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Mucosa-Associated *Escherichia coli* Producing Cyclomodulin Toxins in Colon Cancer and Intestinal Inflammatory Patients

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ABSTRACT

Background: Colorectal cancer (CRC) is the third most common cause of death. In the human intestinal tract, some *Escherichia coli* strains produce cyclomodulin toxins. This study aimed to determine the frequency of cyclomodulin-encoding genes in *E. coli* isolates from patients with CRC and inflammatory bowel disease (IBD) compared to healthy subjects.
Materials & Methods: A total of 120 *E. coli* strains were isolated from colonic mucosa samples during 2016-2017 from Hamadan, Iran. *E. coli* isolates were identified using biochemical tests. Phylogroups of *E. coli* isolates and cyclomodulin toxin-encoding genes were identified by PCR. The results were analyzed by SPSS software.
Findings: The predominant *E. coli* phylogroups were A (52.5%), B2 (52.5%), and A (55%) in the CRC, IBD, and healthy groups, respectively. *E. coli* isolates harboring the *pks* (32.5%) and *cnf1* (27.5%) genes belonged to phylogroup B2 ($p< .001$), and isolates harboring the *pks* gene were more prevalent in CRC patients (30%). The *cnf3* gene had the highest frequency (30.8%) in the *cnf* gene family. The highest prevalence of *cnf1* (27.5%) was observed in *E. coli* phylogroup B2, the highest prevalence of *cdt4* (58.3%) was in phylogroup B1, and the highest prevalence of *cif* (52.5%) was strongly related to phylogroup B2.
Conclusion: The presence of cyclomodulin toxin-encoding genes in *E. coli* isolates was not associated with CRC, and no statistical difference was observed in the level of cyclomodulin-encoding genes in *E. coli* isolates. On the other hand, there is no information about the critical time of host-microbe interaction for tumorigenesis.

Keywords: *E. coli*; *Escherichia coli*, Colorectal, Cancer, Cyclomodulin, PCR

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Introduction

Colorectal cancer (CRC) is the third most common malignancy and the fourth leading cause of mortality and morbidity worldwide [1, 2]. Colorectal cancer is thought to be caused by a variety of bacteria, including pathogenic *Escherichia coli*. Most *E. coli* strains are present in the human gastrointestinal tract as commensals, but some may contain a broad variety of virulence factors [3, 4]. The significance of each virulence factor is determined by the host state, infection site, and genetic power of the strain, which is encoded by the plasmid or chromosome, depending on the strain type [5].

Pks Island is a conserved genomic region found in some CRC-causing *E. coli* strains. Polyketide synthases (PKS) and non-ribosomal peptide synthetase (NRPS) are encoded in this region and are needed for the production of the genotoxin colibactin [6]. Bacterial products play an important role in the development or incidence of colon cancer by stimulating various mechanisms, including pre-inflammatory induction, carcinogenic pathways in epithelial cells, reactive oxygen species production, and genotoxicity. The *pks* gene cluster also exists in the *Enterobacteriaceae* family, such as *Klebsiella pneumonia*e, *Citrobacter koseri*, and *Enterobacter aerogenes* [7-9]. Bacteria possessing the *pks* gene cluster induce the degradation of double-stranded genetic materials in eukaryotic cells and exhibit carcinogenic potential [10].

Based on a phylogenetic assay, *E. coli* strains could be classified into four groups, including A, B1, B2, and D [11, 12]. Pathogenic strains harboring virulence factors belong to groups B2 and D, while other strains belonging to groups A and B1 are faecal *E. coli* strains and are non-pathogenic [13]. The main known virulence factors include colibactin, cytolethal distending toxins (CDT), cytotoxic necrotizing factors (CNFs), and cycle inhibiting factor (CIF), which are classified as cyclomodulins and are genotoxic or modulate cellular proliferation, differentiation, and apoptosis [14, 15]. The most common members of the CNF family include chromosomally-encoded *cnf1* and plasmid-encoded *cnf2* [16]. The CNF1 protein is a 115-kDa toxin that affects urinary tract and meningeal infections and plays a key role in GTPase activation and cell proliferation. It is related to uropathogenic *E. coli* (UPEC) strains [14]. The CIF protein may be produced by certain *E. coli* strains, such as enteropathogenic and enterohemorrhagic *E. coli* [15].

Some of the CIF functions include nuclear elongation and cell division-independent DNA synthesis in infected cells. CDT is produced by some bacteria such as *E. coli* and *Salmonella typhi*; one of the main trigger mechanisms of CDT is to block the cell cycle between the G2 and mitosis phases.

Cyclomodulin toxins are bacterial proteins that modulate host cell processes, including apoptosis, proliferation, and DNA damage [17]. This study uniquely investigated the prevalence of these toxins in *E. coli* isolates from CRC patients, contributing to our understanding of host-microbe interactions in cancer biology. Cyclomodulin toxins, including colibactin, promote DNA double-strand breaks, resulting in genomic instability, which is a hallmark of cancer [18]. These toxins also induce senescence-associated secretory phenotypes (SASPs), releasing pro-inflammatory cytokines and growth factors that enhance epithelial cell proliferation [19]. Other cyclomodulins, like CNF1, activate Rho GTPases, leading to changes in cytoskeletal organization and tumor cell invasion. The CDT toxin disrupts the cell cycle, causing G2/M arrest, which may create a tumor-promoting microenvironment by enhancing inflammatory signaling [20].

Objectives: The objective of this study was

to ascertain the frequency of cyclomodulin toxin-encoding genes in *E. coli* strains isolated from mucosa samples of patients with colorectal cancer and inflammatory bowel disease (IBD) compared to healthy individuals.

Materials and Methods

Patients and sampling: In total, 120 patients who underwent colonoscopy at Shahid Beheshti hospital in Hamadan province (west of Iran) during 2016- 2017 were asked to participate in the study and sign a consent document. Based on pathological analysis of colon mucosal materials, patients were classified into three groups: CRC, IBD, and healthy control groups. A pathologist examined colon biopsy samples of CRC and IBD patients to confirm the diagnosis. Patients were selected based on their confirmed clinical diagnosis by the pathologist. Inclusion criteria included individuals undergoing colonoscopy at Shahid Beheshti hospital. Biopsy samples were collected systematically from the colonic mucosa of all participants to ensure consistency and reduce variability in sampling. Strict aseptic techniques were used throughout the isolation process to minimize contamination. Culture media sterility and bacterial identification were verified using control strains and repeated tests.

Isolation and identification of *E. coli*: Biopsy specimens from the colonic lumen (50-100 mg) were collected during colonoscopy using a syringe connected to the endoscope. Solid components containing bacterial cells were centrifuged at 900 g for 5 min and washed three times with sterile phosphate-buffered saline (PBS) to eliminate any potential faecal contamination in the preparation solution. Blood agar and MacConkey agar (Merck Co, Germany) were used to culture the samples, which were incubated at 37 °C for 24 hours. Biochemical phenotypic methods were used to identify *E. coli* isolates. *E. coli* isolates were selected based on their growth on MacConkey agar and blood agar media and then confirmed by biochemical tests. These isolates were chosen to represent mucosa-associated bacteria, which may differ from the faecal microbiota.

DNA extraction: Total bacterial DNA of *E. coli* isolates was extracted using boiling water. Briefly, five bacterial colonies from a freshly grown bacterial culture on LB (Luria broth) medium were suspended in 150 µL of sterile distilled water and incubated at 95 °C for 15 min. After cooling on ice, bacterial pellets were removed by centrifugation at 15,000 g for 5 min at 4 °C. The supernatant containing bacterial DNA was stored at -20 °C. The purity of the obtained DNA was evaluated using a UV (ultraviolet)-visible spectrophotometer at $OD_{260/280}$, and then gel electrophoresis analysis was done.

PCR analysis: To investigate the phylogenetic grouping of *E. coli* isolates, triplex PCR (polymerase chain reaction) was performed for the *ChuA*, *YjaA*, *TspE*, and *C2* genes. PCR studies using specific primers provided in Table 1 were also used to determine the identity of cyclomodulin toxin-encoding genes (*pks, cdt, cnf,* and *cif*). For PCR reaction, 12 µL of 2X PCR Master Mix (Fermentas, US) containing Taq DNA polymerase (0.5 U/µL), reaction buffer,4 mM $MgCl_2$, 0.4 mM of each dNTP, 1 µL of DNA template, 1 µL of each primer (10 pm), and sterile double-distilled water up to 25 µL for triplex PCR and up to 20 µL for single PCR were added into microcentrifuge tubes. PCR thermal cycling program included pre-denaturation at 94 °C for 5 min, followed by 35 cycles including denaturation at 94 °C for 30 s, annealing at 35 °C for 30 s, and extension at 72 °C for 1 min. For each reaction, the final extension was done at 72 °C for 10 min. Gel

electrophoresis was performed to analyze PCR products using the safe stain [17].

The *chuA* gene is present in all strains belonging to groups B2 (*yjaA* gene positive)

Table 1) List of primers used in this study

Genes Name	Oligonucleotide Primers 5′……3′	Annealing Time (°C)	PCR Product (bp)	Reference
Triplex PCR Primers for Phylogenetic Grouping				
ChuA *ChuA*	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	57	279	(53)
YjaA *YjaA*	F: TGAAGTGTCAGGAGACGCTG R: ATGGAGAATGCGTTCCTCAAC	57	211	(53)
TspE4C2 *TspE4C2*	F: GAGTAATGTCGGGGCATTCA R: CGCGCCAACAAAGTATTACG	57	152	(53)
Colibactin Primers				
Pks *Pks*	F: TCGATATAGTCACGCCACCA R:GTCAAGCGAGCATACGAACA	58	733	(54)
Cytotoxic Necrotizing Factor (CNF) Primers				
CNF1-1s *CNF1*-1as	F: GGGGGAAGTACAGAAGAATTA R:TTGCCGTCCACTCTCACCAGT	52	1112	(55)
CNF2-2s *CNF2*-2as	F: TATCATACGGCAGGAGGAAGCACC R: GTCACAATAGACAATAATTTTCCG	63	1241	(55)
CNF3-3s *CNF3*-3as	F: TAACGTAATTAGCAAAGA R:GTCTTCATTACTTACAGT	63	757	(55)
Cycle Inhibiting Factor (CIF) Primers				
CIF-int-s *CIF*-int-as	F: AACAGATGGCAACAGACTGG R: AGTCAATGCTTTATGCGTCAT	52	383	(55)
Cytolethal Distending Toxin (CDT) Primers				
CDT-I *CDT*-I	F: CAATAGTCGCCCACAGGA R:ATAATCAAGAACACCACCAC	62	411	(55, 56)
CDT-II *CDT*-II	F: GAAAGTAAATGGAATATAAATGTCCG R:TTTGTGTTGCCGCCGCTGGTGAAA	60	556	(55, 56)
CDT-III *CDT*-III	F: GAAAGTAAATGGAATATAAATGTCCG R:TTTGTGTCGGTGCAGCAGGGAAAA	60	555	(55, 56)
CDT-IV *CDT*-IV	F: CCTGATGGTTCAGGAGGCTGGTTC R:TTGCTCCAGAATCTATACCT	60	350	(55)

Table 2) Frequency of Escherichia coli isolates according to phylogroup typing in this study

Groups	Number	Frequency of Phylogroups (%)			
		A No (%)	B1 No (%)	B2 No (%)	D No (%)
Healthy people (normal)	40	22 (55)	5 (12.5)	7 (17.5)	6 (15)
Inflammatory bowel disease patients	40	10 (25)	4 (10)	21 (52.5)	5 (12.5)
Colorectal cancer subjects	40	21 (52.5)	3 (7.5)	12 (30)	4 (10)

and D (*yjaA* gene positive). On the other hand, in all strains belonging to groups A (*TspE4.C2* gene negative) and B1 (*TspE4.C2* gene positive), the *chuA* gene is absent. These features allow the differentiation of groups B2 and D from groups A and B1 [21].

Statistical analysis: Continuous and categorical variables were compared using Mann–Whitney and Chi-square ($\chi 2$) tests, respectively. A p-value < .05 was considered significant. In addition to calculating p-values, effect sizes were determined to quantify the magnitude of differences between groups. Confidence intervals were also reported to assess the precision of these estimates. Statistical analyses were conducted using SPSS software Version 22, ensuring the validity of the applied models.

Findings

In the present study, a total of 120 biopsy samples were collected from patients referred to the hospital. DNA was extracted, and PCR reactions were performed to detect *pks*, *cnf*, *cif*, and *cdt* genes. The isolates were first subjected to PCR analysis to detect their phylogroups.

Phylogroups and their sources: The prevalence of phylogenetic classes A, B1, B2, and D differed significantly in 120 *E. coli* strains isolated from the three groups of CRC, IBD, and healthy subjects (Table 2). The predominant phylogroups of *E. coli* isolates were A (52.5%), B2 (52.5%), and A (55%) in the CRC, IBD, and healthy groups, respectively.

Distribution of *pks, cnf, cdt,* and *cif* genes according to phylogenetic findings: Table 4 reveals a clear heterogeneity in the prevalence of *pks, cnf, cdt,* and *cif* genes with frequencies of 24.2% for *pks* island; 19.2, 6.7, and 30.8% for *cnf*1-3; 32.5, 35, 14.2, and 25% for *cdt*1-4; and 42.5% for *cif*. Strains belonging to phylogroup B2 accounted for the majority of *pks*-positive strains.

The prevalence of the *pks* gene was found to be the highest (30%) in CRC patients, followed by healthy subjects (22.5%) and IBD patients (20%) (Table 3); there was no significant relationship between the study groups and the prevalence of the *pks* gene (p> .05). However, 70% of isolates from CRC patients lacked this gene, indicating that *pks* is not associated with CRC disease.

In this study, the *cnf3* gene was found to be the most frequent (30.8%) in the *cnf* gene family (Table 4). The highest prevalence of *cnf1* (27.5%) was observed in phylogroup

Table 3) Frequency of cyclomodulin related genes in E. coli isolated from study groups

Gene	Healthy Subjects No (%)	Inflammatory Bowel Disease Patients No (%)	Colorectal Cancer Patients No (%)	Total No (%)
pks	9 (22.5)	8 (20)	12 (30)	29 (24.2)
cnf1	5 (12.5)	9 (22.5)	9 (22.5)	23 (19.2)
cnf2	1 (2.5)	5 (12.5)	2 (5)	8 (6.7)
cnf3	11 (27.5)	13 (32.5)	13 (32.5)	37 (30.8)
cdt1	9 (22.5)	17 (42.5)	13 (32.5)	39 (32.5)
cdt2	10 (25)	15 (37.5)	17 (42.5)	42 (35)
cdt3	3 (7.5)	7 (17.5)	7 (17.5)	17 (14.2)
cdt4	12 (30)	12 (30)	6 (15)	30 (25)
cif	13 (32.5)	19 (47.5)	19 (47.5)	51 (42.5)

PKS: Polyketide synthase, CDT: cytolethal distending toxin, CNF: cytotoxic necrotizing factors, CIF: cycle inhibiting factor

Table 4) Frequency of pks, cnf, cdt, and cif genes in E. coli phylogroups

Genes	A No (%)	B1 No (%)	B2 No (%)	D No (%)	Total No (%)
pks	10 (18.9)	1 (8.3)	13 (32.5)	5 (33.3)	29 (24.2)
cnf1	8 (15.1)	2 (16.7)	11 (27.5)	2 (13.3)	23 (19.2)
cnf2	0 (0)	2 (16.7)	5 (12.5)	1 (6.7)	8 (6.7)
cnf3	15 (28.3)	7 (58.3)	10 (25)	5 (33.3)	37 (30.8)
cdt1	18 (34)	4 (33.3)	14 (35)	3 (20)	39 (32.5)
cdt2	23 (43.4)	5 (41.7)	10 (25)	4 (26.7)	42 (35)
cdt3	7 (13.2)	2 (16.7)	6 (15)	2 (13.3)	17 (14.2)
cdt4	13 (24.5)	7 (58.3)	7 (17.5)	3 (20)	30 (25)
cif	21 (39.6)	5 (41.7)	21 (52.5)	4 (26.7)	51 (42.5)

PKS: Polyketide synthase, CDT: cytolethal distending toxin, CNF: cytotoxic necrotizing factors, CIF: cycle inhibiting factor

Table 5) Statistical analysis of correlations between patient groups, E. coli phylogroups, and cyclomodulin toxin genes

		E. coli Phylogroups	Patient Groups	Type of Disease	Sex	PKS	CNF	CDT	CIF
E. coli phylogroups	A B1 B2 D	-	0.036	-	0.06	0.2	CNF2 0.04	CDT4 0.036	0.34
Patient groups	Colorectal cancer patients Inflammatory bowel disease patients Healthy subjects	0.036	-	0.001	0.27	0.55	-	0.17	0.29
Stage of CRC	Stage 1 Stage 2 Stage 3	-	0.001	-	0.36	0.28	CNF1 0.047	0.32	0.81

P< 0.05 is considered as statistically significant.
PKS: Polyketide synthase, CDT: cytolethal distending toxin, CNF: cytotoxic necrotizing factors, CIF: cycle inhibiting factor

B2, while *cnf2* and *cnf3* were more frequent (16.7 and 58.3%) in phylogroup B1 (Table 4). There was a significant relationship between the presence of the *cnf2* gene and phylogroups, with the highest frequency of CNF2 observed in phylogroup B2 (Table 5). The highest prevalence was related to the *cdt* gene family, including CDT1 (35%) in phylogroup B2, CDT2 (43.4%) in phylogroup A, CDT3 (16.7%) in phylogroups B1, and CDT4 (58.3%) in phylogroups B1 (Table 4). The highest prevalence of the CDT4 gene was in phylogroup B1, and there was a significant correlation (p= .036) between the existence

of CDT4 and the studied phylogroups (Table 5). The highest prevalence of *cif* was observed in phylogroup B2 and was significantly associated with sex value (p= .046).

Distribution of *pks, cnf, cdt*, and *cif* genes according to clinical sources: An analysis was conducted on phylogenetic groups and clinical sources of *E. coli* isolates (CRC, IBD, and healthy groups) to ascertain any correlations. The analysis revealed that phylogroup A was the most prevalent phylogroup observed in healthy subjects (55%) and CRC individuals (52.5%) (Table 3).

Furthermore, inflammatory intestinal disease was found to be associated with phylogroup B2 (52.5%). The relationship between gender and the study groups was also investigated. The investigation revealed no statistically significant association between gender and disease condition among the 120 subjects examined (54 males and 66 females).

Discussion

Recently, an increase in the incidence of *E. coli* in colon cancer has been reported [22]. Research has indicated that in various inflammatory illnesses, including IBD, interactions between the host and microbial flora are compromised. Furthermore, colonic inflammation has been shown to facilitate the colonization of intestinal pathogenic microorganisms, including *E. coli* [23, 24]. Putze et al. (2009) examined the presence of the colibactin toxin gene in the *Enterobacteriaceae* family. The results showed that the frequency of this gene was lower in *K. pneumoniae*, *Citrobacter* spp., and *Enterobacter* spp. than in *E. coli* strains [7]. Potential confounding factors, such as dietary habits, antibiotic usage, geographic variation, and host genetic factors, might have influenced the prevalence of cyclomodulin genes among the studied groups [25]. Although these variables were not controlled in the present study, they highlight the complexity of host-microbe interactions and warrant further investigation. Given the role of colibactin in DNA damage and tumorigenesis, inhibitors that target colibactin biosynthesis, such as non-ribosomal peptide synthetase inhibitors, are promising as potential therapeutic agents [26]. Similarly, targeting CNF1 activity with GTPase inhibitors could prevent cellular and cytoskeletal remodeling. These approaches, combined with modulation of the gut microbiota using probiotics or antibiotics, could provide innovative strategies for the prevention and treatment of colorectal cancer [27].

E. coli strains are classified into four important phylogroups based on phylogenetic analysis, including phylogroups A, B1, B2, and D [28]. In the current research, clinical biopsies from patients with colorectal cancer and IBD and healthy people were used to isolate *E. coli* carrying the *pks* gene. The existence of *cdt*, *cnf*, and *cif* gene families was detected in *E. coli* isolates. Cyclomodulin-positive *E. coli* strains are predominantly associated with pathogenic phylogroups B2 and D, while negative strains are more common in commensal phylogroups A and B1. Positive strains exhibit higher levels of DNA damage induction, inflammatory cytokine release, and mucosal adherence. In contrast, negative strains lack these virulence factors, aligning them with non-pathogenic microorganisms in the gut microbiome.

A study by Buc et al. (2013) on 38 colorectal cancer patients and 31 patients with diverticulosis found that most *E. coli* isolates from cancer patients belonged to phylogroup B2 and carried genes encoding cyclomodulin toxins (colibactin and CNF1), while isolates from non-cancer patients belonged to phylogroup A and D [15, 29]. In a study by Raisch et al. (2014), *E. coli* strains isolated from 48 patients were found to belong to

phylogroup B2, with about 86% of them harboring the cyclomodulin toxin-producing genes [30]. In the current study, there was no significant association between the study groups (40 cancer patients, 40 IBD patients, and 40 healthy individuals) and phylogroup B2. The predominant phylogroups were A (52.5%) in CRC, B2 (52.5%) in IBD, and A (55%) in healthy individuals. These findings differ from the findings of previous studies, indicating the predominance of B2 strains in healthy subjects [31, 32].

Dubois et al. (2010) studied 197 *E. coli* strains and found that patients were strongly associated with phylogroup B2, the PKS island was required for colonization, and the *cnf1* gene was required for virulence, with only 1% harboring the *cdtB* and *cif* genes [14]. This study confirms our findings regarding the IBD group, although other groups indicated the predominance of phylogroup A. Raisch et al. (2014) demonstrated that the majority of the specimens were classified under phylogroup B2, and that most strains harbored members of the CNF family [30], which is in contrast to this study results.

In a study conducted in Baghdad province, Mohammed and colleagues (2024) analyzed 112 *E. coli* isolates obtained from various sources and identified phylogroup B2 as the most prevalent (34.8%) phylogroup, followed by groups C (21.4%), D (16.9%), and A (10.7%), while the remaining groups (B1, E, F, and unknown) showed comparatively lower prevalence rates. The study also demonstrated a significant association between phylogroup B2 and both antibiotic resistance and biofilm formation [33]. This study supports our findings regarding the IBD group, indicating that phylogroup B2 was the most common (52.5%), followed by phylogroup A (25%). PKS island-carrying *E. coli* strains are commonly found in the gut microbiome, and DNA changes are linked to cancer; thus, several studies have been conducted to better understand the function of these bacteria in CRC [14, 34]. Research has indicated that these bacteria may synthesize several cyclomodulins, including colibactin [15,35,36]. Research has indicated that colibactin-producing *E. coli* is present in 55–67% of colorectal cancer patients, whereas this prevalence is below 20% in healthy people [15, 35]. It has been reported that colonic mucosa in CRC patients is significantly associated with the presence of *E. coli* harboring PKS island (68%) compared to healthy subjects (22%) [37]. In the current study, most of the *pks* positive strains belonged to phylogroup B2, which is in line with other studies [7, 34, 38]. Most CRC cases (~90%) are sporadic and hence affected by external factors [39, 40]. It is believed that *E. coli* is involved in CRC development. It has been shown for almost 20 years that *E. coli* highly colonizes human CRC biopsies [41]. This study was limited by its cross-sectional design, precluding causal inferences. Additionally, the sample size, although sufficient for initial exploration, might have lacked statistical power to detect subtle differences. Moreover, traditional PCR was used to detect the presence of genes, whereas qPCR provides more precise data on gene expression levels.

The composition of the microbiota is influenced by external variables, including geographic location, [42], which may explain variations in the distribution of PKS islands. The paradoxical findings in this study may be attributed to various variables, including geographical [43] conditions, dietary regimens, host genetic characteristics, and/or the influence of antibiotics on the commensal gut microbiota [38, 44]. Except for the *cif* gene, which was more present in phylogroup B2 (52.5%), the absence of the examined genes in phylogroup B2 was usually greater than their presence. On the other hand, in CRC individuals, the absence of the *pks*, *cnf*, *cdt,* and *cif* genes was significantly more

frequent (p= .001). The expression of these genes does not appear to be linked to CRC. In this research, the presence of the *cif* gene in phylogroup B2, which is extensively utilized in numerous CRC-related tests, surpassed its absence. Recently, a bacterial driver-passenger model has been suggested in CRC development [45]. In this model, some bacteria named "bacterial drivers" trigger the onset of CRC and change the intestinal microenvironment. These changes lead to the proliferation of opportunistic bacteria known as "bacterial passengers". Although cyclomodulin genes in *E. coli* isolates did not show a direct correlation with CRC, understanding their distribution may pave the way for future microbial screening strategies aimed at identifying gut microbiota imbalances as potential biomarkers for colorectal cancer risk. This theory suggests that they have a passenger role, but given the harmful effect of colibactin on DNA, a driver role is also possible. To induce DNA damage and play a driving role, *pks*-positive bacteria should be in close contact with intestinal stem cells [46] and exposed to environmental conditions favorable for colibactin production. Colibactin-producing *E. coli* leads to increased epithelial cell proliferation and tumor invasion in various CRC mouse models [29, 30]. This proposes that they could induce and promote human CRC development [47].

Consequently, despite the high prevalence of *pks*-positive *E. coli* among patients, colibactin production necessitates exposure to certain circumstances. The colibactin toxin functions by inducing cell cycle arrest, which counteracts cancer promotion. One hypothesis for these conflicts is that cell cycle arrest coincides with cellular senescence in diverse cell types [48, 49]. Also, investigating the possibility of the presence of other virulence factors in the bacteria and analyzing mucosa-associated bacteria rather than faecal bacteria may help elucidate this paradox. The temporal dynamics of *E. coli* colonization in tumorigenesis remain unclear. It is hypothesized that cyclomodulin-positive *E. coli* bacteria act as 'bacterial drivers,' initiating DNA damage and inflammation, which prime the colon microenvironment for tumor formation [50]. As the tumor progresses, the microbiota composition changes, with cyclomodulin-negative strains possibly serving as 'bacterial passengers'. Longitudinal studies are required to establish causality and clarify the timing of these interactions [51].

This study had several limitations. First, although the sample size was sufficient for preliminary analysis, it might have lacked statistical power to detect subtle associations. Second, the conventional PCR approach was utilized to ascertain the presence of genes, which does not provide insights into their expression levels or functional activity. Employing quantitative real-time PCR (qPCR) could potentially generate more precise data. Furthermore, dietary patterns, antibiotic usage, and genetic predispositions were not controlled, all of which may influence the gut microbiome composition and *E. coli* prevalence. Finally, the study focused on mucosa-associated *E. coli*, which may not fully represent the entire gut microbiome.

Future studies should employ longitudinal designs to determine the temporal dynamics of *E. coli* colonization and its role in tumorigenesis. The incorporation of qPCR or analogous molecular tools holds promise to offer novel insights into the expression levels and activity of cyclomodulin genes. Moreover, expanding the sample size and including participants with diverse geographic and dietary backgrounds could enhance the generalizability of the findings. Furthermore, exploring other microbial species and their interactions with *E. coli*

could reveal synergistic effects contributing to colorectal cancer progression. Finally, exploring therapeutic approaches, such as the use of probiotics, prebiotics, or specific inhibitors targeting cyclomodulin toxins, could offer novel strategies for the prevention and treatment of colorectal cancer.

Furthermore, personalized medicine must consider tumor biology in conjunction with tumor-associated bacteria, as indicated by this definition. A potentially intriguing adjuvant strategy to reduce cell proliferation in CRC patients colonized with pks-carrying bacteria may involve the use of colibactin synthesis inhibitors [52].

Conclusion

The correlation between cyclomodulin toxin-encoding genes in *E. coli* isolates and colorectal cancer has not been substantiated. Statistical analysis revealed no significant difference in the prevalence of cyclomodulin-encoding genes among *E. coli* isolates between healthy controls and patients. Moreover, the temporal significance of the host-microbe relationship in tumorigenesis remains to be elucidated.

Abbreviations

UPEC: Uropathogenic E. coli; CRC: colorectal cancer; IBD: inflammatory bowel disease; PKS: polyketide synthases; CDT: cytolethal distending toxin; CNF: cytotoxic necrotizing factors; CIF: cycle inhibiting factor.

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