. coli* was detected in 9.44% (111/1175) of biopsy specimens from patients with CRC, with DNA *pks*$^+$-*E. coli* was inversely associated with the stage of tumor process ($p = 0.008$) [16]. Our study revealed a high prevalence of the *clbB* gene of the pathogenicity island of *pks*$^+$-*E. coli* at the I stage of the cancer process.

Conclusion

The results of this study allow us to conclude that CRC in patients of a large metropolitan area is associated with the presence of genes of potentially oncogenic bacteria in the intestinal microbiome, in particular, species-specific genes of periodontal pathogens *F. nucleatum*, *P. gingivalis*, toxin-fragilisin *bft* gene of *B. fragilis*, polyketide synthase *clbB* gene of *Enterobacteriaceae pks* pathogenicity island. The results obtained are consistent with the current ideas about the pathogenetic role of these bacteria and/or their toxin-producing strains in CRC.

Molecular genetic detection of the above genetic determinants of potentially oncogenic MOs can be applied as a method of non-invasive diagnosis of CRC regardless of the localization and morphological type of tumor, both separately and together with other recommended tests.

Given the available data on the association of these bacteria with the stage/progression of CRC, prognosis/survival and resistance to immunotherapy and chemotherapy, we can assume the possibility of their use as non-invasive biomarkers for predicting the course and outcomes of CRC, response to antitumor therapy, and in the future for the development of appropriate cancer prevention measures, including personalized correction of oral and intestinal dysbiosis and/or their sanitation.

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